CHARACTERIZATION OF THE NITROREDUCTASE/ METRONIDAZOLE SUICIDE GENE SYSTEM AS A SAFEGUARD FOR CELL BASED THERAPIES

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ABSTRACT

Cell-based therapies are promising treatment strategies for a variety of disorders ranging from cancer to spinal cord injuries. However, there is a risk of the transplanted cells becoming malignant. As a safeguard against this, suicide gene systems can be implemented so that transplanted cells can be eliminated if necessary by administering a pro-drug. Herpes simplex virus thymidine kinase (HSV-tk) paired with the pro-drug ganciclovir (GCV) is one of the most studied suicide gene systems. However, it can only kill cells that are actively dividing. Here we characterize another suicide gene system, nitroreductase (NTR) with its pro-drug metronidazole (MNZ), to investigate where in the cell cycle the killing occurs, hypothesizing that it could become an ideal candidate for eliminating transplanted cells irrespective of their proliferative status. Murine embryonic stem cells were transfected with vectors expressing either HSV-tk or NTR and treated with the corresponding pro-drug. Confocal imaging and FUCCI (fluorescent ubiquitination-based cell cycle indicator) were used to identify where in the cell cycle the drug was active[2]. MNZ was found to kill both dividing and non-dividing cells whereas GCV killed only the dividing cells. These results suggest that the NTR system may be a valuable addition or complement to HSV-tk.

Les thérapies cellulaires sont des stratégies promettantes en tant que traitements pour une variété de maladies. Celles-ci incluent le cancer et les traumatismes médullaires. Cependant, il y a un risque que les cellules implantées puissent devenir malignes. Afin de prévenir cela, des systèmes de gènes suicides peuvent être utilisés afin d'éliminer les cellules implantées si nécessaires par l'administration d'une prodrogue. La thymidine kinase, une enzyme trouvée chez les patients atteint du virus de l'herpès simplex (HSV-tk), utilisée en conjonction avec la prodrogue ganciclovir (GCV), est un des systèmes de gènes suicides les plus étudiés. Cependant, il peut seulement tuer les cellules qui se divisent activement. Ici, nous caractérisons un autre système de gènes suicidaires, nitroréductase (NTR) avec sa prodrogue metronidazole (MNZ), afin d'étudier à quel point dans le cycle cellulaire la tuerie se déroule. L'hypothèse est que ce système pourrait être un candidat idéal afin d'éliminer les cellules transplantées, peu importe leur statut prolifératif. Des cellules de souche embryonnaires murines ont été transfectées avec des vecteurs qui exprimaient soit HSV- tk ou NTR et traitées avec la prodrogue correspondante. La microscopie confocale et le système FUCCI (pour fluorescent ubiquitination-based cell cycle indicator) ont été utilisés afin d'identifier le point du cycle pendant lequel la drogue était active[2]. Il a été trouvé que MNZ tuait les cellules qui se divisaient et qui ne se divisaient pas, alors que GCV tuait uniquement les cellules qui se divisent. Ces résultats suggèrent que le système NTR pourrait être une addition ou un complément utile à HSV-tk.

KEY WORDS

Suicide gene; nitroreductase; metronidazole; stem cells; cell therapy

INTRODUCTION

Stem cell based therapies hold great promise for the future of regenerative medicine, and could potentially be used to treat a wide variety of diseases, from autoimmunity to neurodegeneration to cancer, as shown in the literature[3]. However, if the differentiation process of pluripotent cells is not perfect, or if random mutations occur during in vitro culture, transplanted cells could become cancerous[1]. Precise control over the presence and proliferation of these cells is therefore essential for assuring the safety of these therapies.

