Laboratory Investigation

In vitro and in vivo growth inhibition of human malignant astrocytoma cells by the farnesyltransferase inhibitor B1620

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Summary

p21-Ras, the protein product of the proto-oncogene Ras is overactivated in malignant astrocytomas despite the absence of mutation. It is known that p21-Ras participates in signaling events from membrane tyrosine kinase receptors and a variety of intracellular biochemical pathways to downstream targets. Signal transduction inhibition by targeting against Ras is now thought to be a promising therapeutic strategy for malignant astrocytomas. This study demonstrates that Ras pathway inactivation by a farnesyltransferase inhibitor, B1620, effectively inhibits in vitro and in vivo growth of human astrocytoma cells, although normal human astrocytes (NHA) derived from fetal brain are resistant to B1620. Anti-proliferative effect of B1620 on in vitro growth of astrocytoma cells was examined by MTT assays and soft agar colony formation assay. B1620 inhibited anchorage-dependent growth of six astrocytoma cell lines with a median effective dose (IC50) ranging from 2.0 to 20.7 \( \mu \text{M} \). However, growth of NHA was not significantly affected by B1620 even at the concentration of 100 \( \mu \text{M} \). All astrocytoma cells showed apoptotic figures after Hoechst 33258 staining, when treated for 5 days at each IC50 concentration against B1620. Anchorage-independent growth of these astrocytoma cell lines was inhibited at a much lower concentration than that of anchorage-dependent growth. Daily treatment of U87 xenograft-bearing athymic mice with B1620 at 100 or 50 mg kg\(^{-1}\) resulted in significant inhibition of tumor growth. A histological study of the B1620-treated tumor tissue showed decreased vascularity with numerous TUNEL-positive apoptotic cells. These results suggest that the mechanism of the growth-inhibitory effect of B1620 is anti-angiogenesis, apoptosis induction and reversion of the transformed phenotype. The potential clinical use of B1620 could be expanded to malignant astrocytomas.

Introduction

Overexpression and activation of receptor tyrosine kinases (RTKs), such as platelet-derived growth factor receptors (PDGFRs) and epidermal growth factor receptor (EGFR) leads to proliferation of human malignant astrocytoma cells [1–3]. EGFR is amplified and often rearranged to a constitutively active form in a significant percentage of glioblastomas [4]. The proto-oncogenes of the Ras family encode the Ras proteins (p21-Ras), which act as important signal transducers downstream of these growth factor RTKs [5,6]. The biological effect of p21-Ras is to induce nuclear transcription via a signaling pathway sequentially involving Raf, MAP kinase kinase (MEK), and mitogen-activated protein kinase (MAPK) [7,8]. p21-Ras comprises a family of GTP-dependent proteins, which exist in an inactive Ras-GDP form or in activated Ras-GTP form. The downstream signals beneath Ras are attenuated when Ras-GTP returns to Ras-GDP, by guanosine triphosphatase (GTPase)-activating proteins, including neurofibromin [9]. p21-Ras requires several post-translational reactions to acquire full biological activity. The first step is farnesylation of a cysteine residue located in the carboxy-terminal tetrapeptide or CAAX motif. Farnesylation causes translocation of Ras to the cell membrane, which is required for full biological activity. Farnesyltransferase inhibitors (FTIs) were developed to inhibit Ras function by inhibiting the first
step in its post-translational modification [10–13]. The strategy targeting Ras using FTIs was initially used specifically to inhibit the function of H-Ras that had been activated by mutation, and FTIs clearly showed an anti-proliferative effect against H-Ras-mutated tumors as expected [12–14]. However, subsequent research has revealed that the strategy may be useful in tumors that have not only N-Ras and K-Ras mutations, but also wild-type Ras [15–18]. Therefore, Ras mutation status in human cancer cell lines appeared not to be predictive of FTI sensitivity, and Ras dependence seems to be more important in FTI sensitivity [9]. Ras is overactivated in malignant astrocytomas despite the absence of mutation [18,19]. Aberrant Ras activation of malignant astrocytomas may be due to an abnormality of the upstream components to Ras, such as aberrant RTKs [2,3,19]. FTIs is now expected to be effective against malignant astrocytomas containing activated RTK signaling pathways [18,19]. In the present study, the authors present experimental data that a methylester prodrug, B1620, effectively inhibits in vitro and in vivo growth of human malignant astrocytoma cells but not normal human astrocytes (NHA).

**Materials and methods**

**Cell culture**

Human malignant astrocytoma cell lines TM-1, TM-31, A172, U87, U251, T98G and normal human astrocyte cell strain (NHA) were used in these experiments. TM-1 and TM-31 were derived from our laboratory [20,21]. TM-31 cells were derived from an NF1 patient. NHA was obtained from Clonetics (Walkersville, MD). Other cell lines were a generous gift from the Riken Gene Bank (Tsukuba, Japan). These cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS).

**Farnesyltransferase inhibitor**

A farnesyltransferase inhibitor, B1620, was provided by Eisai (a gift from Dr. Kentaro Yoshimatsu, Tsukuba, Japan). This drug is a new methylester prodrug, which specifically inhibits post-translational farnesylation of Ras protein (Figure 1). The acid form of B1620 is a potent inhibitor of farnesyltransferase activity in vitro and the concentration needed to inhibit in vitro H-Ras farnesylation by 50% is in the nanomolar range (IC$_{50}$ = 20 nM, unpublished observation). In a whole cell system using H-Ras transformed NIH 3T3 fibroblasts, B1620 inhibited H-Ras post-translational processing by 50% at 39 nM (unpublished observations).

**Anchorage-dependent growth**

An in vitro growth assay of astrocytoma cell lines and NHA against B1620 was carried out using a colorimetric MTT assay as described before [22]. Cells were seeded in 96-well microplates at an initial density of 2500 cell per well in 100 µl of medium without B1620. After incubation for 24 h, the medium was replaced by fresh medium with various concentrations of B1620. The cells were treated for 6 days with B1620. A longer exposure time for B1620 was adopted, because B1620 is a cytostatic drug and usual 4-day treatment of cells showed little cytotoxic effect in a preliminary MTT assay. Then, 50 µl of PBS containing 5 mg ml$^{-1}$ of MTT was added to the culture medium and it was incubated for 4 h. Thereafter, the medium was gently removed and 100 µl of dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO) was added to each well to solubilize a formazan precipitate. Following agitation, the absorbance of each well was measured at a test wavelength of 550 nm.

**Immunoblotting of p21-Ras**

To determine whether the inhibition of astrocytoma cell growth was in fact due to inhibition of p21-Ras processing, Ras localization was determined for both the B1620-treated TM-31 cells and control TM-31 cells, as described before [15]. Exponentially growing TM-31 cells were treated with B1620 at the concentration of IC$_{50}$ (20 µM) for 4 days. The cells were washed with cold