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研究課題名（和文）Investigation of the therapeutic interaction of mesenchymal stem cells with glioblastoma stem-like cells and its biological mechanism

研究課題名（英文）Investigation of the therapeutic interaction of mesenchymal stem cells with glioblastoma stem-like cells and its biological mechanism

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研究成果の概要（和文）：本研究は、膠芽腫(GBM)に対する間葉系幹細胞(MSCs)の作用を、GBM幹細胞(SCs)に対する効果およびテモゾロミド(TMZ)とGSK3β阻害剤との併用効果に着目して検討した。MSCsとGBM-SCsを共培養すると、GBM幹細胞形質が抑制された。GSK3β阻害剤は脂肪組織由来MSCsの患者由来GBM-SCsに対する治療的な細胞間相互作用を高めた。また、TMZとMSCの併用によるGBM治療効果を増強し、GBMマウスモデルの生存期間を延長した。以上の結果より、MSCsとGSK3β阻害剤の併用は有用なGBM治療法と考えられた。

研究成果の概要（英文）：We investigated whether mesenchymal stem cells (MSCs) transplantation benefits to treatment of glioblastoma (GBM) by enhancing anti-tumor effect in combination with GSK3β inhibitors and temozolomide (TMZ). We showed that MSCs co-cultured with GBM cells participate in regulation of GBM stem-like cell (SC) morphology. We examined the interaction between patient-derived GBM-SCs and adipose tissue-derived MSCs under GSK3β inhibition and TMZ and its influence on GBM-SCs. We found that combined treatment by GSK3β inhibitor enhanced therapeutic interaction of MSCs with GBM-SCs, and that combination of low-dose AR-A014418, TMZ and MSC treatment significantly reduced cell viability and showed anti-tumor effect against patient-derived GBM-SCs. Transplantation of MSCs and GSK3β inhibition improved survival in mouse GBM model.

研究分野：　Neuro-oncology

キーワード：　glioblastoma　temozolomide　mesenchymal stem cells　GSK3β

１．研究開始当初の背景

Glioblastoma (GBM) is the most frequent malignant tumor of the brain and is highly unresponsive to the currently available anticancer treatments. The proliferative and invasive activity of GBM [1] hinders curable surgical intervention and makes GBM highly resistant to radiation and chemotherapy [2] with median patient survival showing little improvement over the past 30 years [3]. Consequently, there is a need to develop new treatment modalities represented by molecular target-directed therapies.

Glycogen synthase kinase 3β (GSK3β) is a multi-functional protein kinase that regulates various cellular pathways depending on its substrates for phosphorylation. We have recently demonstrated that GSK3β promotes GBM cell survival and proliferation [4, 5]. Previously it was reported that GSK3β attenuates pluripotency in normal stem cells [6]. Therefore, investigating mechanisms underlying potential tumor promoting role of GSK3β provides new insights into molecular pathways leading to progression of GBM as well as development of novel strategies for GBM treatment. Based on our laboratory studies, the clinical trial of GSK3β inhibitors for GBM treatment (UMIN000005111) has proven their efficacy against the recurrent GBM patients [7].

Mesenchymal stem cells (MSCs) are a mixed population of adult stem cells that are capable for self-renewal and differentiation to generate bone, cartilage, adipose and fibrous connective tissue. MSCs are capable of neurogenic trans-differentiation, giving rise to neurons, astrocytes, or oligodendrocytes [8]. MSCs migrate toward GBM cells *in vitro*, suppress their growth, inhibit angiogenesis and decrease migration of GBM cells [9-12]. GSK3β inhibition stimulates proliferation and prevents differentiation of MSCs; which is opposite to the effects observed in GBM cells [5, 13, 14].

２．研究の目的

Our research group has demonstrated GSK3β as a promising therapeutic target in human GBM. It has been shown that MSCs exert therapeutic effect against GBM and that GSK3β negatively regulates stemness of MSCs. Based on these knowledge, this study attempted to explore whether transplantation of MSCs in combination with GSK3β inhibition and an alkylating agent temozolomide (TMZ) benefits to treatment of GBM and to investigate underlying biological mechanism. This approach provides scientific basis for development of a novel, combined cell-based and molecular target-directed therapy for GBM.

３．研究の方法

(1) Isolation and characterization of MSCs

For *in vitro* study, we isolated GBM stem-like cell culture from patients' GBM tumors, MSCs from human adipose tissues and C57BL mice bone marrow, and primary neural stem cell cultures from C57BL mice. To separate MSCs from other cell types, the differential velocity adherent culture method was used. When replacing the culture medium after 48h, the culture dish was shaken gently, then the culture medium containing the red cells was removed. Isolated MSCs were cultured according to our previously developed method by using growth factor supplemented media in hermetically closed vial of a cylindrical shape with modifications [15]. Expression of markers that serves as a quality control: CD14, CD29, CD31, CD34, CD44, CD45, CD90, and CD105 in these cells were examined by flow cytometry to verify MSC identity of the respective cells.

