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Secretion of functionally active complement factor H related protein 5 (FHR5) by primary tumour cells derived from Glioblastoma Multiforme patients

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ABSTRACT

The complement system is an important humoral immune surveillance mechanism against tumours. However, many malignant tumours are resistant to complement mediated lysis. Here, we report secretion of complement factor H related protein 5 (FHR5) by primary tumour cells derived from Glioblastoma multiforme (GBM) patients. We investigated whether the secreted FHR5 exhibited functional activity similar to factor H, including inhibition of complement mediated lysis, acting as a co-factor for factor I mediated cleavage of C3b, and decay acceleration of C3 convertase. Immunoblotting analysis of primary GBM cells (B30, B31 and B33) supernatant showed the active secretion of FHR5, but not of Factor H. ELISA revealed that the secretion of soluble GBM-FHR5 by cultured GBM cells increased in a time-dependent manner. Primary GBM-FHR5 inhibited complement mediated lysis, possessed co-factor activity for factor I mediated cleavage and displayed decay acceleration of C3 convertase. In summary, we detected the secretion of FHR5 by primary GBM cells B30, B31 and B33. The results demonstrated that GBM-FHR5 shares biological function with FH as a mechanism primary GBM cells potentially use to resist complement mediated lysis.

1. Introduction

Glioblastoma multiforme (GBM) is the most lethal brain tumour in adults with a median survival of less than 15 months (Ohgaki et al., 2004). One of the biggest clinical challenges in the treatment of GBM is the highly aggressive behaviour of cells that prevent complete surgical resection and enhance resistance to conventional radio- and chemotherapy (Ohgaki et al., 2004; Furnari et al., 2007). Even with complete surgical resection, the long-term survival of GBM patients remains very poor due to a high recurrence rate (Ohgaki et al., 2004). Recent findings from The Cancer Genome Atlas confirmed that the immune microenvironment significantly contributes to therapy resistance and is associated with worse survival in GBM (Iglesia et al., 2016). In addition, glioma cells have the capacity to escape immune surveillance, and are involved in immune suppressive activities to promote the malignant transformation of GBM tumours (Chang et al., 2016; Mieczkowski et al., 2015).

GBM is a grade IV astrocytoma, characterised by uncontrolled cellular proliferation, diffuse infiltration, extensive genomic instability, tendency for necrosis, angiogenesis, and resistance to apoptosis. The tumour is characterised by high inter- and intra- morphological heterogeneity, hence the term “multiforme” (Furnari et al., 2007; Liu et al., 2012). GBM is a robust malignant tumour distinguished by its local invasion pattern (Gabrusiewicz et al., 2014; Hermanson et al., 1992). Clinical symptoms may include progressive headaches, seizures and focal neurological deficit (Pace et al., 2009; Faithfull et al., 2005; Oberndorfer et al., 2008; Hermanson et al., 1992). GBM is most often *de novo* (primary GBM), which develops quickly within a three-month

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period before the initial symptoms indicate the onset of the tumour. Primary GBM accounts for approximately 90% of the cases and is predominately found in patients older than 45. The tumour may also develop from a lower grade tumour to a higher malignancy (secondary GBM) over 5-10-years, which is more commonly observed in patients below the age of 45. These subtypes have distinct genetic aberrations but are histologically indistinguishable (Furnari et al., 2007; Maher et al., 2006; Fujisawa et al., 1999). GBM is resistant to destruction by the immune system. Despite aggressive treatment including surgical resection and radiotherapy with concomitant chemotherapy, prognosis remains poor due to GBM recurrence, with a median survival of 14.6 months (Stupp et al., 2005).

The complement system is one of the most potent humoral innate immune protective mechanisms. Complement is activated via three pathways (classical, alternative and lectin). The recognition molecules of each pathway identify non-self and altered self, and the activities of the complement system eventually eliminate the targets (Kohl, 2006). All the three complement pathways converge on the generation of C3 convertase, leading to terminal membrane attack complex (MAC), which is lytic to target cells. The resistance to complement mediated killing by both normal tissue and human malignancies is due to membrane-bound inhibitors including complement receptor 1 (CR1; CD35), membrane cofactor protein (MCP; CD46), decay-accelerating factor (DAF; CD55), and protectin (CD59) (Morgan and Meri, 1994; Gorter and Meri, 1999). CR1 and MCP act as a co-factor for factor I mediated degradation of C3b (Fearon, 1979; Seya et al., 1986), while DAF and CR1 promote the decay of C3 and C5 convertases. Protectin inhibits the formation of the MAC on cell membranes (Sugita et al., 1988; Meri et al., 1990).

