主論文の要旨

Significance of low mTORC1 activity in defining the characteristics of brain tumor stem cells

（脳腫瘍幹細胞の特性定義における低 mTORC1 活性の意義）

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<Background>

Many studies have investigated the role of brain tumor stem cells (BTSCs) in the progression of glioma. BTSCs can be isolated from gliomas by methods analogous to those described for the isolation of neural stem cells in developing mouse brains. BTSC markers including CD133, Sox2, Nanog, Nestin, and Oct4 enable tracking the presence of BTSCs in tissues, although they are variable depending on individual BTSC clones, tumor type, and in vitro culture conditions. A hallmark of BTSCs is their ability to undergo changes in intracellular metabolism and gene expression through multiple mechanisms. The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that forms a large protein complex named mTOR complex 1 (mTORC1). mTORC1 is activated downstream of oncogenic pathways as well as by nutrients. However, despite the fact that several inhibitors targeting the mTORC1 pathway are being developed with many showing promising antitumor activities in animal tumor models, most of them showed limited efficacy in the treatment of cancer patients. Also, controversy remains regarding the significance of the mTORC1 pathway in the maintenance of CSCs.

To the best of our knowledge, the significance of mTORC1 in the maintenance of BTSCs in gliomas has not been reported thus far. In this study, we investigated mTORC1 activity and its kinetics in response to nutrient deprivation and re-feeding in 3 BTSC clones isolated from glioblastoma patients, and compared these results with those from non-BTSCs, which were termed here as DIFFs. We also tested the effects of mTORC1 inhibition on the proliferation of BTSCs and DIFFs.

<Materials and methods>

Three independent BTSC clones were isolated from glioblastoma tissues of patients who underwent surgery at the Nagoya University Hospital after obtaining informed consent. Freshly dissociated tumor cells were incubated with BTSC medium comprising Neurobasal Medium and the B-27 and N-2 supplements (Invitrogen), supplemented with human recombinant basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (20 ng/ml each; R&D Systems). The differentiation of BTSCs was induced by culturing them in high-glucose Dulbecco's Modified Eagle’s Medium (DMEM) (Wako) containing 10% fetal bovine serum (FBS). DIFFs cultured for 10 generations were used in all experiments. qPCR analysis was performed using an ABI7300 thermal cycler and THUNDERBIRD SYBR qPCR Mix (Toyobo), according to the manufacturers’ instructions. All tumor samples (5 cases each for glioblastoma, anaplastic astrocytoma, and diffuse astrocytoma samples) were obtained at the time of surgery, after patients gave informed consent.

<Results>

All BTSCs examined exhibited a sphere-like morphology while replacement of the

complete growth medium with DMEM containing 10% FBS and subsequent culture for 48 hours induced the differentiation of BTSCs into DIFFs (Fig. 1A). Western blot, qPCR results showed that the expression of BTSC markers such as Sox2, CD44v9, and Oct4 was downregulated, while that of differentiation markers such as GFAP, OSP, and β3-tubulin was upregulated following differentiation (Fig. 1B, C). We found that mTORC1 activity in BTSCs was significantly lower than that found in DIFFs across all tested clones in their respective complete growth media (Fig. 1D).

To test whether the low activity of mTORC1 in BTSCs is achieved intrinsically or extrinsically, we starved the BTSCs and DIFFs of nutrients by replacing the complete growth medium with DMEM lacking serum, amino acids, and glucose (Fig. 2A). The data implied that BTSCs might have an intrinsic mechanism to suppress mTORC1 activity. Moreover, mTORC1 reactivation following re-feeding with DMEM supplemented only with EGF (Fig. 2B) after 3 hours starvation was much more modest in BTSCs than in DIFFs.