Suicide genes are genetic tools that enable the elimination of cells in vivo by the systematic administration of specific drugs to which the largest cells exhibit sensitivities. Although initially proposed

as a cancer treatment in the 1980s[4], they can also be used to improve the safety of cell transplantation. Transplants harbouring a suicide gene can be efficiently and selectively removed from the recipient by the administration of a specific drug[5]. Several suicide gene systems have been discovered, each acting through different mechanisms, with their own set of advantages and disadvantages. For instance, to prevent cancer from occurring from transplantation, a system that kills all rapidly multiplying cancerous cells would be necessary. For other situations, such as repairing breakages in trauma injuries, a system that can kill all transplanted cells after the breakage has been healed would be more advantageous.

The original and currently most studied suicide system, the herpes simplex virus thymidine kinase (HSV-tk), eliminates actively dividing cells. HSV-tk has a high affinity for ganciclovir (GCV)[6]. GCV and various other cellular kinases phosphorylate HSV-tk into a triphosphate derivative, which mimics guanosine and is able to incorporate itself into DNA. However since it is not a nucleoside, DNA synthesis is halted, leading to cell apoptosis[7]. This eliminates dividing cells, as they are killed once they undergo mitosis. Since TK can only kill dividing cells, it may not be appropriate for other kinds of cell therapies. There is an urgent need to find alternatives that can also kill quiescent cells, for these other types of cell therapies. The nitroreductase (NTR)/metronidazole (MNZ) system is considered a strong contender for anticancer strategies. Genetically modifying tumor cells to express the NTR gene activates the MNZ prodrug[8]. NTR is a common enzyme produced by E.coli and other bacterial cells, and MNZ is a common antibiotic often used with other antibiotics to treat infectious diseases. Similarly to the effect of GCV on TK transfected cells, MNZ was found to inhibit the proliferation of NTR transfected T-cells[8]. Previous experiments with NTR allude to the fact that this could be a marketable suicide system, but to our knowledge, there is no information precisely defining where in the cell cycle the NTR/MNZ system is active. The aim of this work is to investigate the precise temporal action of NTR/MNZ within the cell cycle. Finding a suicide system that is capable of eliminating all grafted cells irrespective of their proliferative state is crucial for the field of cell therapies.

MATERIALS AND METHODS

Molecular cloning of transgenes

Vectors expressing TK.007 (TK), an efficient variant of HSV-tk[9], and Ntro, a high-expression variant of nitroreductase[10], were constructed using sequences from Addgene. AttB sites were added to the ends of TK and Ntro for site-specific recombinases to recognize and cleave for later amplification. Their cDNA sequences were PCR-amplified. TK and Ntro were cloned into pDONR entry vectors using the Gateway BP reaction. A Gateway LR reaction was used to insert TK and Ntro cDNA respectively into a TAB destination vector containing the beta-Geo selectable marker, allowing for positive selection with G418 and confirmation of transgene expression level by lacZ staining.

Transfection of transgenes into murine embryonic stem cells (ESC)

The Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) labels cell cycle stages with different fluorescent colors[2]. We used this to determine the cell cycle stage that the TK and Ntro systems were capable of eliminating cell expressing their respective transgenes. Murine ESC (C57BL/6) were transfected with the suicide genes and the FUCCI2 transgene using JETPRIME (PolyPlus). A total of 2 ug of DNA was transfected. Transfected cells were selected with G418 and puromycin for 7 days, while adding doxycycline to activate the TetO promoter. Colonies from the TK- and Ntro-transfected cells were picked into four 96-well plates and passaged into triplicates. LacZ staining, and fluorescent microscopy was used to select clones with the strongest and widest transgene expression. Selected clones were clonally expanded and used for experiments.

Treatment of transgenic ESC line with prodrugs to induce cell death

TK and Ntro expressing cells were grown under differentiating conditions and treated with 100 ug/ml, 150 ug/ml, 200 ug/ml, and 400 ug/ml of MNZ, and 2 uM, 3 uM, 4 uM, and 8 uM of GCV respectively, for seven days. A dosage curve for GCV and MNZ were conducted prior to final imaging to obtain the appropriate concentration and time for the final experiment. A timeline of seven days was chosen based on the number of days it took to kill almost all the TK transgenic cells treated with GCV – elimination of all TK cells would make FACS analysis impossible. Confocal fluorescent microscopy images were taken daily at the same location on each well. FACS analysis was used to determine the cell cycle position based on FUCCI expression.

