

# Hypothesis: are neoplastic macrophages/microglia present in glioblastoma multiforme?

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## ABSTRACT

Most malignant brain tumours contain various numbers of cells with characteristics of activated or dysmorphic macrophages/microglia. These cells are generally considered part of the tumour stroma and are often described as TAM (tumour-associated macrophages). These types of cells are thought to either enhance or inhibit brain tumour progression. Recent evidence indicates that neoplastic cells with macrophage characteristics are found in numerous metastatic cancers of non-CNS (central nervous system) origin. Evidence is presented here suggesting that sub-populations of cells within human gliomas, specifically GBM (glioblastoma multiforme), are neoplastic macrophages/microglia. These cells are thought to arise following mitochondrial damage in fusion hybrids between neoplastic stem cells and macrophages/microglia.

Key words: fusion, glioblastoma multiforme, glioma, macrophage, microglia, phagocytosis.

## INTRODUCTION

It has recently been suggested that many types of human cancers (lung, breast, colon, kidney, etc.) contain neoplastic cells with mesenchymal/macrophage properties (Huysentruyt and Seyfried, 2010). These neoplastic macrophages are often the most highly invasive and metastatic cells within the tumour. It is not clear if similar kinds of cells are part of the malignant-cell population in human GBM (glioblastoma multiforme). GBM is the most common form of primary brain cancer in adults and represents ~65% of all newly diagnosed malignant gliomas

(Ohgaki and Kleihues, 2005; Stupp et al., 2009). GBM portends an extremely poor outcome with only approx. 10% of patients surviving 5 years after diagnosis, and a median survival of ~12 months (Krex et al., 2007; Preusser et al., 2011). The poor prognosis is due largely to the highly invasive nature of this tumour. GBM invades throughout the brain and often produces multi-centric secondary lesions at sites distant from the primary tumour (Scherer, 1940; Rubinstein, 1972; Laws et al., 1993). Complete surgical resection of GBM is extremely rare. Radiation therapy, which enhances the necrotic microenvironment, often results in further tissue damage and more aggressive tumours (Lakka and Rao, 2008; Kargiotis et al., 2010; Seyfried et al., 2010, 2011). Thus, effective therapeutic options are desperately needed for GBM patients.

GBM is classified as either primary or secondary. Primary GBM arise *de novo* without any prior evidence of a low-grade tumour, whereas secondary GBM arises from malignant progression of a lower grade glioma (Ohgaki and Kleihues, 2009). A defining characteristic of GBM is the 'secondary structures of Scherer' which include diffuse parenchymal invasion, perivascular growth, subpial surface growth and invasion along white matter tracks (Scherer, 1940; Shelton et al., 2010b). While systemic metastasis of GBM is uncommon due to early patient death, GBM can be extremely metastatic especially if the neoplastic cells gain access to extraneural sites (Hoffman and Duffner, 1985; Rubinstein, 1972; Ng et al., 2005; Taha et al., 2005; Frank et al., 2009; Zhen et al., 2010; Gotway et al., 2011). These findings indicate that GBM is not only highly invasive within the CNS (central nervous system), but can also invade outside the CNS.

GBM generally contain multiple morphologically diverse cell types that express neural, glial and myeloid markers (Rubinstein, 1972; Wood and Morantz, 1979; Seyfried, 2001; Yuan et al., 2004; Huysentruyt et al., 2008). In fact, mesenchymal cells with characteristics of TAM (tumour-associated macrophages) and/or microglia can comprise up to

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**Abbreviations:** AIF1, allograft inflammatory factor 1; CNS, central nervous system; GFAP, glial fibrillary acidic protein; GBM, glioblastoma multiforme; IL, interleukin; MDSC, myeloid-derived suppressor cell; MNGC, multinucleated giant cell; PXA, pleomorphic xanthoastrocytoma; ROS, reactive oxygen species; RTG, retrograde; TAM, tumour-associated macrophage; VM, vasculogenic mimicry.

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70% of some GBM (Morantz et al., 1979). It has been difficult to determine with certainty, however, the origin of all the mesenchymal and macrophage-like cells that appear within the GBM (Tso et al., 2006; Ricci-Vitiani et al., 2010). Based on numerous similarities between macrophages and invasive neoplastic cells in non-CNS tumours (Huysentruyt and Seyfried, 2010), we propose that some neoplastic cells within the GBM arise from transformed macrophage/microglia. As cells of the myeloid/macrophage lineage naturally embody the capacity to invade (Mallat et al., 2005; Banaei-Bouchareb et al., 2006), we suggest that some of the highly invasive mesenchymal-type cells within GBM may arise from resident or infiltrating myeloid cells of the tumour stroma that then become neoplastic during disease progression (Huysentruyt and Seyfried, 2010).

Our hypothesis comes from information on the VM (vasculogenic mimicry) mouse model of GBM which consists of highly invasive tumours (Shelton et al., 2010b). The VM tumours arise spontaneously in the brains of inbred VM mice (Fraser, 1971, 1986; Shelton et al., 2010b). The neoplastic cells in the invasive VM brain tumours express multiple properties of macrophages and microglial cells and the VM-M3 and VM-M2 tumours have been established as a model system for human GBM (Huysentruyt et al., 2008; Huysentruyt et al., 2010; Huysentruyt and Seyfried, 2010; Shelton et al., 2010b). We recently reviewed evidence showing that many invasive and metastatic human cancers also express multiple properties of mesenchymal myeloid cells (Huysentruyt and Seyfried, 2010). If many non-neural metastatic tumours arise from mesenchymal myeloid type cells, what about malignant tumours of the CNS? The goal of this commentary is to highlight similarities between myeloid cells and the invasive-cell populations of GBM, and to discuss possible mechanisms by which neoplastic cells could express these properties.