In addition to membrane bound regulators, the activity of the complement system is efficiently controlled by soluble factor H (FH), which is predominantly synthesised by the liver (Davies et al., 1989; Weiler et al., 1976). FH regulates the alternative pathway by acting as a cofactor for factor I-mediated cleavage of C3b, competes with factor B to bind to C3b, and promotes the dissociation of C3 convertase, C3bBb (Fearon and Austen, 1977; Whaley and Ruddy, 1976). FH is a 150 kDa soluble protein consisting of 20 complement control protein modules (CCPs) (also called short consensus repeats; SCR), each composed of about 60 amino acids (Ripoche et al., 1988). The complement regulatory activity is located in CCP1–4 (Jokiranta et al., 2000; Sharma and Pangburn, 1996; Gordon et al., 1995; Kuhn and Zipfel, 1996), which is also one of the three C3b binding sites. A second C3b binding site may be localised on CCP 8–15 and the third binding site exists on CCP 19–20 (Jokiranta et al., 2000, 2005).

FHR5 is a protein of the FH family, which includes FH, FH like protein 1 (FHL-1), and FHR1-5, which are structurally and immunologically related. FHL-1 is an alternatively-spliced product of the FH gene, whereas FHR1-5 are each encoded by separate genes. FHR5 exists as a glycosylated protein with a plasma concentration of approximately 5 μg/mL (Skerka et al., 2013) The FHR5 gene is located downstream of the FH gene on a distinct segment of human chromosome 1q32 within the Regulation of Complement Activation (RCA) gene cluster (Jokiranta et al., 2005; Clark and Bishop, 2015). FHR5 is the largest human FHR protein (~65 kDa) and is composed of 9 CCPs. All FHR proteins contain CCPs which are similar in sequence to CCP 6, 19 and 20 of FH (Ohgaki et al., 2004; Furnari et al., 2007; Iglesia et al., 2016; Chang et al., 2016; Mieczkowski et al., 2015). However, FHR5 is the only FHR to contain additional CCPs, which are similar to CCP10–14 of FH. CCP3-7 of FHR5 show 46%, 75%, 57%, 48% and 71% amino acid identity with CCP10–14 of FH, respectively (Jokiranta et al., 2005). The extensive sequence similarities between FHR1-5 and FH allow FHRs to be recognised by polyclonal anti-FH antibodies. The C-terminal FHR5 CCP8 and 9 share sequence identity of 64% with CCP19 and 42% with CCP 20 of FH whereas there is a more distant sequence homology between FHR5 CCP1-2 and CCP6 and CCP7 of FH (Medjeral-Thomas and Pickering, 2016; McRae et al., 2001). FHR5 has the ability to bind to C3b, heparin, C-reactive protein, and iC3b (the cleavage product of C3b). The function of FHR5 is not well understood (Csincsi et al., 2015). Weak co-factor activity for cleavage of C3b by factor I and decay-acceleration activity of C3bBb convertase have been attributed to FHR5 (Jokiranta et al., 2005; McRae et al., 2005). However, studies have also reported that FHR5 can deregulate complement by competing with FH for binding to surface polyanions and C3b (Csincsi et al., 2015; Goicoechea de Jorge et al., 2013).

Here, we report that primary tumour cells, directly isolated from GBM patients post-surgery, secrete FHR5 in culture (GBM-FHR5). Our results show that the primary GBM-FHR5 is functionally active and regulates complement by acting as a co-factor for factor I mediated cleavage of C3b, and degrading (decaying) C3 convertase.

2. Materials and methods

2.1. Isolation of primary GBM Cells from tumour samples

Primary GBM cells were obtained directly from surgical resections of three patients at the John Radcliffe Hospital, Oxford, UK following local ethical board approval. Briefly, a small piece of each tumour tissue was removed and placed into Accutase (Sigma-Aldrich) for 15 min to dissociate into single cells. All primary cells used in this study, namely B30, B31 and B33, were grown in DMEM-F12 medium (Life Technologies) supplemented with 10% v/v heat inactivated fetal calf serum (FCS; Life Technologies), and 1% penicillin/streptomycin (Life Technologies) at 37 °C in 5% v/v CO_2. The cells were passaged up to three times.

2.2. FHR5 purification from B30, B31 and B33 cells

2×10^6 B30, B31, or B33 cells were grown in 75 cm^3 culture flasks in serum free DMEM/F12 with penicillin/streptomycin until the cells were 80% confluent. The culture supernatant was centrifuged for 5 min at 2000 rpm to remove cells in suspension. The supernatant was dialysed overnight against buffer containing 25 mM Tris−HCl pH 7.5, 140 mM NaCl, 0.5 mM EDTA, pH 7.5 and passed through an anti-FH Sepharose column (CNBr-activated Sepharose to which rabbit anti-human FH IgG fraction was coupled) (MRC Immunochemistry Unit, Oxford) (Soames and Sim, 1997).

The column was then washed with 10 bed volumes of the same buffer. Bound protein was eluted with 3 M $MgCl_2$, adjusted to pH 6.8. The eluted fractions were dialysed overnight against distilled water followed by 4 h dialysis against 10 mM potassium phosphate, 140 mM NaCl, 0.5 mM EDTA, pH 7.5. The final protein concentration was measured with a nanodrop spectrophotometer, reading at 280 nm. FH was purified as a control protein from normal human serum using the same technique. Procedures are based on those in Yu et al. (2014), although a different antibody preparation was used.

