NEOPLASIA
www.neoplasia.com

Volume 19 Number xx Month 2017 pp. 519–529 519

The Cell Cycle Inhibitors p21 Cip1 and p27 Kip1 Control Proliferation but Enhance DNA Damage Resistance of Glioma Stem Cells

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Abstract

High-grade gliomas are the most prevalent and lethal primary brain tumors. They display a hierarchical arrangement with a population of self-renewing and highly tumorigenic cells called cancer stem cells. These cells are thought to be responsible for tumor recurrence, which make them main candidates for targeted therapies. Unbridled cell cycle progression may explain the selective sensitivity of some cancer cells to treatments. The members of the Cip/Kip family $p21^{Cip1}$ and $p27^{Kip1}$ were initially considered as tumor suppressors based on their ability to block proliferation. However, they are currently looked at as proteins with dual roles in cancer: one as tumor suppressor and the other as oncogene. Therefore, the aim of this study was to determine the functions of these cell cycle inhibitors in five patient-derived glioma stem cell–enriched cell lines. We found that these proteins are functional in glioma stem cells. They negatively regulate cell cycle progression both in unstressed conditions and in response to genotoxic stress. In addition, $p27^{Kip1}$ is upregulated in nutrient-restricted and differentiating cells, suggesting that this Cip/Kip is a mediator of antimitogenic signals in glioma cells. Importantly, the lack of these proteins impairs cell cycle halt in response to genotoxic agents, rendering cells more vulnerable to DNA damage. For these reasons, these proteins may operate both as tumor suppressors, limiting cell proliferation, and as oncogenes, conferring cell resistance to DNA damage. Thus, deepening our knowledge on the biological functions of these Cip/Kips may shed light on how some cancer cells develop drug resistance.

Neoplasia (2017) 19, 519–529

Introduction

Gliomas are the most common type of primary brain neoplasms, accounting for approximately 30% of central nervous system tumors and 80% of all malignant brain tumors. Within this group, glioblastoma multiforme, a high-grade glioma (World Health Organization grade IV glioma) [1,2], is characterized by elevated intratumoral heterogeneity, diffuse infiltration throughout the brain parenchyma, and resistance to traditional therapies, which inevitably leads to tumor recurrence and the demise of the patient [3]. The high rate of cancer relapse suggests that current therapies do not eradicate all malignant cells. In this regard, a

Abbreviations: CSC, cancer stem cell; DDR, DNA damage response; GSC-ECLs, glioma stem cell–enriched cell line; NR, nutrient restriction.

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Received 18 January 2017; Revised 28 March 2017; Accepted 3 April 2017

1476-5586
http://dx.doi.org/10.1016/j.neo.2017.04.001

subpopulation of tumor cells called cancer stem cells (CSCs) has been identified in gliomas and in many other cancers. These cells are characterized by their capacity of self-renewal and by their enriched tumorigenic potential [4–6]. Furthermore, CSCs are also known for their ability to differentiate into both rapidly proliferating progenitor-like tumor cells and more differentiated tumor cells that define the histological features of the tumor entity [7]. Importantly, CSCs appear to be more resistant towards radio- and chemotherapy than the highly proliferative progenitors that coexist within the tumor. To effectively eradicate CSCs and thus avoid cancer recurrence, it is critical to target their essential functions. Increased resistance of glioblastoma multiforme cells to radiotherapy was suggested to be due to the DNA damage response (DDR), which is preferentially activated in CSCs as compared to non-CSC counterparts [8–10].

The DDR is a network of signaling pathways that is able to sense and repair DNA lesions. The activation of DDR also modulates other cellular processes, including cell cycle checkpoint regulation and programmed cell death. DDR induces cell cycle arrest to allow repair of DNA lesions; however, if too much damage has been sustained, DDR triggers cell death to avoid the generation of deleterious mutations [11]. The progression along the cell cycle requires the activation of different cyclin-dependent kinases (CDKs). Tight CDK regulation involves CDK inhibitors (CKIs) which ensure the correct timing of CDK activation in different phases of the cell cycle. The CKIs include the INK4 family and the Cip/Kip family. In mammals, the Cip/Kip family consists of three proteins, p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}. As a critical negative regulator of the cell cycle, p21^{Cip1} binds to and inhibits both CDK/cyclin complexes and PCNA [12,13]. Therefore, based on its capacity to block cell proliferation, p21^{Cip1} may act as a tumor suppressor [14,15]. However, evidence has revealed novel functions of p21^{Cip1}, such as the control of cell migration, regulation of apoptosis, and maintenance of stem cell pools, among others [13,16–18]. In fact, p21^{Cip1} can acquire an antiapoptotic gain of function in the cytoplasm, pointing to a dual role for p21^{Cip1} as both a tumor suppressor and an oncogene [19,20]. Also, p21^{Cip1} induction is essential for the onset of cell cycle arrest in DDR, giving cells time to repair critical damage [21]. Despite the absence of p21^{Cip1} in some cancer types, its overexpression or cytoplasmic localization correlates with poor prognosis in malignant tumors of the skin, pancreas, breast, prostate, ovary, cervix, and brain [18]. Similarly, p27^{Kip1} may act as a tumor suppressor through inactivation of cyclin/CDK complexes in the nucleus, but tumorigenic properties of p27^{Kip1} have also been proposed, especially when located in cytoplasm [20,22]. Cytosolic p27^{Kip1} promotes cell proliferation via interaction with cyclin D/CDK4 [23] and cell migration via inhibition of RhoA/ROCK signaling [24]. In many cancers, p27^{Kip1} expression is reduced in the nucleus and exhibits different degrees of cytoplasmic localization [25]. High nuclear and low cytoplasmic expression of p27^{Kip1} has been associated with a better prognosis in high-grade astrocytoma [26]. Moreover, an inverse correlation between p27^{Kip1} immunoreactivity and the Ki-67 labeling index was observed in patients with malignant gliomas [27].

