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Local bystander effect induces dormancy in human medullary thyroid carcinoma model *in vivo*

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ABSTRACT

The extent of local bystander effect induced by fusion yeast cytosine deaminase::uracil phosphoribosyltransferase (yCD) in combination with 5-fluorocytosine (5FC) was evaluated in xenogeneic model of human medullary thyroid carcinoma (MTC). This approach to gene-directed enzyme/prodrug therapy (GDEPT) induces strong bystander cytotoxicity. Effector yCD-TT mixed with target EGFP-TT cells in a ratio 2:9 could achieve significant tumor regression and 14-fold decrease in serum marker calcitonin upon 5FC administration. Histopathological analysis unraveled that antitumor effect resulted in tumor dormancy and proliferation arrest of remaining tumor cell clusters *in vivo*. yCD/5FC combination represents another GDEPT approach to achieve tumor growth control in MTC.

1. Introduction

Gene-directed enzyme/prodrug therapy (GDEPT) approaches were developed to limit host toxicity by introducing new catalytic functions into tumor cells in order to sensitize them to otherwise inert prodrugs [1–3]. Several suitable gene/prodrug combinations were proven efficient in preclinical antitumor testing. However, clinical trials have shown only limited extent of tumor control due to several reasons. Critical problem to overcome in cancer gene therapy remains to achieve gene delivery in sufficient cell numbers in order to target substantial proportion of tumor cells and mediate tumor regression [4]. Gene transfer efficiencies in clinical settings are unlikely to exceed more than 10% of target tumor cell population. Therefore an indirect effect of the treatment which causes cytotoxicity in neighboring non-transgenic cells within the tumor being called bystander effect is desirable in the context of enzyme/prodrug gene therapy. Bystander effect results in more cell deaths and higher cytotoxicity in comparison to the situation if only transgenic cells were killed by direct action of enzymatic conversion (suicide effect) [5].

GDEPT may elicit both local and distant bystander effect. The latter involves an intense systemic anti-tumor inflammatory infiltration and it is immune mediated. Distant bystander effect is considered to be one of the important factors for the clinical success of gene therapy *in vivo* [1]. On the other hand, local bystander effect is limited by the tissue penetration capacity of the prodrug to achieve sufficient concentrations and subsequently the ability of activated metabolites to spread into adjacent nontransduced cells [6]. Soluble toxic metabolites can be transferred by either passive diffusion or active transport, via apoptotic vesicles or gap junctions. Frequent deregulation in gap junction intercellular communication severely compromises the efficiency of the approaches using highly charged cytotoxic metabolites such as ganciclovir-triphosphate in GDEPT combination of herpes simplex virus thymidine kinase and ganciclovir [5]. Combination of bacterial or yeast enzyme cytosine deaminase with prodrug 5-fluorocytosine produces toxic effect via cytotoxic drug 5-fluorouracil, which can freely diffuse across the cell membranes, and thus induces significant local bystander effect [7]. Moreover, the fusion of the yeast cytosine deaminase with the uracil phosphoribosyltransferase has significantly improved the conversion efficiency of 5FC to 5FU and its metabolites further improving the antitumor effect in many preclinical models [7–10].

Although medullary thyroid carcinoma (MTC) constitutes only 2–4% of all thyroid cancer diagnosis, it has generally proven to be refractory to cytotoxic therapy [11]. Systemic therapy in patients with inoperable metastatic disease requires prolonged treatment

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with switching between different agents [12]. However, based on efficiency and toxicity not all patients can tolerate this therapy. Therefore novel multikinase inhibitors were tested in series of trials and have brought significant progress in treating patients with advanced progressive MTC during the past 5 years [13]. Messina and Robinson have recognized a potential role of gene therapy in MTC treatment summarizing also several studies using cytoreductive enzyme/prodrug combinations [14]. Our recently published data have extended the scale of effective GDEPT combinations against MTC to the therapeutic combination employing fusion yeast cytosine deaminase::uracil phosphoribosyltransferase (yCD) in combination with 5-fluorocytosine (yCD/5FC) [9]. The absence of gap junction intercellular communication was observed in model MTC cells, and therefore the approach relying on passive metabolite diffusion was proposed to be employed to increase cytotoxicity. Potent cytotoxic effect was confirmed *in vitro*, and antitumor efficiency *in vivo*. Even though transgenic and non-transgenic tumor cells were mixed in a ratio 1:1 to induce tumor xenotransplant growth in immunodeficient mice, we have observed tumor inhibition of 92.5% indicative of strong suicide and local bystander effect *in vivo*.