2.3. SDS-PAGE

SDS-PAGE was performed using the Laemmli (1970) method to observe the proteins purified from primary GBM cells and to assess the purity of human FH. The samples were added to 2 x treatment buffer with or without the reducing agent i.e. beta mercaptethanol (Sigma-Aldrich) in a 1:1 v/v ratio and were denatured for 10 min at 100 °C. Standard Protein markers (Thermo-Scientific) and the denatured samples were loaded onto the gel and separated via electrophoresis for 90 min at 120 V. The gel was stained overnight on a rotary shaker with Coomassie blue stain solution (0.1% w/v Coomassie blue (Sigma-Aldrich), 10% v/v acetic acid, 20% v/v methanol, followed by de-staining for 2 h using de-stain solution (10% v/v acetic acid, 20% v/v methanol) to visualise the bands.

2.4. Western blot

0.2×10^6 cells/mL of primary GBM cells B30, B31, B33 and cell line H2 (control: glioblastoma cell line; Gasque et al. (1992) (kindly provided by Prof Seppo Merri from University of Helsinki) were plated in 12 well plates in the presence of DMEM-F12 and 1% penicillin/streptomycin to obtain serum-free culture supernatant. Cells were grown at 37 °C in 5% CO_2 until 80% confluency was reached. The culture supernatant was centrifuged for 5 min at 10,000 rpm. Each supernatant was run on a 12% SDS-PAGE gel under reducing conditions. The protein bands were transferred to a nitrocellulose membrane in 1 x transfer buffer (25 mM Tris-HCl, 190 mM glycine, 20% v/v methanol pH 8.3) at 70 V for 2 h at 4 °C. After blocking non-specific binding sites overnight with 2% w/v bovine serum albumin (BSA) at 4 °C, the membrane was incubated for 1 h at room temperature (RT) with primary polyclonal rabbit anti-human FH (10 μg/mL IgG; MRC Immunochemistry Unit, Oxford) in 1 x PBS (Thermo-Scientific). The membrane was washed using 1 x PBS + 0.05% Tween20 (PBST) and incubated with secondary anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (5 μg/mL) (Sigma Aldrich) for 1 h at RT. After washing, bound antibodies were detected and visualised using 3,3′diaminobenzidine (DAB; Sigma Aldrich). A similar blot was also probed with goat anti-FHR5 polyclonal antibodies (R&D; cat. no. AF3845-SP).

2.5. ELISA

The culture supernatant (100 μL) from 0.2×10^6 B30, B31 or B33 primary cells/mL grown in serum free media was dispensed in triplicates in a microtiter plate, with the addition of another 100 μL of carbonate bicarbonate buffer (Sigma-Aldrich) to each well. Human FH and BSA (100 μg/mL) were added to separate wells as positive and negative control proteins. After overnight incubation at 4 °C, the wells were washed with 1 x PBST and non-specific binding sites were blocked by incubation with 2% BSA for 2 h at 37 °C. The wells were washed again, the primary polyclonal rabbit anti-human FH antibody (10 μg/mL IgG) in PBS was added, and incubated for 1 h at 37 °C. After washing, bound antibodies were detected using anti-rabbit IgG-HRP secondary antibody (5 μg/mL) in PBS for 1 h at 37 °C. Wells were washed and o-Phenylenediamine (Sigma-Aldrich) was added in the dark. The A_{450} nm was measured using a microplate absorbance reader (Bio-Rad).

2.5.1. Haemolytic assay

Sheep erythrocytes (TCS Bioscience) were washed with PBS-5 mM EDTA. The cell concentration was adjusted to 1×10^9 cells/mL using DGVB-Mg-EGTA (2.5 mM sodium barbital, 71 Mm NaCl, 7 mM $MgCl_2$, 10 mM EGTA, 0.1% w/v gelatin, 2.5% w/v glucose pH 7.4). The presence of EGTA and $MgCl_2$ allows activation of the complement alternative pathway only. Serum depleted of FH family proteins (FH, FHL-1, and FHR1-5) was made by passing normal human serum (TCS Bioscience) through a polyclonal anti-FH Sepharose column in the presence of 0.1 mM EDTA at 4 °C and freezing the flow-through immediately at −80 °C (McRae et al., 2005). A dot blot was performed on the serum that had been passed through the column using polyclonal anti-human FH and anti-rabbit IgG-HRP (1:1000) antibodies to ensure FH family proteins were absent. 40 μL of FH family depleted serum was diluted in 160 μL of DGVB-Mg-EGTA followed by a 2-fold serial dilution on a microtiter plate on ice and 100 μL of sheep erythrocytes were added to each well. Following incubation for 30 min at 37 °C and centrifugation (5000 rpm, 10 min, 4 °C), the supernatants were removed, and the absorbance was measured at 541 nm. To determine percentage haemolysis, 100 μL of sheep erythrocytes with 100 μL of water was included separately to determine total cell lysis and 100 μL of sheep erythrocytes with 100 μL of DGVB-Mg-EGTA was included as a blank.