Although p21^{Cip1} and p27^{Kip1} were initially considered as tumor suppressors, it rapidly became clear that the situation was not so simple. It appears that the loss of the regulatory mechanisms governing Cip/Kip proteins may lead to the specific loss of its tumor suppressor function while maintaining the oncogenic ones, favoring cancer development. In this context, we wondered whether p21^{Cip1} and p27^{Kip1}function as tumor suppressors or oncogenes in patient-derived glioma stem cell–enriched cell lines (GSC-ECLs) [28]. To address this issue, we investigated the role of p21^{Cip1} and p27^{Kip1} in the response of GSC-ECLs to camptothecin (CPT), a potent and specific inhibitor of eukaryotic DNA topoisomerase I that induces DNA double-strand breaks. Initially, we examined the expression pattern of p21^{Cip1} and p27^{Kip1} in five GSC-ECLs. After CPT exposure, we observed a marked increase in the expression levels of these Cip/Kips, which displayed a predominant nuclear localization. Finally, by small interfering RNA (siRNA)–mediated downregulation of both p21^{Cip1} and p27^{Kip1}, we determined that these CKIs confer protection against CPT in a cell line–dependent manner. This protection may be due, at least in part, to the ability of these inhibitors to halt cell cycle progression. Therefore, the significance of cell cycle regulators in the pathobiology of these tumors is of interest, and the roles of p21^{Cip1} and p27^{Kip1} need to be elucidated further.

Materials and Methods

Culture of Human Glioma–Derived Cells and Treatments

Brain tumor–derived cultures were isolated from biopsies and established as previously described [28]. GSC-ECLs were cultured in serum-free medium consisting of neurobasal medium supplemented with B27, N2, 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 2 mM L-glutamine, 2 mM nonessential amino acids, 50 U/ml penicillin/streptomycin (all from Invitrogen, Carlsbad, CA), 20 μg/ml bovine pancreas insulin, and 75 μg/ml low-endotoxin bovine serum albumin (Sigma, St. Louis, MO) and plated onto Geltrex-coated plates (10 μg/ml) (Invitrogen, Carlsbad, CA). Cells were routinely grown to confluence, dissociated using Accutase, and then split 1:2 to 1:3. Medium was replaced every 2 to 3 days. To induce differentiation, cells were cultured for 14 days in the same medium without bFGF and EGF.

To induce genotoxic stress, cells were incubated in 1 μM CPT (Sigma, St. Louis, MO). To generate nutritional stress, cells were incubated in neurobasal medium without supplements.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. cDNA was synthesized from 500 ng of total RNA using MMLV reverse transcriptase (Promega, Madison, WI). Quantitative PCR studies were carried out using SYBR Green-ER™ qPCR SuperMix UDG (Invitrogen, Carlsbad, CA). Primers used were the following: p21^{Cip1} forward 5′-ATGACAGATTTCTAC CACTC-3′, reverse 5′-AAGACACACAAACTGAGAC-3′; p27^{Kip1} forward 5′-GGCTAACTCTGAGGACAC-3′, reverse 5′-TTCTTCT GTTCTGTTGGC-3′; and RPL7 forward 5′-AATGGCGAGGA TGGCAAG-3′, reverse 5′-TGACGAAGGCGAAGAAGC-3′. All samples were analyzed using an ABI PRISM 7500 Sequence Detector System (Applied Biosystems, Foster City, CA) and were normalized to RPL7 gene expression.

Immunostaining and Fluorescence Microscopy

Cells were analyzed for *in situ* immunofluorescence. Briefly, cells were rinsed with phosphate-buffered saline (PBS) and fixed in PBS with 4% formaldehyde for 25 minutes. After two washes with PBS with 0.1% bovine serum albumin (PBSA), cells were permeabilized with 0.1% Triton X-100 in PBSA with 10% normal goat serum for 30 minutes, washed twice, and stained with the corresponding primary antibodies. Fluorescent secondary antibodies were used to localize the antigen/primary antibody complexes. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and examined under a Nikon