The aim of this study was to analyze the extent of local bystander effect induced by yCD/5FC with limited numbers of yCD-transduced cells present within the tumor mass. The proportion of effector-to-target tumor cells corresponded to the proportion necessary to achieve complete target tumor cell eradication in *vitro*. In order to track the target cells, these were modified to stably express the EGFP protein. Our xenogeneic model enables to evaluate local bystander effect in the absence of inflammatory anti-tumor responses *in vivo*. 5FC administration resulted in 71.4% tumor growth inhibition, 14-fold decrease in calcitonin serum levels and histopathologically confirmed tumor dormancy. These preclinical data provide evidence for potent local bystander toxicity and support the observation that yCD/5FC system is very efficient in tumor growth control in chemotherapy-resistant MTC model. We suggest that the GDEPT approach utilizing yCD/5FC combination might prove useful to overcome low antitumor efficiency and high systemic toxicity of 5FU.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased form from Sigma–Aldrich®, St. Louis, MO, if not stated otherwise.

2.2. Cells

Epithelial adherent TT cell line (ATCC. No. CRL-1803™) derived from human medullary thyroid carcinoma was purchased from ATCC and cultured in RPMI medium (Roswell Park Memorial Institute, PAA Laboratories GmbH) supplemented with 10% fetal bovine serum (FBS, Biochrom AG), 10.000 IU/ml penicillin (Biotika, Part. Lupca, Slovakia), 2 mM glutamine and 5 μg/ml streptomycin (Sigma, St. Louis, MO). Transgenic TT cells expressing fusion yeast cytosine deaminase::uracil phosphoribosyltransferase yCD or enhanced green fluorescent protein (EGFP) were prepared as described elsewhere in detail [9].

All cells were expanded according to standardized protocol and kept in humidified atmosphere and 5% CO_2 at 37 °C.

2.3. Calcitonin expression analysis

Parental TT and/or transduced TT cells were collected by accutase solution (PAA Laboratories GmbH), washed with PBS solution and pelleted (5–7 × 10^6 cells per pellet). These were frozen in RNAlater Stabilization Reagent (Qiagen, Hilden, Germany) and stored at −80 °C until use.

RNA was isolated with NucleoSpin® RNA II Kit (Macherey–Nagel). Any traces of DNA were removed by RNase-free DNase I treatment (10 U/μg RNA) in the presence of 20 U RiboLock solution (Thermo Fisher). Total RNA was screened for complete DNA removal by PCR and then reverse transcribed using RevertAid™ H Minus First Strand cDNA Synthesis Kit and oligo-dT18 primer (Thermo Fisher).

2.3.1. GAPDH housekeeping gene protocol

Quantitative PCR was performed with Master Mix containing: 1× Maxima® Probe qPCR Master Mix, final concentration of 5.5 mM $MgCl_2$ (Thermo Fisher), 0.4 μM of GAPDH primers (sense 5′-GAA GGT GAA GGT CGG AGT C-3′ and antisense 5′-GAA GAT GGT GAT GGG ATT TC-3′), 0.1 μM GAPDH dual labeled probe (5′-Hex-CAA GCT TCC CGT TCT CAG CC-BHQ1-3′ (Metabion, Martinsried, Germany) and 100 ng of cDNA. Thermal cycling conditions were as follows: DNA polymerase activation (50 °C for 2 min), initial denaturation step (95 °C for 10 min) followed by 40 cycles (95 °C for 15s, 60 °C for 60s and HEX fluorescence aquire). Correct amplification of 226 bp product was verified on 1% agarose gel.