On ice, 20 μL of depleted serum that caused 50% haemolysis, was mixed with varying concentrations of GBM-FHR5 (0.2–4 μM), 100 μL of sheep erythrocytes and sufficient DGVB-Mg-EGTA (to bring the mix to 200 μL). Human FH (0.2–4 μM) was used instead of FHR5 as a control. The samples were incubated for 30 min at 37 °C followed by immediate centrifugation at 4 °C. The absorbance at 541 nm was measured for each supernatant and the percentage haemolysis was calculated.

2.6. Co-factor assay

The Factor I co-factor activity assay was adapted from Brandstatter et al. (2012). Briefly, 3 μg of C3b (0.55 μM)(Complement Technology), 45 ng of factor I (4 nM)(Complement Technology) and 0.39–78 μg (0.2–4 μM) of FHR5 or 0.9–18 μg (0.2-4μM) FH were added to 10 mM sodium phosphate, pH 6.0 to a final volume of 30 μL. Samples without FH or FHR5 were included as controls. The samples were incubated at 37 °C for 10 min and separated under reducing conditions via SDS-PAGE and subjected to Coomassie blue staining.

2.7. Decay acceleration activity

The decay acceleration assay was modified from Brandstatter et al. (2012). Briefly, a microtiter plate was coated with 50 μL containing 250 ng of C3b in 50 mM carbonate bicarbonate buffer, pH 9.6 overnight at 4 °C. Generation of nickel chloride stabilised C3 convertase was achieved by the addition of 400 ng of Factor B (Complement Technology) and 25 ng of factor D (Complement Technology), in the presence of 2 mM nickel chloride (Sigma-Aldrich), 25 mM NaCl and 4% w/v BSA to a total volume of 150 μL. The samples were incubated for 2 h at 37 °C. After extensive washing with PBS, varying concentrations of FHR5 (1.3 to 26μg, resulting in concentration 0.2–4 μM) were diluted in PBS to a total volume of 100 μL and added directly to each well then incubated for 30 min at 37 °C. Samples with and without FH or FHR5 were included as controls. After extensive washing, intact complexes (not decayed) were detected with goat anti-human factor B antibody (40 μg/mL; Complement Technology) in PBS for 1 h at 37 °C. The wells were washed and rabbit anti-goat IgG-HRP (1.5 μg/mL) (Thermo-Scientific) in PBS was added to each well and incubated for 1 h at 37 °C, followed by washing. To each well, o-Phenylenediamine was added for 5 min in the dark and the A450 nm was measured.

3. Results

3.1. Secretion of FHR5 by B30, B31 and B33 GBM cells

We first examined the presence of FH family proteins in the culture supernatant of B30, B31 and B33 primary GBM cells. Using western blot analysis, we were able to detect FHR5 in serum free culture supernatant of primary GBM cells, demonstrating that these cells secreted FHR5, migrating at ~65 kDa under reducing conditions (Fig. 1A). H2 supernatant (Fig.1B) differed from the primary cells supernatant as bands were displayed for FH at 150 kDa and FH like -1 (FHL-1) at 42 kDa. FH, FHL-1 and FHR1–4 were undetectable in B30, B31 and B33 culture supernatant. The identity of FHR5 was a established by use of an anti-FHR5 polyclonal antibody (Fig. 1C). Subsequently, we affinity-purified the protein using a polyclonal anti-human factor H Sepharose column (Fig. 2). In order to eliminate interference with the presence of FH in fetal calf serum, the culture supernatant was obtained from cells grown in serum-free media. A prominent band was visualised at ~65 kDa under reducing conditions, the molecular weight for FHR5. No higher or lower bands were present, indicating that there were no impurities or other FH family proteins eluted. The lack of additional bands also confirmed that the protein was not a cleaved product of full length FH. In addition, the purified protein continued to migrate at ~65 kDa even under non-reducing conditions (Fig. 2B), ruling out the possibility of the purified protein being C4b binding protein (with which polyclonal anti-FH also cross-reacts). A protein yield of 400 μg for B30, 300 μg for B31 and 300 μg for B33 was obtained from 50 mL serum-free DMEM/F12 with penicillin/streptomycin collected from the cells at 80%

