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Toxicity and delivery methods for the linamarase/linamarin/glucose oxidase system, when used against human glioma tumors implanted in the brain of nude rats

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ABSTRACT

Glioblastoma multiforme (GBM) is one of the deadliest forms of cancer, with an average survival time of approximately 1 year despite aggressive surgery, radiotherapy and chemotherapy. Here, we report a preclinical study by which the two main energy pathways of the tumor cells, oxidative phosphorylation and aerobic glycolysis, are simultaneously disrupted. The therapy is based on a plant gene encoding a β-glucosidase, linamarase (*lis*), which react with the substrate linamarin (lin) producing cyanide. We also use glucose oxidase (GO) to enhance oxidative stress and to induce cell death in the tumor. To test *in vivo* this suicide gene therapy system (*lis*/lin/GO), we used an orthotopic model of the human U87MG glioma cells, genetically modified to express the *lis* gene, and stereotactically implanted into the brains of nude rats (*rnu*/*rnu*). Despite its genetic condition, 6% of the animals immunorejected the xenotransplanted cells giving false curative results. We tried several delivery methods with limited success. The therapeutic cocktail, at dosages that perhaps eliminated the brain tumors, is too toxic for the animal causing its premature death.

1. Introduction

1.1. Glioblastomas in general

Glioblastomas (GBMs) are life-threatening, invade surrounding normal brain tissue, and usually grow rapidly with respect to other malignancies. This makes gliomas a major challenge for clinical intervention. Also, the majority of patients with this illness respond very poorly to current treatments and the mean survival time following tumor resection is less than 18 months. Therefore, there is a critical need to develop novel therapeutic approaches for this disease. The U87MG intracerebral glioblastoma model is particularly relevant to human tumors because it has a certain capacity for infiltration along white matter tracts. There are the migrating glioma cells, away from the original focus, the ones that presumably kill the patient. Brain tumors have some unique biologic features, such as high mitotic activity on an essentially postmitotic background and no tumor spread outside of the central nervous system.

1.2. Gene therapy

GBMs have been treated by gene therapy since the late 1980s, initially in numerous laboratory studies and later on, also in clinical trials [1–6]. Despite the failure of most clinical gene therapy protocols to produce a significant and unequivocal benefit to cancer patients, the trials have contributed to a continuous improvement of vector systems, delivery methods and clinical procedures. Enzymes which render tumor cells susceptible to certain prodrugs forms the basis of a gene therapy approach in which the gene for the enzyme is targeted to cancer cells and the

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substrate is systemically delivered [7]. Some of these gene therapeutic strategies using prodrug-converting enzymes are promising because of their bystander effect; the linamarase/linamarin/glucose oxidase (*lis*I/lin/GO) system is one of them [8].

1.3. The linamarase system

Linamarase is an enzyme originally purified from cassava (*Manihot esculenta*) [9] and localized in the cell walls of cassava leaf tissue. The corresponding plant gene *lis*, encodes a cyanogenic β-glucosidase that hydrolyzes the cyanogenic glucoside linamarin (2-hydroxy isobutyronitrile-β-D-glucopyranoside) into acetone, cyanide and glucose. Cyanogenesis protects the plant against herbivory. When expressed in mammalian cells, linamarase is exported and in the presence of linamarin, cyanide is produced in the extracellular environment [8]. The cyanide diffuses freely across cellular membranes, inhibits the cytochrome c oxidase of the mitochondrial respiratory chain, blocks the oxidative phosphorylation, induces ATP depletion and causes cell death.

1.4. Previous work

We have previously demonstrated that rat glioblastoma C6 cells carrying the linamarase gene, mixed with naive C6 cells and exposed to linamarin, induce generalized cell death. Cells expressing *lis* efficiently export linamarase, whereas linamarin enters cells poorly by endocytosis; as a result most of the cyanide is produced outside the cells. As few as 10% of C6*lis*-positive cells produce enough cyanide to eliminate an entire glioma cell culture in 96 h [8]. This bystander mechanism does not preferentially kill toxic metabolite producer cells compared to bystander cells, thus maybe allowing production of sufficient cyanide to cause tumor regression *in vivo*. We have successfully used adenoviral vectors encoding linamarase (adeno*lis*) to transduce a variety of tumor cells and a radical cell death was achieved when treated with linamarin. Nevertheless, although the observed cyanide production was sufficient to eliminate cancer cells *in vitro*, the system achieved a poor antitumor effect *in vivo*. In an attempt to increase the therapeutic potential, we have combined the *lis*/lin system with a non-toxic amount of glucose oxidase. Some studies have used GO for therapeutic purposes by inducing oxidative stress in tumor cells [10]. GO catalyzes the conversion of glucose to gluconic acid, producing hydrogen peroxide and generating more oxidative damage. Also, the addition of GO would counteract the increment in glucose production as a result of linamarin hydrolysis. The combination of the three components *lis*/lin/GO created a synergistic effect that accelerated cell death by 24 h in cell cultures, and triggered a potent autophagy [11]. We have tried quite successfully, to eradicate dog and human glioma tumors from the flanks of nude mice using the *lis*I-lin/GO cocktail [11,12]. A significant regression of tumors derived from the human glioma cell line U87MG and the human breast cancer cell line MCF7 was achieved when the therapeutic gene *lis* was either inserted in the genome of the cancer cells or delivered to the grown tumors by adenovectors (adeno*lis*) direct injections [12]. Others have also showed that adenovirus-mediated linamarase/linamarin treatment results in a significant inhibition of hepatocellular carcinoma in nude mice [13]. In this study, as previously, we have combined a subtoxic amount of GO with the *lis*/lin system in order to increase the cellular stress to induce neoplastic cell death. Now, we applied the system to human glioma tumors developed in the brain of immuno-compromise animals.

1.5. The brain as the target organ

We know that the brain is a delicate organ, isolated from general circulation and characterized by the presence of a rather impermeable endothelial barrier. Delivery of therapeutic agents to this organ has been an ongoing challenge to clinicians and scientists for many years and still is far from perfect [14]. The blood–brain barrier (BBB) severely limits the entry of many large or small-charged molecules, and a more direct approach based on catheter techniques coupled with subcutaneous osmotic pumps could be considered more pertinent. In this study, we have used the two modalities of drug delivery, local and systemic, and a combination of both.

2. Materials and methods

2.1. Cell lines and cell culture

U87MG*lis* cells were obtained by stable transfection of U87MG cells with the plasmid pIRES-P carrying the linamarase gene and the puromycin resistance gene *pac* [11]. Transfection by lipofectamine was followed by puromycin selection (1 μg/ml). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum and non-essential amino acids (L-Ala 44 mM, L-Asp 45 mM, L-Glu 40 mM, L-Pro 30 mM); also with 63.2 mg/ml penicillin, 0.1 mg/ml streptomycin or gentamycin, and 4 mM glutamine. Cells were cultured at 37 °C, 5% CO_2, and 97% relative humidity.

2.2. Lin/GO cell viability assay

U87MG-*lis* cells were cultured in M96 plates (5000 cells/well) and treated with 500 μg/ml of linamarin (Toronto Research Chemicals, Canada) in combination with 5.5 mEU/ml of glucose oxidase (from *Aspergillus niger*; Sigma, St. Louis, USA) for 48 h at 37 °C. It was necessary to seal the M96 plates with parafilm and one clip on each corner of the plate, to avoid cyanide release. Cell survival determination was performed using the MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma), which is a standard colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan in living cells giving a purple color. The absorbance of dye was measured in an automated microplate reader at 490 nm.