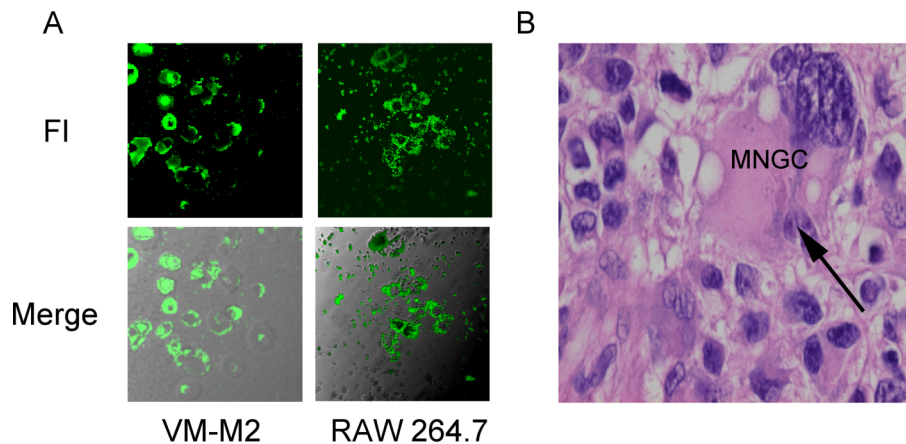
## MYELOID CELLS AND INVASIVE CANCER

Myeloid cells have long been considered the origin of human metastatic cancers; however, this hypothesis has received little attention in the cancer field (Munzarova and Kovarik, 1987; Rachkovsky et al., 1998; Huysentruyt and Seyfried, 2010; Pawelek, 2000; Vignery, 2005; Pawelek and Chakraborty, 2008). Although large numbers of cells with macrophage and myeloid properties are found in most malignant cancers including GBM, these cells are generally considered part of the tumour stroma and not part of the neoplastic population (Seyfried, 2001; Mantovani et al., 2002). These cells are often referred to as TAM, and are thought to facilitate tumour development and malignant progression (Seyfried, 2001; Bingle et al., 2002; Lewis and Pollard, 2006; Talmadge et al., 2007; Pollard, 2008). The tumour stroma is a complex microenvironment that generally comprises malignant tumour cells and host infiltrating cells that include immune and epithelial cells (Bissell and

Hines, 2011). The most commonly accepted view is that tumour-derived chemoattractants signal monocytes to extravasate out of the bloodstream and infiltrate the tumour mass (Bottazzi et al., 1983; Murdoch et al., 2004). Once in the tumour microenvironment, enhanced inflammation and angiogenesis establish the pre-metastatic niche, thus contributing to tumour progression (Seyfried, 2001; Bingle et al., 2002; Lewis and Pollard, 2006; Talmadge et al., 2007; Pollard, 2008). However, little consideration has been given to the possibility that microglial or TAM subsets might actually be part of the neoplastic cell population within GBM (Graeber et al., 2002; Watters et al., 2005). As cells of macrophage/microglial origin can express multiple mesenchymal morphologies depending on the microenvironment, how is it possible to be sure that none of these stromal cells are part of the neoplastic cell population?

Macrophages and microglia are among the most versatile cells of the body with respect to their ability to migrate, to change shape and to secrete growth factors and cytokines (Gordon, 1999; Stossel, 1999; Burke and Lewis, 2002; Huysentruyt et al., 2008). Macrophages fluctuate between two activation states depending on the microenvironment. Macrophages can become classically activated (M1 activation) in response to pathogens and pro-inflammatory molecules, resulting in the generation of NO and ROS (reactive oxygen species) (Sica et al., 2002, 2006; Biswas et al., 2008; Mantovani and Sica, 2010; Qian and Pollard, 2010). In contrast, macrophages can become alternatively activated (M2 activation) in response to anti-inflammatory molecules such as IL-4 (interleukin-4) and IL-10 and are the major wound-healing cells (Gordon, 2003; Biswas et al., 2008). Interestingly, the two macrophage subpopulations play separate roles in tumorigenic processes (Biswas et al., 2008). M1 macrophages, located at sites of chronic inflammation, contribute to neoplastic transformation through the release of cytotoxic and DNA damaging pro-inflammatory molecules (Biswas et al., 2008). Macrophages with an M2 phenotype contribute to angiogenesis and metastasis within the established tumour (Mantovani et al., 2002; Sica et al., 2006; Biswas et al., 2008; Huysentruyt and Seyfried, 2010; Mantovani and Sica, 2010). However, recent evidence suggests that TAMs may be a more complex subtype that can share features of both activation states (Ojalvo et al., 2009, 2010; Qian and Pollard, 2010).