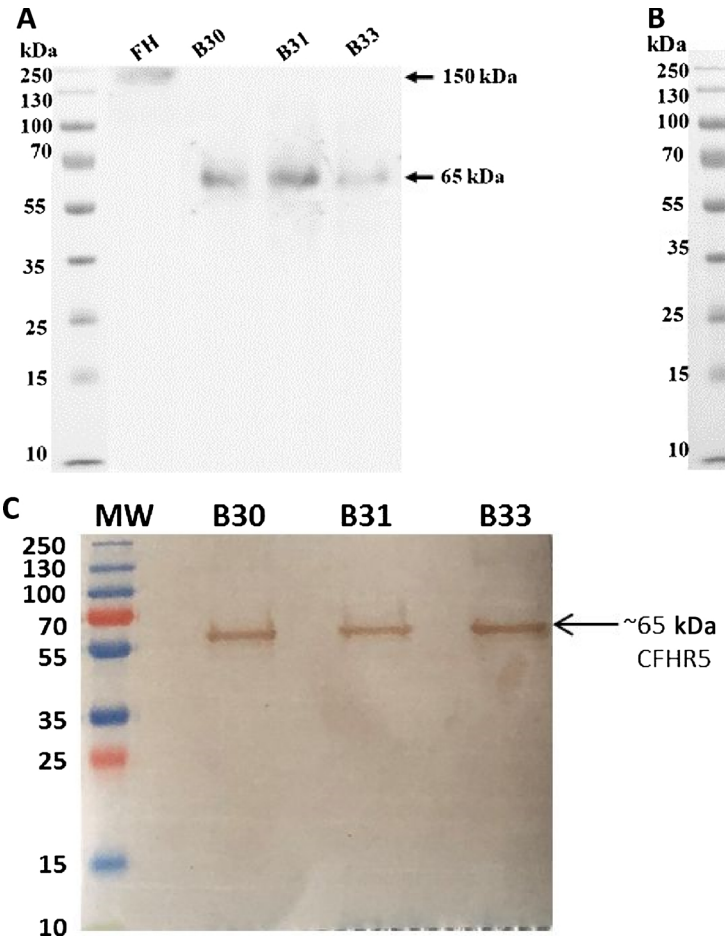

Fig. 1. Western blot analysis of serum free primary GBM supernatant. 0.2×10^6 (A) B30, B31, B33 (B) and H2 cells (control) Cells were grown for 48 h at 37 °C with 5% v/v CO_2 in the absence of fetal calf serum. The culture supernatant was collected and an SDS-PAGE was run (reduced samples). FH was included as a control. The samples were transferred onto nitrocellulose membrane and probed with polyclonal rabbit polyclonal anti-FH (10 µg IgG/mL) and anti-rabbit IgG-HRP (5 µg/mL) antibodies for 1 h. The detected protein band was visualised using 3,3′diaminobenzidine. (c) The purified proteins were also probed with goat anti-FHR5 polyclonal antibodies (1:1000; R&D: cat. no. AF3845-SP).

confluence. Using ELISA, we analysed the protein secretion at regular intervals of cell growth. There was a significant time dependent increase in FHR5 secretion over 48 h of growth (Fig. 3). These data show that FHR5 is secreted by primary GBM cells B30, B31 and B33, but FH and other members of the FH family are not.

3.2. GBM-FHR5 inhibits complement-mediated lysis of sheep erythrocytes

Sheep erythrocytes have cell surfaces rich in sialic acid and are used as the standard non-activator for human complement analysis. The ability of FHR5 from B30, B31 and B33 cells to recognise sialic acids and inhibit activation of the complement system was assessed by replacing serum depleted of FH family proteins (confirmed by dot blot) with purified GBM-FHR5. We first carried out the haemolytic assay in serum depleted of FH family proteins which demonstrated that sheep erythrocytes were lysed in a dose dependent manner. When sheep erythrocytes were incubated with varying concentrations of FHR5, haemolysis was reduced dose dependently (Fig. 4). 4 µM of FHR5 was needed to achieve complete inhibition of cell haemolysis, whereas 1 µM of FH was needed to reduce lysis to zero.

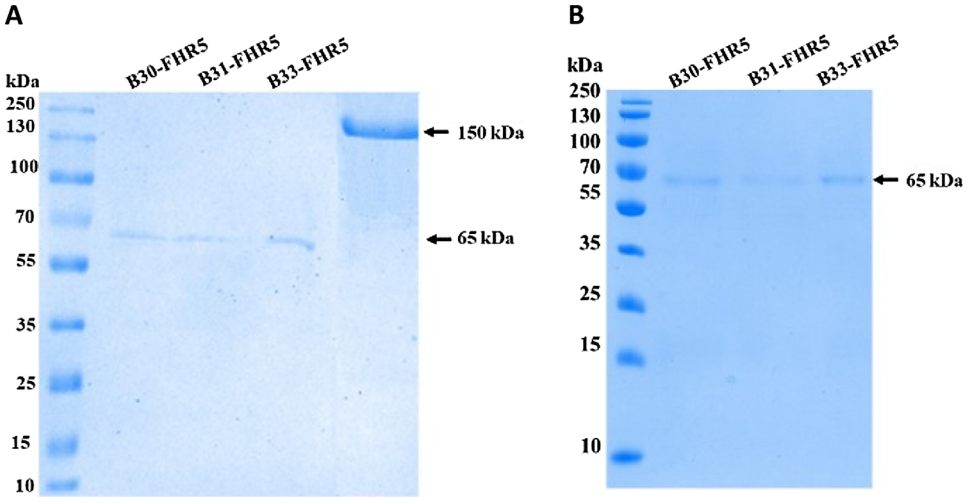

Fig. 2. Purification of FHR5 from primary GBM cells. The supernatant from cultured B30, B31 and B33 cells were dialysed against wash buffer I overnight. The dialysate was passed through a polyclonal anti-human FH Sepharose column. The bound protein was eluted and dialysed against distilled water over night and dialysis buffer I for 4 h. (A) A reduced SDS-PAGE. (B) A non-reduced SDS-PAGE. FH is shown as a control in A.