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RESULTS

Dosages of 100 ug/ml of MNZ and 2 uM of GCV were selected for killing

Initial experiments were conducted with different concentrations of MNZ and GCV, respectively, to see what range yielded the best results, while using the cells transfected with the other suicide gene as a negative control. 100 ug/ml of MNZ, and 2 uM of GCV were shown to be appropriate base concentrations.

Proliferation of TK transgenic cells decreased in the presence of GCV

Images from the confocal microscope show a decrease in proliferating TK transgenic cells when GCV was administered. At day 0 of GCV treatment, the number of total cells and the ratio between dividing and non-dividing cells for all drug concentrations were roughly the same. After seven days of treatment, we observed a dramatic decrease in TK transgenic cells, specifically those that were proliferating (Fig.1). Using the data from FACS analysis, it appears that there is a decrease of dividing cells and a subsequent relative increase of non-dividing cells. While the ratio of dividing to non-dividing cells after seven days of no drug treatment was roughly 5:2, the ratio changed to be roughly 1:3 dividing to non-dividing when GCV was administered. This ratio was approximately maintained throughout the different concentrations of GCV (Fig.3).

The number of both proliferating and non-proliferating Ntro transgenic cells decreased in the presence of MNZ

Confocal microscopy shows a decrease in Ntro transgenic cells when MNZ is administered. At day 0 of MNZ treatment, the total number of cells and the ratio between dividing and non-dividing cells for all drug concentrations is roughly the same. After seven days of MNZ treatment, there are fewer cells than in the no-drug control. The ratio between dividing and non-dividing cells is relatively the same in treated versus non-treated cells. Proliferating cells appear to be smaller in size than the non-proliferating cells (Fig.2). Using data from the FACS analysis, the percentage of proliferating and non-proliferating cells remains very well preserved throughout the different concentrations, at approximately a 4:3 ratio. At the 400 ug/ml concentration of MNZ, there are nearly equal proliferating cells as non-proliferating cells (Fig.4).

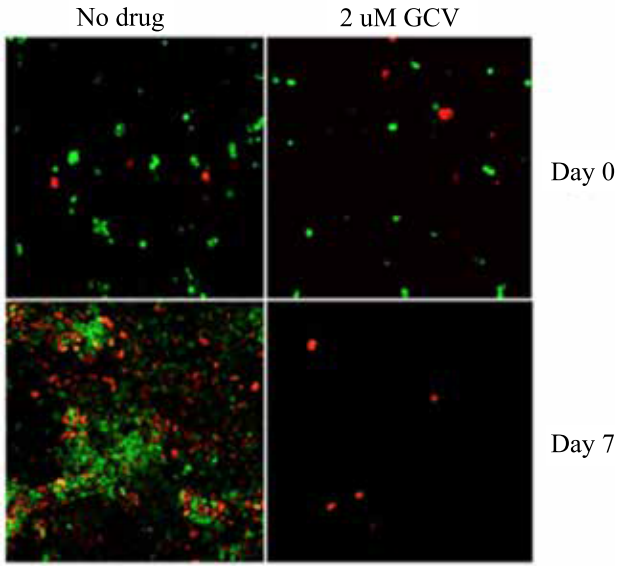

Figure 1: GCV treated TK/FUCCI transgenic ESC lines at 5x magnification imaged on day 0 and day 7 of treatment, in comparison to the no drug treatment control. Green cells represent the dividing cells, and red cells represent the non-dividing cells2. The TK transgenic ESC line undergoing GCV treatment showed a considerable decrease in the number of total cells, specifically proliferating cells.