Ineffective anti-tumour immunity resulting from reduced T-cell function is a hallmark of many cancers including GBM (Waziri, 2010; Raychaudhuri et al., 2011). Recently, it has been suggested that another myeloid cell type, MDSCs (myeloid-derived suppressor cells) are partially responsible for GBM-related immune suppression (Raychaudhuri et al., 2011). MDSCs are a heterogeneous population of immature myeloid cells that accumulate in tumour-bearing hosts where they suppress T-cell function, resulting in ineffective anti-tumour immunity (Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg, 2010; Raychaudhuri et al., 2011). This cell type accumulates in the blood, lymph nodes and spleen in patients with solid tumours and has been reported to represent approx. 5% of the total tumour mass in various experimental tumour-model systems



**Figure 1** Phagocytic behaviour of malignant glioma cells  
 (A) Phagocytic behaviour of the VM-M2 and macrophage RAW 264.7 cell lines was assessed from merging (Merge) the fluorescence (FI) images and the differential interference contrast images after feeding the cells fluorescent beads. The image indicated that the malignant VM-M2 cells were phagocytic. (B) MNGC in a human GBM containing engulfed cell debris (arrow). (A) From Huysentruyt et al. (2008), reproduced with permission from Wiley, © 2008 Wiley-Liss, Inc. (B) Provided by A. Persson.

(Yang et al., 2004). Raychaudhuri et al. demonstrated that patients with GBM have increased MDSC counts in their blood when compared with healthy controls and these cells likely promote T-cell immune suppression in this patient population (Raychaudhuri et al., 2011). While the phenotype and function of MDSCs are believed to be distinct from those of TAMs, MDSCs can differentiate into TAMs within the tumour micro-environment thus further contributing to tumour progression (reviewed in Gabrilovich and Nagaraj, 2009). To our knowledge, it has not been demonstrated if MDSCs contribute to GBM tumour mass.

## PHAGOCYTOSIS A DEFINING BEHAVIOUR OF MACROPHAGES AND INVASIVE CELLS

Phagocytosis, involving engulfment and ingestion of extracellular material, is a defining property of macrophages/microglia and of other professional phagocytes (Burke and Lewis, 2002). In order to maintain tissue homeostasis, macrophages phagocytose cellular debris, apoptotic cells and pathogens (Burke and Lewis, 2002). Phagocytosis is also expressed in numerous invasive and metastatic cancers including breast, lung, liver, pancreatic, skin and brain (reviewed in Huysentruyt and Seyfried, 2010). It has been suggested that invasive tumour cells use phagocytosis to facilitate migration through the extracellular matrix and into surrounding tissues (Bjerknes et al., 1987; Coopman et al., 1998).

The phagocytic/cannibalistic behaviour of tumour cells was first described over a century ago when foreign cell bodies were identified within human cancer cells (reviewed in Steinhaus, 1981). These cells were commonly described as

'signet-ring' and 'birds-eye' cells due to the peripheral displacement of their nuclei from engulfed materials (Fais, 2007). Although numerous micro-organisms use cannibalism as a feeding mechanism, cellular cannibalism is an exclusive property of malignant tumour cells (Fais, 2007). Both human and murine cancers have been shown to phagocytose tumour cells, erythrocytes, leucocytes, platelets, apoptotic cells and extracellular particles (reviewed in Huysentruyt and Seyfried, 2010). Interestingly, phagocytic tumour cells are observed in malignant gliomas, especially in GBM (Figure 1) (Youness et al., 1980; Bjerknes et al., 1987; Nitta et al., 1992; Zimmer et al., 1995; Chang et al., 2000; Persson and Englund, 2009; van Landeghem et al., 2009).

We previously identified two spontaneous brain tumours (VM-M2 and VM-M3) in the inbred VM mouse strain that are highly invasive in brain (Huysentruyt et al., 2008; Shelton et al., 2010b). These VM brain tumours express multiple properties of macrophages including phagocytic activity and are a model for human GBM (Huysentruyt et al., 2008; Shelton et al., 2010b). The phagocytic phenotype in these cells is remarkably similar to that of observed in the RAW 264.7 macrophage cell line (Figure 1A) (Huysentruyt et al., 2008). In a similar study to ours that examined the phagocytic activity of four rat glioma cell lines with varying degrees of *in vivo* invasiveness, Bjerknes et al. demonstrated that the invasive glioma cells phagocytosed bacteria, red blood cells, zymosan particles and glia cell fragments (Bjerknes et al., 1987). Furthermore, the two most invasive rat glioma cell lines displayed the highest level of phagocytosis. The authors noted that the phagocytic behaviour correlated with the amount of rat brain destruction during tumour cell invasion, suggesting that phagocytic activity may be connected to the excretion of lysosomal enzymes (Bjerknes et al., 1987). Lysosomal enzyme secretion and phagocytosis are macrophage-specific behaviours. Invasive glioma cell lines that display macrophage/microglia-like behaviours suggest a

myeloid origin. Chang et al. (2000) also demonstrated that the glioma cell lines U87, U251 and SF268 exhibit phagocytic behaviour against apoptotic glioma cells. These findings further demonstrate that invasive glioma cells can exhibit macrophage-like phagocytic behaviour.

Although astrocytes have been described as semi-professional phagocytes, their phagocytic ability is limited. It could be suggested that the phagocytic behaviour observed in the GBM might arise from transformed astrocytes. However, the recent findings of Persson and Englund (2009) suggest that the phagocytic activity observed in human GBM are properties of malignant macrophages/microglia, rather than properties of malignant astrocytes. Utilizing paraffin-embedded GBM material, they identified numerous CD68-positive phagocytic macrophage/microglia throughout brain tumour specimens. Double-immunostaining with CD68 and the tumour cell marker hTERT (human telomerase reverse transcriptase) was used to verify that the CD68-positive cells were in fact part of the malignant GBM cell population (Persson and Englund, 2009). These findings support the hypothesis that neoplastic macrophages/microglia can exist within GBM.