2.3.2. Calcitonin gene protocol

Calcitonin primers (sense 5′-TGC GGT AAT CTG AGT ACT TGC ATG C-3′ and antisense 5′-CCA ACC CCA ATT GCA GTT TGG-3′) were designed using Vector NTI software (Invitrogen) according to human calcitonin precursor mRNA calcitonin sequence (GenBank ID: X00356.1). The final reaction Master Mix contained: 1× Maxima® SybrGreen qPCR Master Mix (Fermentas) supplemented with 2.5 mM $MgCl_2$, 0.18 μM of each primer and 100 ng of cDNA. Thermal cycling conditions: DNA polymerase activation (50 °C for 2 min), initial denaturation (95 °C for 10 min), followed by 35 cycles of (95 °C for 15 s, 63 °C for 60 s and acquire of SybrGreen fluorescence at 72 °C, 10 s). Correct amplification of 91 bp product was verified on 2.5% agarose gel and as a one sharp melting peak at 78 °C during melting in range 65–86 °C.

2.3.3. Statistical analysis and validation

All samples were tested in parallels in four independent qPCR runs for GAPDH and qPCR runs for calcitonin expression. Data were normalized to housekeeper expression. Normalized expression for each sample relative was determined by ΔΔCt method using CFX Manager software 1.5 (Bio-Rad).

2.4. Calcitonin EIA assay

Calcitonin EIA Assay (Alpco Diagnostics) was used to quantify the protein levels. The immunoassay utilizes two mouse antibodies: biotinylated Ab against human Calcitonin 11-23 and peroxidase labeled Ab against Calcitonin 21-32. Quantification of calcitonin secretion was performed in two independent experiments, all samples in parallel according to manufacturer instruction. Briefly, 100 μl of calcitonin standards (calibrators), controls and samples in parallels were mixed with 50 μl of each antibody in streptavidin-coated microplate in a dark on an orbital rotator. It was incubated for 4 h at room temperature, washed away very carefully and incubated with TMB substrate for 30 min at room temperature in a dark with rotation. Reaction was stopped and optical density of each sample was measured at 450 and 405 nm. Concentration was derived from calcitonin standard curve. Calcitonin secretion was expressed as pg of calcitonin per 1 μg of total proteins in cell culture media as determined by Lowry method or pg of calcitonin per 1 ml of mouse serum. Standard cell culture medium or mouse serum from control mouse was used as background control.

2.5. Proliferation analysis

One to 1.5 × 10^5 cells per well in 6-well plates were plated in duplicates, which ensured unrestricted exponential growth for the duration of the assay. Population doubling time (PDT) was calculated as an average of three measurements for the TT cells according to the formula $G = t\log 2/\log \mathrm{Nt} - \log \mathrm{No}$, where t = time period, Nt = number of cells at time t, No = initial number of cells.

In order to directly compare proliferation rates for EGFP-TT and yCD-TT cells, these cells were then labeled with 5 μL of Vybrant DiI (Invitrogen, Carlsbad, CA) diluted in 1 ml PBS per 1 × 10^6cells for 20 min at 37 °C. One part of these cells was proliferation-arrested by 10 μg/ml Mitomycin C (Mit-C, Kyowa, Hakko Ltd., UK) diluted in culture medium for 3 h and washed. Proliferating and arrested cells were kept in standard culture medium for 14 days. Cells were then detached by accutase solution, washed and fluorescence intensity was determined by flow cytometry. DiI fluorescent staining does not interfere with EGFP fluorescence, therefore this method is suitable for EGFP-TT stably expressing fluorescent reporter protein.

2.6. Xenotransplant growth and animal treatments in vivo

A 6–8 weeks old athymic nude female mice (Balb/c nu/nu) were used in accordance to the institutional guidelines under the approved protocols. Tumors were induced with 1.1 × 10^7 EGFP-TT cells resuspended in serum-free RPMI diluted 1:1 with ECM gel (cat. No. #1270, Sigma–Aldrich®, St. Louis, MO) s.c. Animals with growing xenotransplants (n = 5–6/group) were randomly divided into two groups for 5FU treatment, which was started on day 9. Treated animals received the dose of 10 mg 5FU/kg/day i.p. every other day for 22 days. Preliminary experiments have shown that this was the maximum tolerated dose in our experimental setting.

In independent series of experiments, tumors were induced by 2 × 10^6 effector transgenic yCD-cells TT mixed with 9 × 10^6 target transgenic EGFP-TT (ratio effector-to-target cells 2:9) injected subcutaneously. Cells were resuspended in