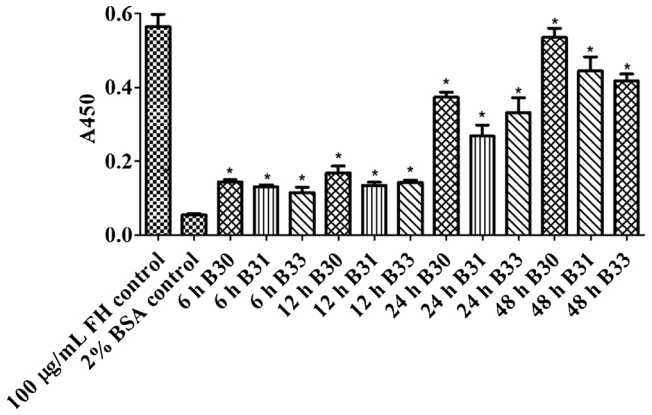

Fig. 3. FHR5 secreted by primary GBM cells. 0.2 × 10 (Liu et al., 2012) primary GBM cells B30, B31 and B33 were grown in the absence of fetal calf serum for 6 h, 12 h, 24 h and 48 h. The culture supernatants were coated onto microtitre plates in 0.05 M carbonate bi-carbonate buffer, wells were blocked with 2% BSA and probed with polyclonal anti-FH (10 µg IgG/mL) and anti-rabbit IgG-HRP (5 µg/mL) antibodies. FH (100 ug/mL) and 2% w/v BSA were coated on plates as controls. The experiments were carried out in triplicates. Data were analysed between the mean A450 for culture supernatants and the mean A450 for 2% BSA. *P < 0.05, error bars represent standard deviation.

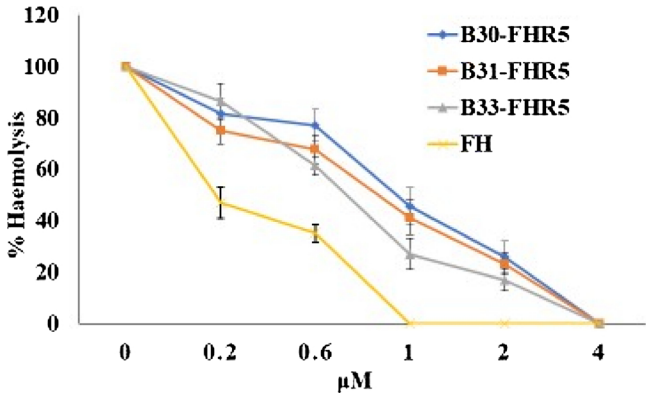

Fig. 4. Inhibition of haemolysis by FHR5. The volume 20 µL of FH family depleted serum capable of giving 50% haemolysis was mixed with DGVB-Mg-EGTA, sheep erythrocytes and varying concentrations of FHR5 or FH (0.2–4 µM). Haemolysis observed is displayed as percentage of the lysis of FH family depleted serum without additions. The experiments were carried out in triplicates. Error bars represent standard deviation.

3.3. GBM-FHR5 shows co-factor activity for factor I-mediated cleavage of C3b

A co-factor assay was performed to measure the ability of FHR5 to act as a co-factor for factor I-mediated cleavage of C3b. Factor I, C3b and GBM-FHR5 or FH were mixed and incubated to allow FHR5 or FH to bind to C3b and enable factor I mediated cleavage. Co-factor activity for factor I was visualised by SDS-PAGE analysis through the loss of or reduced intensity of C3b α- chain and the appearance of two cleaved inactivated C3b (iC3b) fragments at 68 kDa and 43 kDa (Fig. 5A-D). Reactions containing 0.6–4 µM of GBM-FHR5 mediated factor I cleavage of C3b. However, at 0.2 µM (Fig. 5A-C) cleavage was not detected whereas reactions containing 0.2 µM of FH did display C3b cleavage in the presence of factor I (Fig. 5D). In the absence of FHR5 and FH, factor I alone was unable to cleave C3b.

3.4. GBM-FHR5 has decay acceleration activity for C3 convertase

To establish whether GBM-FHR5 had decay acceleration activity as a mechanism to inhibit the alternative pathway, a dissociation assay was carried out. A modified ELISA technique was used to determine the ability of FHR5 to exert decay acceleration activity. Nickel chloride-stabilised C3 convertase was formed and subsequently treated with FHR5 and the control protein, FH. The addition of GBM-FHR5 was successful in dissociating Bb fragment from C3bBb(Ni^{2+}) in a dose-dependent manner (Fig. 6), as shown by the inverse correlation between FHR5 concentration and intact C3 convertase.

4. Discussion

The function of human FHR5 is currently controversial and only limited data is available. There are studies which show that FHR5 has a complement "activating" role (by competing with the down-regulatory effect of FH) (Goicoechea de Jorge et al., 2013) whereas it is also reported that FHR5 can inhibit complement (McRae et al., 2005). An earlier study by Junnikkala et al demonstrated the expression of FH and FHL-1 in GBM H2 cell line, which were found to regulate complement activation (Junnikkala et al., 2000). We therefore investigated whether primary GBM cells would express the same factor H family proteins and if the endogenous product would inhibit or activate complement.

