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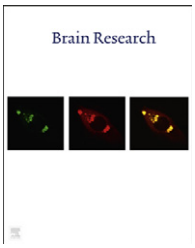

Research Report

Stabilin-1 expression in tumor associated macrophages

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ABSTRACT

Glioblastoma multiforme is a very aggressive and common form of brain tumor. Current therapies consist of a combination of surgical removal, chemotherapy and radiation therapy. These drastic treatments still leave a current prognosis of median survival of less than 1 year. Lack of effectiveness of these treatments has left researchers looking for alternative forms of treatment. A significant alternative currently being investigated is the use of the immune system to potentially target and eliminate tumor cells directly. Stabilin-1, a scavenger receptor expressed by macrophages, has the potential in inhibiting tumor growth by binding and internalizing secreted protein acidic and rich in cysteine (SPARC). SPARC is known to be upregulated in the tumor microenvironment and is involved in extracellular matrix remodeling, cell proliferation and migration. Decreasing SPARC expression using siRNA has been shown to decrease tumor invasiveness and survival. We investigated the phenotype of stabilin-1 expressing immune cells in the tumor environment and demonstrated a transient population of alternatively activated macrophages expressing stabilin-1 in the tumor environment and the disappearance of that population as the tumor progresses.

1. Introduction

Glioblastoma multiforme is the most common and aggressive form of CNS tumors. Though current therapies including surgical removal, chemotherapy and radiation seem to extend the survival of the affected patients, the current prognosis still has a median survival of less than 1 year. Surgical removal and radiation therapy crudely target large regions of the brain while chemotherapy targets all dividing cells of the body, thus these methods tend to leave the patient with considerable side effects (Sughrue et al., 2009). Moreover, the lack of effectiveness of these treatments has left researchers looking for alternative forms of treatment. One significant alternative is the use of the immune system, with its high specificity, it has the potential to target and eliminate the tumor cells directly; though knowledge of the immune environment within and around gliomas is lacking. Furthermore, the markers unique to glioma cells and the extent of immunosuppression in the glioma milieu remain largely unknown.

Matricellular proteins are secreted into the extracellular space and interact with cell surface receptors, proteases and structural proteins such as collagen (Bornstein and Sage, 2002). Secreted protein acidic and rich in cysteine, osteonectin (SPARC) is a matricellular protein which promotes cell migration as it facilitates an intermediate stage of adhesion as opposed to the strong adhesion of most matricellular proteins (Bornstein and Sage, 2002). The role of SPARC in cell

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migration makes it an important factor during normal development, wound healing and tissue remodeling (Workman and Sage, 2011; Framson and Sage, 2004; Brekken and Sage, 2001). SPARC is upregulated by glioma cells, and is associated with increased tumor metastasis and proliferation (Golembieski et al., 1999; Schultz et al., 2002; Rich et al., 2003; Schittenhelm et al., 2006). This may be partially due to the increase of several proteases, which leads to tissue degradation and allows room for infiltrative tumor cells to migrate (Golembieski et al., 2008). Targeting and decreasing SPARC expression with siRNA has proven to decrease tumor invasiveness and tumor cell survival (Seno et al., 2009; Shi et al., 2007).

Recently, the scavenger receptor stabilin-1 was identified as the first known receptor for SPARC. When bound, SPARC is internalized by stabilin-1 and rendered to the endosomal pathway where it is subsequently degraded (Kzhyshkowska, 2006). Stabilin-1 is expressed on the surface of alternatively activated macrophages (AAMØ) which participate in wound healing and in the anti-inflammatory process (Kzhyshkowska et al., 2004; Park et al., 2009; Palani et al., 2011; Mosser and Edwards, 2008). Although SPARC is known to exist in the tumor environment, the presence or location of its receptors are not yet known. The capacity of stabilin-1 expressing macrophages to clear SPARC has the potential to be significant in the control of glioma growth and proliferation.