In addition to GBM, other invasive CNS tumours contain neoplastic phagocytic cells. For example, phagocytic cells were observed in the bone marrow of a patient with a highly invasive and metastatic medulloblastoma, which involved invasion and metastasis to brainstem, bone marrow, lymph nodes and spinal cord (Youness et al., 1980). After extensive characterization of the phagocytic cells via histopathology, electron microscopy and cytogenetics, the authors stated that the phagocytic cells were unquestionably metastatic medulloblastoma cells. Interestingly, this tumour metastasized outside the CNS after excision of the primary tumour from the right cerebellar region (Youness et al., 1980). While extracranial metastasis of CNS tumours is uncommon, invasive CNS tumours can metastasize throughout the body if they gain access to extraneural sites (Rubinstein, 1972; Hoffman and Duffner, 1985; Vural et al., 1996; Taha et al., 2005). In fact, several reports show that GBM can be highly metastatic (Hoffman and Duffner, 1985; Ng et al., 2005; Taha et al., 2005; Frank et al., 2009; Zhen et al., 2010; Gotway et al., 2011). It therefore appears that this case of metastatic medulloblastoma exhibited the macrophage/microglia characteristics seen in some GBM.

While it might be difficult to demonstrate a myeloid origin of invasive GBM, the evidence reviewed here suggests that subpopulations of neoplastic GBM cells display the phagocytic behaviour of macrophages/microglia. As microglia are the resident macrophages of the brain, we propose that subpopulations of the malignant GBM cells could arise from microglia/macrophages (Morantz et al., 1979; Seyfried, 2001). Human GBM contain mixtures of numerous neoplastic cell types, many of which have mesenchymal properties, do not express GFAP (glial fibrillary acidic protein) and are of unknown origin (Duffy, 1983; Ohgaki and Kleihues, 2009; Han et al., 2010). Indeed, the original 19th century observations of Virchow (1863/1865) described glioblastomas

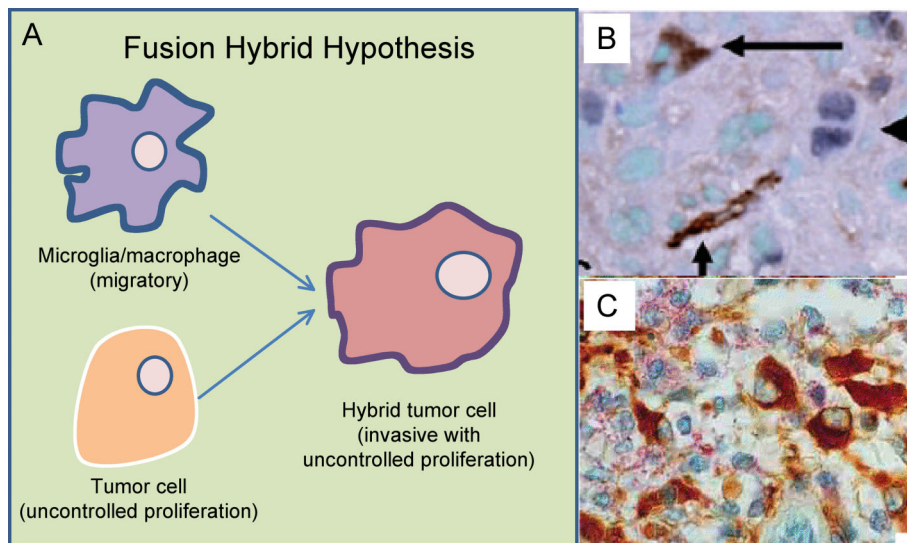
as gliosarcomas of mesenchymal origin (Scherer, 1940; Zagzag et al., 2008). While numerous mesenchymal cells are frequently seen in GBM, the specific classification of all tumour cell types within human GBM remains ambiguous at best (Yates, 1992; Tso et al., 2006; Fan et al., 2007). According to our hypothesis, some of these neoplastic mesenchymal cells could arise from transformed macrophages/microglia.

Novel therapeutic approaches to GBM management become possible if the phagocytic behaviour can be targeted. Gollapudi and co-workers demonstrated that tumour cells undergo apoptosis after 'feeding' tumour cells with anti-tumour agents (Ghoneum and Gollapudi, 2004; Ghoneum et al., 2005, 2007, 2008). They showed that various tumour cells, grown either *in vitro* or *in vivo*, underwent a yeast-induced apoptosis after engulfing *Saccharomyces cerevisiae*. Recent studies indicate that similar approaches could have therapeutic efficacy in GBM. Glioblastoma patients underwent treatment with a magnetic nanoparticle-based immunotherapy where nanoparticles were engulfed by cells within the GBM (van Landeghem et al., 2009). To identify the phagocytic cells within the tumour, these investigators stained post-mortem fixed GBM sections with either the macrophage marker, CD68, or with the astrocyte/CNS stem cell marker, GFAP (van Landeghem et al., 2009). The authors reported that most of the cells engulfing the magnetic nanoparticles were CD68-positive. It was not possible from their study, however, to determine if the CD68-positive cells were also neoplastic since CD68 would stain both transformed macrophages/microglia as well as non-neoplastic TAM.