(2) Effect of MSCs on GBM cells and underlying molecular mechanism

GBM stem-like cells and MSCs were labeled with a fluorescent linker to provide fate mapping of the cells in consequent co-culture experiments and after transplantation to GBM mouse model. We then examined effects of MSCs, GSK3β inhibition, TMZ and various combination of them against GBM stem-like cells by cell proliferation assay, isobologram method[5], median dose-effect analysis[5], time-lapse microscopy and by measurement of O^6-methylguanine DNA methyltransferase (MGMT) promoter methylation and gene expression. MGMT promoter methylation is a favorable prognostic factor in patients with GBM, implicated in chemosensitivity to TMZ [16]. Previously I reported that the molecular mechanism underlying combined effect of GSK3β inhibition and TMZ against GBM was GSK3β inhibition-induced decrease in MGMT expression via c-Myc-mediated MGMT promoter methylation [5]. To address our working hypothesis of interaction between GBM stem-like cells and MSCs via GSK3β-mediated signaling, we investigate (a) c-Myc, MGMT expression and its promoter methylation; and (b) c-Myc and DNA (cytosine-5)-methyltransferase (DNMT) 3A binding to MGMT promoter according to our previous study [5].

(3) Animal model study

We examined effects and underlying mechanism of transplantation into brain parenchyma of fluorescence-labeled MSCs against GBM in C57BL mice bearing mouse GBM cells generated by using GBM line EPNT-5 (Institute for Cytology, Saint-Petersburg). This cell line was induced in C57BL mouse by 7,12-dimethyl-benzanthracene and represents human GBM. We observed survival as well as consequence of neurological state and cognitive functions by open field analysis in mice following transplantation of the respective cells. We evaluated morphology and cellular interaction

phenomena in GBM locus in this GBM model by conventional and luminescent microscopy of brain cryosections, respectively. I have investigated the effects of co-culture with mice MSCs on mice EPNT-5 GBM cells by fluorescence microscopy.

４．研究成果

(1) Isolation and characterization of MSCs

Isolated MSCs were cultured. Before their utilization for experiments, flow cytometry for a set of cell surface markers that serves as a quality control was performed to verify MSC identity. Flow cytometry results for expression of CD14, CD29, CD31, CD34, CD44, CD45, CD90, and CD105 confirmed MSC identity of the isolated cells (Figure 1).

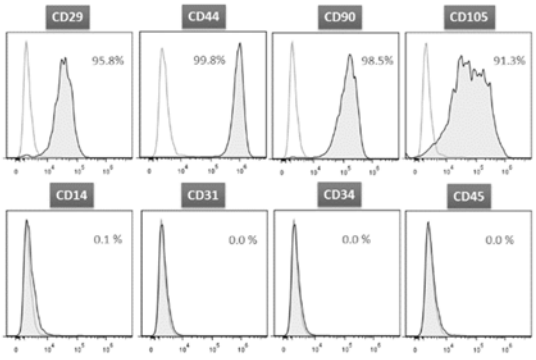

Figure 1. Detection of the expression of cell surface markers in human MSCs by flow cytometry. Cells were stained with respective antibodies. Note that isolated human MSCs are positive for CD29, CD44, CD90 and CD105 while they are negative for CD14, CD31, CD34 and CD45.

(2) Effect of MSCs on GBM cells and underlying molecular mechanism

Our experiments showed that MSCs co-cultured with GBM cells participated in regulation of GBM stem-like cell morphology and behavior.

We then examined the interaction between patient-derived GBM stem-like cells and adipose tissue-derived MSCs in the presence and absence of GSK3β inhibition and TMZ and its influence on GBM stemness phenotype.

Isobologram analysis was performed to evaluate whether low-dose AR-A014418 and MSC treatment potentiates TMZ effect against GBM cells. The combination of low-dose AR-A014418, TMZ and MSC treatment significantly reduced GBM cell viability.

(3) Mouse model study

Our experiments showed that transplantation of MSCs and GSK3β inhibition enhances the anti-tumor effect for treatment of experimental GBM. Survival analysis data were supported by histologic analysis data of brain samples from respective animals with orthotopic EPTN-5 GBM model.

These observations suggesting implications of GSK3β signaling in regulation of MSC/GBM interactions encouraged us to continue investigation of the biological mechanisms by which MSCs regulate GBM stemness phenotype under the control by GSK3β. The results of the study could be used for the development of novel methods for glioblastoma treatment by GSK3β inhibitor.

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５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計１件）

1. Domoto T, **Pyko IV**, Furuta T, Miyashita K, Uehara M, Shimasaki T, Nakada M, Minamoto T. Glycogen synthase kinase-3β is a pivotal mediator of cancer invasion and resistance to therapy Cancer Sci 107: 1363-72, 2016 (peer-reviewed).

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5. Domoto T, **Pyko IV**, Uehara M, Bolidong D, Minamoto T. Aberrant glycogen synthase kinase (GSK)3β participates in tumor-promoting autophagy in colorectal cancer proliferation. The 9th International Conference on the International Society of Gastroenterological Carcinogenesis November 17~18, 2017 Kumamoto, Japan.
6. **Pyko IV**, Domoto T, Nakada M, Minamoto T. Sensitizing patient-derived glioblastoma stem-like cells to temozolomide by glycogen synthase kinase 3β inhibition. The 75th Annual Meeting of the Japanese Cancer Association. October 06~08, 2017, Yokohama, Japan.

〔図書〕（計　０件）

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〔その他〕
ホームページ等

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