Macrophages display a wide spectrum of activation states which are generalized as either classically activated (M1) or alternatively activated (M2) (Mosser and Edwards, 2008). In contrast to the microbicidal, inflammatory classically activated macrophages; M2 macrophages are anti-inflammatory and are involved in tissue repair through upregulation of CXCR3, IL-10, arginase-1, the mannose receptor MMR and the scavenger receptor stabilin-1 (Mosser and Edwards, 2008; Kzhyshkowska, 2006; Kzhyshkowska et al., 2004). During glioblastomas, a population of macrophages associates with the tumor: tumor associated macrophages (TAMs). This population of macrophages comprises a significant proportion of the tumor, accounting for up to 50% of the tumor mass (Solinas et al., 2009). The phenotype of TAMs is not yet well defined, though it is generally accepted that TAMs are primarily M2 activated. Furthermore, TAMs display pro-tumor functions: promoting tumor cell survival, proliferation and metastasis (Mantovani et al., 2002; Luo et al., 2006; Solinas et al., 2009; Gordon and Martinez, 2010; Vasievich and Huang, 2011; Zhang et al., 2011). Indeed, high levels of TAMs are often associated with a worse prognosis (Bingle et al., 2002; Zhang et al., 2011).

In this study, we investigated the phenotype of tumor associated macrophages in an attempt to clarify their activation status and whether or not they expressed stabilin-1. Here we demonstrate a significant increase of total lymphocyte and macrophage infiltration in the glioma injected hemisphere (GL) 2 weeks post-injection, a two fold increase in stabilin-1 and MMR expression compared to the naïve hemisphere at day 7 post-injection and reduction of stabilin-1 expression in the glioma hemisphere by day 14 post-injection.

These data demonstrate the presence of stabilin-1 at the site of initial glioma growth but then a downregulation of stabilin-1 as the tumor progresses. It is well known that tumors can cause phenotypic changes in infiltrating immune cells and thus may be responsible for the change in stabilin-1 expression. Increasing this receptor expression on infiltrating macrophages may help in the control of glioma growth.

2. Results

2.1. *Scavenger receptor stabilin-1 is expressed in early glioma*

A model of glioma was used in this study where GL-26 cells were injected intra-cranially and allowed to grow for either 7, 14 or 21 days (Fig. 1A, B). Injection of 90,000 GL-26 cells in the caudate putamen (CPu) leads to a stable tumor which initially grows in the needle tract before expanding in the border between the striatum and the external capsule (ec). By day 14, the needle tract is mostly closed and the tumor expands from the dorsal portion of the caudate putamen to the exterior of the cortex (Fig. 1A) and by day 21, the tumor has expanded to both hemispheres (Fig. 1B).

To determine if stabilin-1 is present in the tumor environment, expression of stabilin-1 was measured using RT-qPCR at day 7 post-injection in the right striatum. Furthermore, as stabilin-1 has been shown to be predominantly expressed on AAMØ, expression of MMR was also measured to test if AAMØ were present in our tumor model. Both stabilin-1 and MMR transcripts are up-regulated 1.912 ± 0.6391 and 1.881 ± 0.5023 times (Fig. 1C) respectively in the injected hemisphere (GL) when compared to the non-injected hemisphere (NI).

At day 14, although high levels of MMR are maintained on the injected hemisphere, the expression of stabilin-1 was reduced (Fig. 1C). Indeed, at day 14 post-glioma injection, MMR expression increased 3.265 ± 1.423 fold higher in the injected hemisphere whereas stabilin-1 expression was reduced to equal levels in both hemispheres (1.031 ± 0.4456 fold higher in the injected hemisphere compared to non-injected hemisphere).

Immunofluorescent staining at day 7 post-injection for stabilin-1 and tomato lectin at the site of the tumor reveals both membrane and cytoplasmic stabilin-1 within the tumor tissue (Fig. 1D). Tomato lectin is known to stain for macrophages, blood vessels and microglia. These images suggest a small population of infiltrating macrophages which express stabilin-1 within gliomas.

These data demonstrate the presence of a growing glioma at days 7 and 14 post-injection which is associated with increased MMR expression and early (day 7) stabilin-1 upregulation in the injected hemisphere.

2.2. *Stabilin-1 expression on macrophages decreases with tumor growth*

To determine the cell types which express stabilin-1 in the tumor environment, infiltrating macrophages, lymphocytes and local microglial populations were analyzed at days 7 and 14 post-glioma injection by flow cytometry. At day 7 post-injection, there were no differences in total mononuclear cell