To our knowledge, this is the first study to show that primary GBM cells (B30, B31 and B33), obtained directly from three different GBM patients who underwent brain surgery, were found to secrete FHR5, but not FH. We were able to detect the presence of FHR5 through western blot as the polyclonal anti-FH antibody detected a band at 65 kDa, which corresponds to FHR5. The primary anti-FH polyclonal antibody used can detect FH family proteins as they all share high sequence homology, especially FHR5, as it is the only FHR to share sequence homology with CCP 10-14 of FH (Skerka et al., 2013). FH, FHL-1 and CFHR1–4 were not detected in the culture supernatant of the three primary GBM cells examined in this study. The secretion of FH family proteins was also studied in the H2 cell line to compare the difference between primary cells and a cell line. As expected, H2 cells were found to secrete FH and FHL-1 as previously demonstrated by Junnikkala et al. (2000). The difference in FH family expression between both cell types, highlight the importance of studying primary cancer cells. The absence of FH from primary GBM culture is of interest as it is known to be the most abundant soluble complement inhibitor. We were able to rule out the possibility of the detected protein being a cleaved product of FH as the purification process only produced a single band at 65 kDa for each primary GBM cell (Fig. 2) whereas a cleaved product would show up as having more than one band. Since the purified protein continued to run at 65 kDa on SDS-PAGE under non-reducing conditions, it became evident that the isolated protein was not C4BP (Fig. 2B).

Protein production of FHR5 was supported by an ELISA, which demonstrated that over a 48-h growth period, the concentration of FHR5 gradually increased: with more primary GBM cells present, the greater the quantity of FHR5 secreted. The significant increase in FHR5 secretion over 48 h as well as the lack of FH suggested FHR5 may have a role in the control of complement activation in tumour cells. A previous study by Gasque et al demonstrated that certain glioma cells express FH mRNA; however, its role in complement resistance was not studied (Gasque et al., 1992). They had also shown that an oligodendrocyte cell line (HOG) was able to secrete FH upon IFN-α cytokine stimulation. However, in our study, we observed that primary GBM B30, B31 and B33 produced FHR5 without the addition of cytokines. As FHR5 has a high sequence homology with FH, we wanted to establish whether it was functionally active and could potentially inhibit activation of the complement alternative pathway.

We have demonstrated, via in vitro assays, that purified FHR5 from primary GBM cells is biologically active and our study support a function of FHR5 as a down-regulator of complement. The functional activity was assessed using various techniques including the haemolytic assay. The assay revealed that all three primary GBM-FHR5 exhibited dose-dependent inhibition of sheep erythrocyte lysis (Fig. 4). It is likely