## FUSOGENICITY

Fusogenicity involves the merging of two distinct plasma membranes and is a defining characteristic of macrophages and microglia (Vignery, 2000; Duelli and Lazebnik, 2003; Huysentruyt and Seyfried, 2010). Cell fusion is a highly regulated process that is essential for fertilization, skeletal muscle and placenta formation (Duelli and Lazebnik, 2003). Outside of normal developmental processes, fusion events are normally restricted to cells of myeloid origin (reviewed in Duelli and Lazebnik, 2003). Macrophages often fuse during osteoclast formation and wound healing in order increase cell volume to facilitate engulfment of large extracellular materials (Vignery, 2000, 2005). Macrophages also undergo heterotypic fusions with numerous cell types. It is currently believed that macrophage heterotypic fusion is a mechanism by which macrophages can 'heal' a defective cell or tissue (Vignery, 2000; Camargo et al., 2004a, 2004b; Vignery, 2005; Powell et al., 2011). There is also considerable evidence demonstrating that macrophages fuse with tumour cells (reviewed in Huysentruyt and Seyfried, 2010). These properties would also be expected in microglia since microglia are the resident macrophages of the CNS.





**Figure 2** **Fusogenic properties of malignant glioma cells**  
 (A) The fusion hybrid hypothesis suggests that a macrophage × tumour-cell hybrid will express genetic and functional traits of both parental cells. (B) A bi-nucleated cell in human GBM expressing the macrophage fusion protein CD98 (arrows). (C) Human PXA demonstrating double immunoreactivity of the glial marker GFAP (brown) and macrophage CD68 (red). (B) From Takeuchi et al. (2008), reproduced with permission from Wiley, © 2007 Japanese Society of Neuropathology. (C) From Matyja et al. (2003), reproduced with permission from Folia Neuropathologica © Termedia.

Nearly a century ago, Aichel first suggested that fusions between somatic cells and leucocytes could result in aneuploidy and malignant hybrids (reviewed in Rachkovsky et al., 1998). Sixty years later, Mekler and Warner proposed that fusions between myeloid cells and tumour cells would produce daughter cells endowed with the invasive properties of the myeloid cell as well as the unlimited proliferative potential of the tumour cell (reviewed in Rachkovsky et al., 1998). The fusion hybrid hypothesis has emerged as a credible alternative to the epithelial mesenchymal transition for the origin of invasive and metastatic cancers (Huysentruyt and Seyfried, 2010; Pawelek and Chakraborty, 2008). Indeed, Pawelek and co-workers showed that fusions between non-metastatic cells and macrophages result in cells with the ability to invade and metastasize (Rachkovsky et al., 1998; Rachkovsky and Pawelek, 1999; Chakraborty et al., 2000, 2001, 2004; Pawelek, 2000, 2005; Handerson et al., 2005; Yilmaz et al., 2005; Pawelek and Chakraborty, 2008). Additionally, they provided evidence demonstrating that these fusion events occur spontaneously both *in vitro* and *in vivo* in a variety of animal models and in human cancers (Rachkovsky et al., 1998; Chakraborty et al., 2000, 2004; Yilmaz et al., 2005). Fusions between tumour cells and macrophage/microglia, with subsequent nuclear fusion, could produce novel phenotypes in the absence of new mutations since the tumour-macrophage hybrids express genetic and functional traits of both parental cells (Figure 2A) (Rachkovsky et al., 1998; Powell et al., 2011). Tumour-macrophage hybrids could also account in part for immune surveillance evasion seen in GBM and other metastatic cancers (Huysentruyt and Seyfried, 2010).

GBM is composed of neoplastic cells with features that vary from small round cells to MNGCs (multinucleated giant cells) (Homma et al., 2006). MNGCs arise from the fusion of two or more macrophages (Vignery, 2000, 2005). Recent reports suggest that MNGCs in GBM could arise through fusions of peripheral macrophages or microglia with glioma cells (Alvarez-Dolado et al., 2003). A recent study demonstrated that CD98, a protein that regulates monocyte fusion events, was expressed on the surface of MNGCs in 15/16 GBMs (Figure 2B) (Takeuchi et al., 2008). These findings suggest that MNGC formation in GBM is similar to what occurs during normal monocyte/macrophage fusion events (Takeuchi et al., 2008). Microglia and bone-marrow derived cells can fuse with CNS cells resulting in MNGCs (Alvarez-Dolado et al., 2003; Ackman et al., 2006). The MNGCs in GBM often express GFAP, a protein expressed on CNS stem cells and astrocytes, suggesting that this cell population contains macrophage/microglia and CNS stem cell/astrocyte hybrids (Takeuchi et al., 2008). Patient prognosis is generally worse when GBMs contain large numbers of MNGCs and other macrophage-like cells when compared with GBMs containing fewer of these cells (Deininger et al., 2003). This situation is similar to that seen in non-CNS tumours in that prognosis is generally worse for malignant tumours with higher rather than lower numbers of macrophages (Leek et al., 1996; Homma et al., 2006; Shabo et al., 2009; Huysentruyt and Seyfried, 2010).

