EDITORIAL

Ethylnitrosourea-induced gliomas: a song in the attic?

Yuji Ikeno[a, *]

[a] *Barshop Institute for Longevity and Aging Studies and Department of Pathology, The University of Texas Health Science Center at San Antonio; Geriatric Research Education and Clinical Centers (GRECC), Audie L. Murphy VA Hospital (STVHCS), San Antonio, TX 78229, USA.*

Abstract

It is essential to seek the underlying molecular mechanisms of glioma development, and critical to discover interventions that reduce the incidence and attenuate the growth of gliomas using a well-established *in vivo* experimental model because glioma is clinically one of the most difficult malignant tumors to treat. Ethylnitrosourea (ENU)-induced glioma in the rat has been extensively utilized as an experimental brain tumor model since the mid-1960s, however, the scientific value of ENU-induced glioma has been underappreciated mainly due to the recent development of transgenic mouse glioma models. Because of the pathophysiological characteristics, which are similar to the high grade human malignant gliomas, ENU-induced glioma is an excellent *in vivo* model to: a) examine the cell origin, development, and pathophysiology of gliomas; b) investigate anti-tumor effects of calorie restriction (CR) and its underlying mechanisms; and c) discover new preventive and/or therapeutic interventions of glioma. Further exploration of genetic changes during initiation, malignant transformation of glial cells, and progression of glioma as well as CR's anti-tumor effects on cellular processes using cutting edge technology, *e.g.*, spatial transcriptomics, could provide more insight and a deeper understanding of the pathophysiology of gliomas.

Keywords: Ethylnitrosourea, glioma, gliogenesis, calorie restriction, spatial transcriptomics

Ethylnitrosourea (ENU)-induced glioma

Glioma is not a common malignant tumor compared to other types of cancers. However, it is clinically one of the most difficult malignant tumors to treat because of its extensive infiltrative nature to surrounding central nervous system (CNS) parenchyma, which makes complete surgical resection extremely challenging, and their resistance to chemotherapy and other treatments. If it is not treated, the survival for glioblastoma (most malignant glioma) patients after diagnosis is approximately 6-9 months, and even with treatment, glioma is one of the malignant tumors that has a poor prognosis. Therefore, it is essential to seek the underlying molecular mechanisms of glioma development, and it is critical to also discover interventions that reduce the incidence and attenuate the growth of gliomas using a well-established *in vivo* experimental model.

N-ethyl-N-nitrosourea (ENU)-induced glioma in the rat has been extensively utilized as an experimental brain tumor model since the mid-1960s [1-5]. ENU is an alkylating agent and a highly potent mutagen. When a single dose of ENU (50 mg/kg body weight) is intravenously injected to pregnant rats on day 15 of gestation, all offspring from ENU injected pregnant rats develop glioma. The continuous profile of tumor development of ENU-induced glioma has been well characterized which makes the ENU-induced glioma an excellent *in vivo* model to seek the mechanisms of glioma development and examine the effects of interventions such as calorie restriction (CR). ENU-induced glioma starts as hyperplasia, characterized by a few or several abnormal cells forming a cluster with no destructive nature. At 10-12 weeks of age, a cluster of abnormal cells shows further growth (larger than that for hyperplasia, but less than 500 μm in diameter) with higher cell density and a mild destructive nature to surrounding CNS parenchyma, and progress as a microtumor (< 1 mm), medium sized (1-2 mm), and gross tumor (2 mm <) with age. Histologically, a gross tumor is classified into two types: a) oligodendroglioma, which shows isomorphic proliferation of small round cells similar to a human

oligodendroglioma; and b) anaplastic glioma showing cellular atypism and pleomorphism, and structural change such as necrosis, hemorrhage, and endothelial proliferation. Specific cell type and origin of glioma induced by ENU is still controversial because of the existence of glial fibrillary acidic protein (GFAP) positive cells within the tumor, which has a cellular morphology that resembles an oligodendrocyte. However, most investigators agree that major neoplastic cells are immature glial cells committed to an oligodendrocyte lineage [2-5] and maintain GFAP expression potential [2, 3, 6].

Effects of CR on ENU-induced glioma development

Since the original discovery by McCay and colleagues, CR has become well known for anti-aging effects [7-9]. Anti-aging actions of CR is correlated to a reduction in tumor incidence and growth. CR's anti-tumor effects have been further demonstrated using several experimental model systems, including spontaneous lymphomas in p53-deficient mice [10], breast cancer in DBA mice [11], spontaneous tumors in Fischer 344 (F344) rats [12], transplantation of cultured cells or tumors [13], and induced carcinogenesis [14-19]. However, previous studies have been unable to fully uncover the exact underlying mechanisms of the anti-tumor effects of CR. In addition, some of the experimental models previously used have several weaknesses as follows: a) as the incidence of each spontaneous tumor is not high (15-20%), a large number of experimental animals are required to examine the particular tumor or organ; b) the time course of tumor onset and growth during the lifespan of the animal is not established in spontaneous tumors; c) the interactions among mutation, oncogenes and transformation is not established in all of the spontaneous and induced tumors; and d) carcinogen challenge or tumor cell transplantation after CR induction could be due to the difference in sensitivity against the carcinogen and transplanted tumor cells, and normal cell proliferation activities between CR and *ad libitum* (AL) animals.