To examine the biological significance, we tested the effect of the mTORC1-specific inhibitor rapamycin between BTSCs and DIFFs. We found that rapamycin treatment did not affect the sphere-formation efficiency of all BTSC clones (Fig. 3A), which is consistent with results showing no apparent effect of rapamycin on BTSC proliferation (Fig. 3C). Considering that BTSCs comprise heterogeneous populations and the possibility that their proliferation depends on intercellular communications, we also examined the sphere-formation efficiency from multiple (200) dissociated cells (Fig. 3B) with no effect of rapamycin on sphere formation. In contrast, the proliferation of DIFFs was significantly dependent on mTORC1 activity (Fig. 3D), as observed in many types of cancer cell lines.

We next examined the effect of mTORC1 inhibition on the differentiation of BTSCs. Adding rapamycin to the differentiation medium attenuated complete differentiation of BTSCs to DIFFs, as shown by the remaining expression of stem cell markers Sox2, CD133, and Nestin, as well as downregulation of β3-tubulin and GFAP after inducing differentiation (Fig. 4).

BTSCs and DIFFs with temozolomide treatment revealed higher viability in BTSCs than DIFFs (Fig. 5A, B).

Finally, we examined the correlation between mTORC1 activity and BTSC markers by immunohistochemical staining of human glioma tissues. Heterogeneity in Sox2 and phospho-S6 production (Fig. 6A) was observed. We confirmed our findings by further staining glioma specimens including diffuse astrocytoma, anaplastic astrocytoma, and glioblastoma (Fig. 6B, C) with double staining for Sox2 and phospho-S6 [diaminobenzidine (DAB, brown) and alkaline phosphatase (AP, red)]. The data showed that no apparent cross reactivity occurred between either double-staining procedure (Fig. 6B), which was confirmed by the reverse combination of secondary antibodies (Fig. 6C). The data showed an almost complete inverse correlation between Sox2 expression and S6 phosphorylation. Consistent with the data found with cultured BTSCs, most Sox2-positive cells were negative for phospho-S6,

supporting the view that BTSCs exhibit low mTORC1 activity even in tumor tissues (Fig. 6D). Staining with another BTSC marker CD44v9, also resulted in a low correlation between BTSC marker expression and mTORC1 activation in tumor cells (Fig. 6F). Intriguingly, frequency of Sox2/CD44v9-negative and phospho-S6-positive tumor cell populations, but not Sox2/CD44v9-positive and phospho-S6-negative BTSC populations, correlated with the grade of glioma (Fig. 6E).

<Discussion>

In this study, we showed that BTSCs exhibited low basal mTORC1 activity and different kinetics during activation and deactivation from DIFFs, in an in vitro culture system. BTSCs showed less dependency on mTORC1 activity for their proliferation than did DIFFs at least in in vitro conditions, although the significance of the data in the context of human glioma has remained unproven at present. Nonetheless, it was interesting to observe an inverse correlation between the expression of BTSC markers and mTORC1 activation in tumor cells of human glioma tissues. Although preliminary, we believe that these data shed new insight into the different metabolic states between BTSCs and DIFFs and provide clues for the development of novel strategies that specifically target BTSCs (Fig. 5C).

One issue that needs to be addressed is that our present findings seem to contradict previous reports in CSCs in other types of cancers. The high activity of mTORC1 in CSCs appears to be compatible with the concept of a perivascular niche for CSCs that is rich in nutrients and growth factors that help them proliferate. In contrast, data from another study showed that the forced activation of mTORC1 in leukemic stem cells led to their aberrant differentiation and depletion, suggesting that mTORC1 activity may be kept low during the maintenance of CSCs. An attractive hypothesis that could explain these clinical data and our present findings is that some CSCs reside in nutrient-poor (hypoxic or necrotic) niches, where oxygen and nutrient supplies obtained by diffusion from nearby vessels are depleted by the growing cancer cells, and show low mTORC1 activity and resistance to mTORC1-targeted or conventional therapies (Fig. 5C).

<Conclusion>

In this study, we detected lower mTORC1 levels in BTSCs than in DIFFs in both cultured cell and human tumor samples and inhibition of mTORC1 will not effect the stemness of BTSCs.

Abbreviations: BTSCs, brain tumor stem cells; DIFF, differentiated BTSC; mTORC1, mammalian target of rapamycin complex 1.