The large size and unique phenotype of MNGCs make them easily detectable in brain tumour biopsy specimens (Figure 1B) (Rubinstein, 1972). However, it can be difficult to detect fusion events among tumour cells and myeloid cells with subsequent nuclear fusions. Utilizing a double immunostaining technique,

**Table 1** Malignant glioma cell expression of myeloid antigens

Myeloid antigen	References
CD98	Takeuchi et al. (2008)
AIF-1 (Iba-1)	Deininger et al. (2000) and Huysentruyt et al. (2008)
CD14	Deininger et al. (2003)
HLA-class II	Rossi et al. (1987) and Matyja et al. (2003)
CD68	Leenstra et al. (1995), Matyja et al. (2003), Strojnik et al. (2006), Huysentruyt et al. (2008) and Strojnik et al. (2009)
CD11b	Leenstra et al. (1995) and Huysentruyt et al. (2008)
HAM56	Leenstra et al. (1995)

Deininger et al. (2000) identified a subset of glioma cells that were positive for both GFAP and AIF1 (allograft inflammatory factor 1), a microglial marker also referred to as Iba1. GFAP+/AIF1+ cells were localized near areas of tumour growth and were indicative of spontaneous *in vivo* fusion events (Deininger et al., 2000). It is well documented that TAM aid tumour progression by promoting angiogenesis, invasion and metastasis (reviewed in Seyfried, 2001; Lewis and Pollard, 2006). However, fusion events between tumour cells and tissue macrophage/microglia would also accelerate tumour progression since resulting daughter cells would inherit the invasive/migratory potential of microglia and unlimited proliferative potential of the tumour cell. Hence, a microglial/glioma cell hybrid could rapidly acquire an invasive phenotype in the absence of novel mutations.

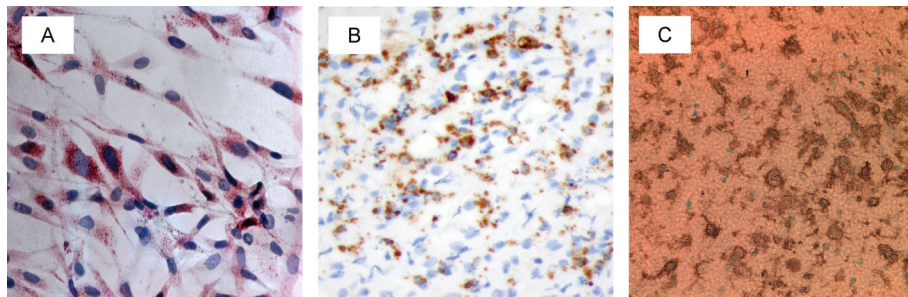
The macrophage-like characteristics and invasive properties of the murine VM-M2 and VM-M3 brain tumours are more similar to human GBM than to most previously described experimental mouse brain tumour models (Shelton et al., 2010b). If fusion hybridization of neoplastic cells with macrophages/microglia could be the origin of invasive cells within human GBM, why are these cell types rarely found in rodent brain tumour transplant models? Most macrophage/microglia seen in chemically induced brain tumours are derived from resident microglia and TAM (Ecsedy et al., 1998). We suggest that the mouse or the rat brain would respond to a tumour implant as if it were an acute infection. This would involve invasion of TAM and activation of local microglia. This is not what usually occurs in the development of a human GBM, where neoplastic transformation is a protracted process. For example, GBM can arise many years after head wound trauma (Gruss et al., 1993; Sabel et al., 1999). It is therefore possible that conditions for macrophage/microglia fusogenicity are greater in the microenvironment of human brain than in the microenvironment of the rodent brain during malignant transformation.

## GLIOMA CELL EXPRESSION OF MYELOID ANTIGENS

Macrophages and microglia express unique antigens that can help identify these cells in various tissues (Roggendorf et al.,

1996; Guillemin and Brew, 2004). A list of some of these antigens is shown in Table 1. Numerous studies have reported high numbers of cells in GBM that express antigens seen only in macrophages/microglia (Rossi et al., 1987; Roggendorf et al., 1996; Leung et al., 1997; Badie and Schartner, 2000; Graeber et al., 2002). As mentioned above, most cells within GBM that express macrophage antigens are generally considered host-infiltrating TAM, and are not considered part of the neoplastic tumour cell population (Roggendorf et al., 1996; Seyfried, 2001). However, several reports show that macrophage/microglial antigens are expressed on neoplastic cells within GBM (Figure 3) (Rossi et al., 1987; Leenstra et al., 1995; Graeber et al., 2002; Deininger et al., 2003; Strojnik et al., 2006). These findings in GBM are consistent with findings reported for other human cancer types including breast, lung, skin, ovarian, pancreatic, rectal and renal (Rossi et al., 1987; Leenstra et al., 1995; Deininger et al., 2003; Matyja et al., 2003; Strojnik et al., 2006, 2009; Colman et al., 2010) and reviewed in Huysentruyt and Seyfried (2010). The expression of macrophage antigens in neoplastic cell subpopulations within GBM would support our hypothesis that malignant microglia are a component of human GBM.