To further test the effects of CR on tumor development and seek the possible underlying mechanisms of anti-tumor actions of CR, our laboratory has utilized the ENU-induced glioma in rats [20]. The development of tumors, especially chemically induced tumors, is considered a multistage process that can be divided into three distinct stages, *i.e.*, initiation, promotion, and progression [21]. In the ENU-induced glioma, initiation of tumors is controlled by the injection of ENU at day 15 of gestation, but monitoring the incidence and growth of tumors over time can provide insight into the effects of CR on promotion and progression. The advantages of this model are its extremely high rate (100%) of tumor induction and the certainty of the occurrence of multiple tumors per brain. Additionally, the continuous profile and time course of tumor progression in this experimental model have been well documented as described above. Thus, this model is

an ideal *in vivo* model to critically evaluate whether CR attenuates tumor incidence and/or growth as well as to seek underlying mechanisms to attenuate glioma development *in vivo*.

Our results showed that the number of gliomas did not change with age in the AL groups; however, the average size of the gliomas was significantly larger in the old group compared to that of the younger groups. Immuno-histochemical analysis showed increased accumulation of lipid peroxidation products, oxidized protein, glycated end products, heme oxygenase-1 (HO-1), and thioredoxin 1 (Trx1) with the growth of gliomas. The CR group reduced both number and size of gliomas, and tumors exhibited less accumulation of oxidative damage, decreased formation of glycated end products, and a decreased presence of HO-1 and Trx1 compared to the AL group. Gliomas of the CR group also showed less proliferating cell nuclear antigen (PCNA) positive and more single-stranded DNA (ssDNA) positive cells which are correlated to the suppressed tumor growth. Furthermore, the anti-tumor effects of CR were associated with decreased hypoxia inducible factor-1 alpha (HIF-1α) levels in normal brain tissue. Our results demonstrated the anti-tumor effects of CR in gliomas, which were accompanied by reduced accumulation of oxidative damage, decreased formation of glycated end products, decreased presence of HO-1 and Trx1, reduced cell proliferation, increased apoptosis, and decreased levels of HIF-1α [20].

ENU-induced glioma meets modern technology: in search of mechanisms

In spite of much endeavor, the exact mechanism of malignant transformation and multi-step carcinogenesis process of ENU-induced glioma has not been fully uncovered. This is most likely due to technical limitations to follow the target cells of ENU during the carcinogenesis and accompanied molecular/biochemical changes.

Recent advancement of transcriptome experiments provides researchers with a very powerful tool to explore molecular signatures that play important roles in tumor development/progression. In particular, spatial transcriptome analysis provides extremely important information for cancer research because this technique can map the analytes, *e.g.*, RNA data from their spatial localization on tissue sections, which allows us to further analyze the molecular changes associated with malignant transformation and cancer progression [22].

As described above, development of ENU-induced glioma started as hyperplasia followed by sequential progress of early neoplastic proliferation (ENP), microtumor, medium sized and gross tumor (oligodendroglioma and anaplastic glioma). Using this cutting edge technology, *i.e.*, spatial transcriptomics, we will be able to obtain the molecular signatures that allow us to trace the transformed glial cells and discover the gene expression changes that play important roles in each step of ENU-induced glioma development (from malignant transformation of glial cells

to glioma progression) and also determine the responsible molecular changes by CR's anti-tumor actions.

Conclusion

The scientific value of ENU-induced glioma has been underappreciated and underutilized mainly due to the recent development of transgenic mouse glioma models and the clinical importance of glioblastoma, which is the most common malignant glioma in humans. Although the histopathological features of ENU-induced glioma are different from the human malignant glioma, *i.e.*, the glioblastoma, some of the pathophysiological characteristics, *e.g.*, proliferative activity, invasiveness, vascularization, and blood-brain barrier disturbances, are similar to the high grade human malignant gliomas. Therefore, ENU-induced glioma is an excellent in vivo model to: a) examine the cell origin, development, and pathophysiology of gliomas; b) investigate anti-tumor effects of CR and its underlying mechanisms; and c) discover the new preventive and/or therapeutic interventions of glioma. Further exploration of genetic changes during initiation, malignant transformation of glia, and progression of glioma as well as CR's anti-tumor effects on cellular processes using cutting edge technology, *e.g.*, spatial transcriptomics, could provide more insight and a deeper understanding of the pathophysiology of gliomas. More importantly, results from the further study of ENU-induced glioma could be clinically significant for developing a new intervention to attenuate the occurrence and growth of gliomas in humans.

Declarations

Conflicts of interest: Yuji Ikeno is a member of the Editorial Board of *Aging Pathobiology and Therapeutics*. All authors declare no conflicts of interest and were not involved in the journal's review or decision of this manuscript.

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Availability of data and materials: Not applicable.

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