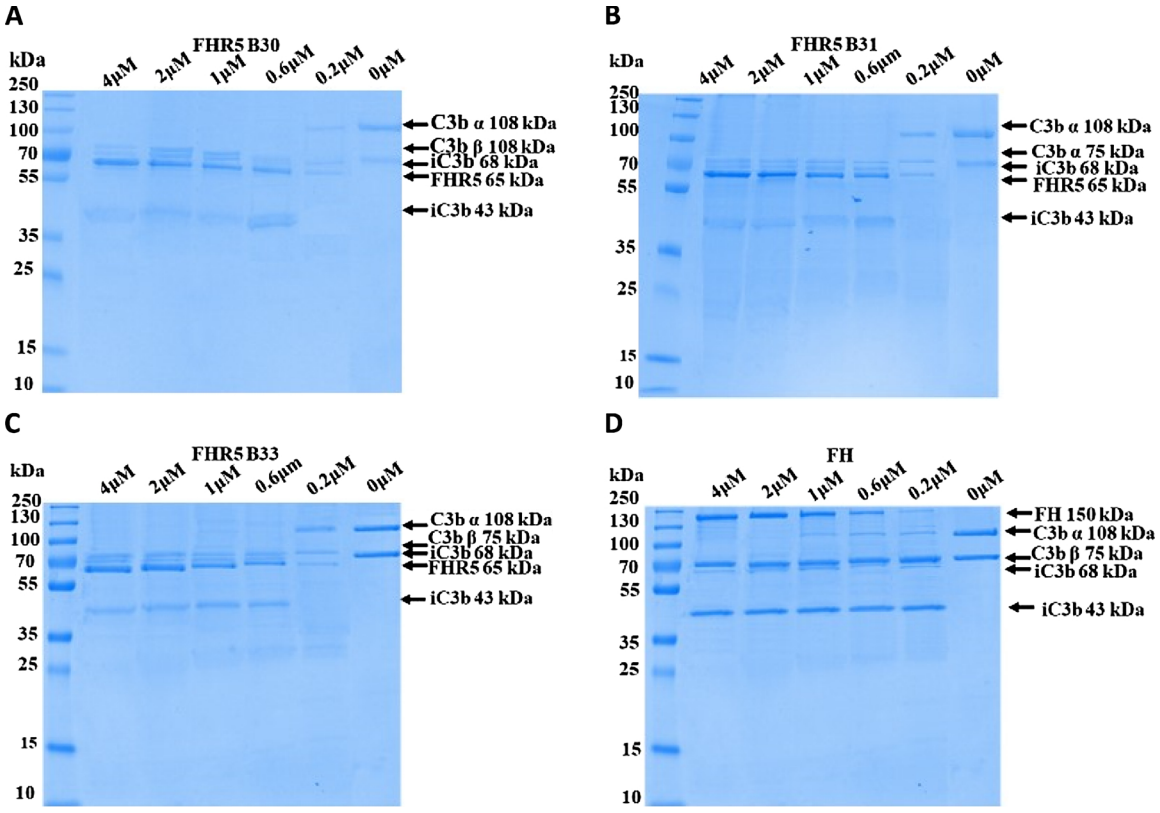

Fig. 5. GBM-FHR5 has factor I-cofactor activity. Varying doses between 0.2–4 μM of GBM-FHR5 were incubated with 3 μg of C3b, 45 ng of Factor I in 10 mM sodium phosphate (pH 6.0) for 10 min at 37 °C. Reactions without GBM-FHR5 or FH and were included as controls. Cleavage of C3b was analysed by SDS-PAGE using Coomassie blue staining.

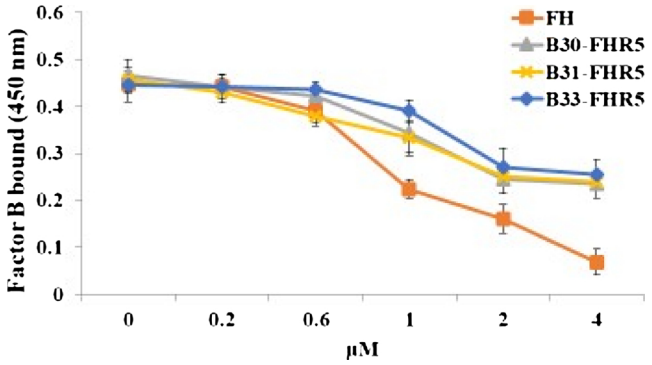

Fig. 6. FHR5 promotes decay acceleration activity on C3 convertase. C3 convertase was generated in microtiter plates with 250 ng of C3b, 400 ng of factor B, 25 ng of factor D, 2 mM nickel chloride, 25 mM NaCl and 4% BSA for 2 h at 37 °C. Varying concentrations of FHR5 (0.2–4 μM) were added and incubated for 30 min at 37 °C. FH and samples without FH and FHR5 were included as controls. Intact C3 convertase was detected by goat anti-human factor B and rabbit anti-goat IgG-HRP antibody. The colour was developed with o-Phenylenediamine. The experiment was repeated in triplicates. Error bars represent standard deviation.

that the implication of FHR5 inhibiting complement activity is to promote tumour survival as tumour cells readily exploit many strategies to overcome immune attack (Jurianz et al., 1999).

Since GBM-FHR5 down-regulated complement lysis of sheep erythrocytes, we wanted to investigate the mechanism through which this inhibitory effect was brought about. Co-factor activity of recombinant FHR5 has been reported (McRae et al., 2005). Therefore, we wanted to study whether this activity was present in GBM-FHR5. GBM-FHR5 was able to act as a co- factor for factor I mediated cleavage of C3b (Fig. 5).

It was clear from SDS-PAGE analysis that the presence of FHR5 had enabled factor I to cleave C3b into inactive iC3b fragments of 68 and 43 kDa. This demonstrated that co-factor activity is a mechanism by which primary GBM-FHR5 can inhibit complement activation.

Human FH displays decay accelerating activity as a mechanism to inhibit activation of the alternative pathway (Brandstatter et al., 2012). We therefore examined the ability of GBM-FHR5 to decay C3 convertase. The inverse correlation depicted on the graph between FHR5 concentration and intact C3 convertase showed that FHR5 was able to decay C3 convertase dose dependently although the activity was less than that of FH (Fig. 6). This was interesting as McRae et al. (2005) were unable to demonstrate recombinant FHR5 decay acceleration activity using the solid phase assay; however, primary GBM-FHR5 were able to show decay acceleration using this assay (McRae et al., 2005).

It is interesting that GBM FHR5 downregulates activation of complement lysis, acts as a co-factor for factor I mediated cleavage of C3b and accelerates the decay of C3 convertase as these functions had been attributed to CCPs 1–4 of FH. FHR5 does not share homology with FH CCP 1-4 which suggests these biological activities may also be associated with CCPs 10–14 or 19–20 of FH as these CCPs share high sequence homology with FHR5.

In summary, our study shows that primary GBM cells B30, B31 and B33 secrete FHR5, which share biological function with human FH. Primary GBM-FHR5 is biologically active and can inhibit complement activation, act as a cofactor for factor I mediated cleavage and degrade C3 convertase in-vitro. These findings warrant additional studies to evaluate the relevance in in-vivo immune surveillance. The elucidation of FHR5 functional activity would potentially help to design efficient complement mediated immunotherapies against GBM.

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