Deininger et al. (2003) utilized double labelling experiments to demonstrate that neoplastic cells located in regions of infiltrative glioma growth were positive for both GFAP and the macrophage marker CD14. It is currently thought that TAM, localized at the leading edge of the tumour, facilitate local tumour cell invasion (Lewis and Pollard, 2006). However, the invading cells were CD14+/GFAP+ suggesting that these cells were part of the invading tumour cell population and not TAM. It is possible that these cells arise through fusion of tumour cells with macrophages/microglia, thus representing a neoplastic hybrid-cell population. In contrast to the Deininger et al.'s study (Deininger et al., 2003), most studies of macrophages in brain tumours have not used double labelling to distinguish neoplastic cells from non-neoplastic cells (Rossi et al., 1987; Shinonaga et al., 1988; Morimura et al., 1990; van Landeghem et al., 2009). Consequently, it is often difficult to determine with certainty the origin of all cell types that express macrophage/microglia antigens in GBM. However, the human glioblastoma cell line U138 expressed the macrophage marker Ki-M1P (CD68) in an *in vitro* environment where there were no contaminating TAMs (Figure 3A) (Paulus et al., 1992). This finding is similar to what we showed for the murine VM-M2 and VM-M3 cell lines (Huysentruyt et al., 2008). A few investigators



**Figure 3 Malignant glioma cells express macrophage-specific antigens**  
 (A) Expression of the macrophage marker CD68 (Ki-M1P) in the human GBM cell line U138 ( $\times 475$ ). (B) CD68 expression in most tumour cells of a human GBM ( $\times 400$ ). (C) Expression of the macrophage/microglia marker Iba1 in tumour cells of the VM-M2 tumour, a mouse model of human GBM ( $\times 630$ ). (A) From Paulus et al. (1992), with kind permission from Springer Science+Business Media: Acta Neuropathologica, Ki-MIP as a marker for microglia and brain macrophages in routinely processed human tissues, 84, 1992, 538–544, W Paulus, W Roggendorf and T Kirchner, © Springer-Verlag, 1992. (B) From Strojnik et al. (2009), reproduced with permission from International Institute of Cancer Research. (C) From Huysentruyt et al. (2008), reproduced with permission from Wiley, © 2008 Wiley-Liss, Inc.

have entertained the possibility that some macrophage/microglial-type cells in GBM might be part of the neoplastic cell population. Several previous studies show, however, that subpopulations of neoplastic macrophage-like cells are present in most human cancers (reviewed in Huysentruyt and Seyfried, 2010). It would therefore be important to consider the possibility that some GBM cell subpopulations, previously identified as TAM, are in fact neoplastic cells.

Like GBM, PXA (pleomorphic xanthoastrocytoma) contains cells of multiple morphologies (Matyja et al., 2003). As in GBM, MNGC are also a characteristic cell type in PXA (Matyja et al., 2003). In an attempt to further characterize this cell subtype, Matyja et al. (2003) evaluated the co-expression of glial and macrophage markers in the various tumour cell populations in eight cases of PXA. The authors identified a population of neoplastic cells that expressed GFAP and either HLA-class II or CD68 within the cytoplasm (Matyja et al., 2003). Many of these neoplastic cells were large and lipid-laden suggesting a mesenchymal origin. According to our hypothesis, neoplastic microglia/macrophages could give rise to PXA following fusion hybridizations.

Expression of macrophage markers has been reported in high-grade gliomas including GBM (Rossi et al., 1987; Leenstra et al., 1995; Strojnik et al., 2006). Leenstra et al. (1995) examined macrophage characteristics in six malignant glioma cell lines. All the cell lines examined were classified as malignant gliomas based upon DNA flow cytometry and GFAP expression. Interestingly, all six glioma lines co-expressed macrophage markers and GFAP. In contrast, macrophage antigens were not found in cultured astrocytes isolated from healthy brain tissue, suggesting that the macrophage markers expressed by malignant astrocytes were not artefacts of an *in vitro* culture environment (Leenstra et al., 1995). In a separate study, immunohistochemical analysis revealed that 40% of grade III astrocytomas and GBM tumour specimens contained neoplastic cells with macrophage markers (Rossi et al., 1987). The U87 GBM model has also been shown to express both CD68 (Figure 3B) and glia markers when grown either *in vitro* or *in vivo* (Strojnik

et al., 2006). Strojnik et al. (2009) evaluated the prognostic significance of CD68 expression in malignant human gliomas. It was clear from their study that the presence of high levels of CD68-positive tumour cells was predictive of reduced survival. CD68 was expressed by both microglia and tumour cells (Strojnik et al., 2009). The macrophage/microglia marker, Iba1, is also expressed by the VM-M2 mouse GBM tumour cells (Figure 3C) (Huysentruyt et al., 2008). Taken together, these findings support our hypothesis and indicate that some neoplastic GBM cells could be of macrophage/microglial origin.

## NUMEROUS HUMAN CANCERS EXPRESS MACROPHAGE PROPERTIES

Evidence presented above suggests that subpopulations of the invasive GBM tumour cells include neoplastic cells with multiple characteristics of macrophages/microglia. Recent studies show that macrophage properties and antigens are expressed in the neoplastic cells of numerous human metastatic cancers including bladder, brain, breast, carcinoma of unknown primary, endometrial, fibrosarcoma, gall bladder, liver, lung, lymphoma, melanoma, multiple myeloma, ovarian, pancreatic, rectal, rhabdomyosarcoma and renal cancers (reviewed in Huysentruyt and Seyfried, 2010). Commonly, these macrophage characteristics are observed in the invasive and metastatic cell populations, further highlighting the possibility that the highly-invasive tumour cells may be of mesenchymal origin. Indeed, these observations led to our recent prediction that the metastatic cells in most human cancers are of myeloid origin (Huysentruyt and Seyfried, 2010). As microglia are mesenchymal cells of myeloid origin, we suggest that neoplastic microglia can represent an invasive cell population in GBM. This shared property among numerous invasive/metastatic tumours could have broad therapeutic implications. For example,

any therapy that is able to reduce invasion and metastasis of one tumour type would likely be effective in treating tumours with macrophage characteristics.

## POSSIBLE MECHANISMS

How could resident microglia or TAM become part of the neoplastic cell population in GBM? We recently reviewed evidence indicating that all cancer, regardless of tissue or cellular origin, is primarily a disease of impaired cellular energy metabolism (Seyfried and Shelton, 2010). Otto Warburg first proposed that all types of cancers arise from irreversible damage to cellular respiration (Warburg, 1931, 1956). Persistent injury to oxidative phosphorylation will require compensatory non-oxidative energy metabolism to maintain viability (Seyfried and Shelton, 2010; Seyfried et al., 2010; Shelton et al., 2010a). The mitochondrial stress response or RTG (retrograde) signalling from the mitochondria to the nucleus is required to up-regulate the oncogenes needed to sustain glycolysis and non-oxidative mitochondrial energy production (Seyfried and Shelton, 2010; Seyfried et al., 2010). However, persistent RTG activation leads to eventual nuclear genomic instability and other recognized hallmarks of cancer (Butow and Avadhani, 2004; Singh et al., 2005; Seyfried and Shelton, 2010). Mitochondria energy production is often damaged as a consequence of mutagens, hypoxia, inflammation, ROS or inherited mutations (Kiebish et al., 2008). We recently showed that mitochondria isolated from murine gliomas contain numerous lipid defects supporting previous findings that respiratory energy production is dysfunctional in tumour mitochondria (Kiebish et al., 2008, 2009; Ordys et al., 2010; Seyfried and Shelton, 2010). Macrophages/microglia home to mitochondria-damaging environments in response to inflammation, infection, wound repair and tumorigenesis (Giulian et al., 1989; Chettibi, 1999; Bingle et al., 2002; Graeber et al., 2002; Lewis and Murdoch, 2005; Martin and Leibovich, 2005; Lewis and Pollard, 2006). Inflammation and hypoxia in the tumour's microenvironment can damage macrophage mitochondria (Frost et al., 2005; Navarro and Boveris, 2005). It is therefore possible that mitochondria could become dysfunctional when macrophages/microglia respond to various chronic tissue injuries, resulting in a persistent RTG response with eventual malignant transformation (Seyfried and Shelton, 2010). Hence, we suggest that some invasive GBM cells could arise from resident tissue microglia or TAM that have suffered mitochondrial damage during tumour initiation or progression.

Focal hypoxia combined with persistent inflammation and stem cell proliferation could produce macrophage-microglia or macrophage-stem cell fusions. Respiratory damage could either proceed or follow fusion events thus initiating the path to frank neoplasia. Additionally, damaged mitochondria could also be passed from one cell to another via cytoplasmic inheritance during cellular-fusion events (Seyfried and Shelton, 2010). Although normal mitochondria are known

to suppress tumorigenesis in fusion hybrids (Seyfried and Shelton, 2010), persistent inflammation in the tumour microenvironment could contribute to continued mitochondrial damage in the fused hybrids. This would enhance the Warburg effect, unbridled proliferation and genomic instability. As microglia naturally embody the ability to migrate throughout the brain (Graeber and Streit, 1990; Amat et al., 1996), we suggest that transformed cells of macrophage/microglia origin would possess invasive potential.

Currently, the cause of macrophage and tumour cell hybridization is unknown. Various stages of the macrophage response to tumour development could increase the probability for cell-fusion events. The host immune system treats tumours as unhealed wounds (Dvorak, 1986; Seyfried, 2001; Bissell and Hines, 2011). Macrophages home to hypoxic tumour areas in the process of wound healing. The failure of macrophages to completely digest apoptotic cells could result in macrophage × tumour hybrid cells (Pawelek, 2000). It is therefore possible that macrophages fuse with tumour cells in an attempt to 'heal' the tumour, as macrophages are known to fuse with non-myeloid cells during tissue repair. Radiation is commonly used as a primary therapy for most brain tumours. However, radiation therapy enhances the incidence of fusion hybrid formation and could result in a more aggressive and invasive tumours (Shabo et al., 2009; Seyfried et al., 2011). This could account in part for the exacerbating effects of radiation therapy on GBM progression (Seyfried et al., 2010).

The macrophage fusion hybrid hypothesis could potentially explain many of the distinguishing characteristics seen in invasive and metastatic GBM tumour cells. These macrophage-reprogramming strategies may account for some of the cellular heterogeneity seen in human GBM since macrophage × CNS cell hybrids would likely retain the histology of the CNS fusion partner. Tumour hybrids could also account for the cancer cell aneuploidy and chromosomal abnormalities often seen in tumour cells (Steinhaus, 1981; Pawelek, 2000). Further studies will be needed to determine the extent to which fusion hybridization might contribute to the invasive properties seen in GBM.

## CONCLUDING REMARKS

GBM is a highly complex and lethal tumour. Unfortunately, the current therapies available to GBM patients are largely ineffective and often have a negative impact on the patient's quality of life. In this commentary, we aim to highlight the possibility that the most aggressive and invasive cells in human GBMs are neoplastic macrophages/microglia. While further experimentation is needed to confirm the gene expression and immunohistochemical data reviewed here, we believe that novel therapeutic approaches could be developed targeting the macrophage characteristics of invasive GBM cells. It would be of particular interest to determine if the CD68+ cell population of human GBM has neoplastic properties.



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