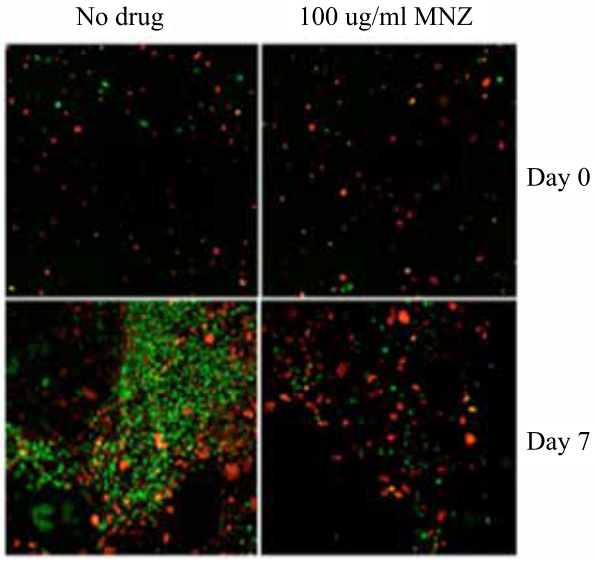

Figure 2: MNZ treated Ntro/FUCCI transgenic ESC lines at 5x magnification imaged on day 0 and 7 of treatment, in comparison to the no drug treatment control. Green cells represent the dividing cells, and red cells represent the non-dividing cells2. The Ntro transgenic ESC line undergoing MNZ treatment showed a decrease in the number of total cells, killing both proliferating and non-proliferating cells.

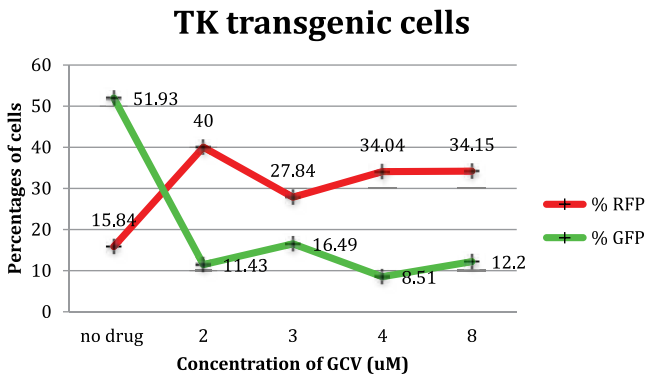

Figure 3: The percentage of RFP positive TK transgenic cells (not dividing) and GFP positive cells (dividing) seven days after treatment with various concentrations of GCV. There is a general decrease of GFP positive dividing cells, and a relative increase of RFP positive non-dividing cells.

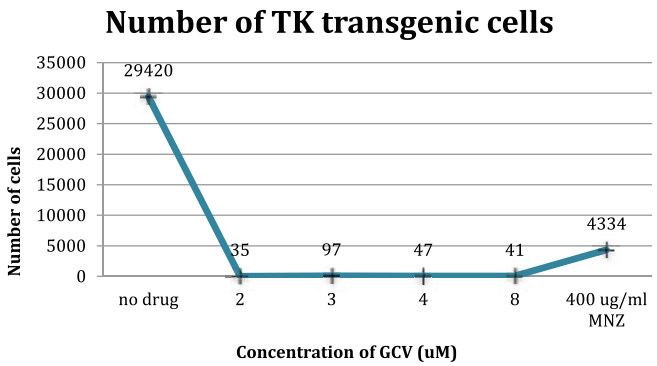

Figure 5: The total number of TK transgenic cells seven days after GCV treatments of differing concentrations. There is an evident decline in the number of cells when GCV is administered.

GCV efficiently kills TK transgenic cells

Seven days after drug treatment, the number of remaining live cells was determined via flow cytometry. We show that the quantity of TK transgenic cells dramatically decreases when GCV is administered: 300 times less compared to the untreated control. There was approximately the same number of cells in the different concentrations of GCV. There are six times less cells

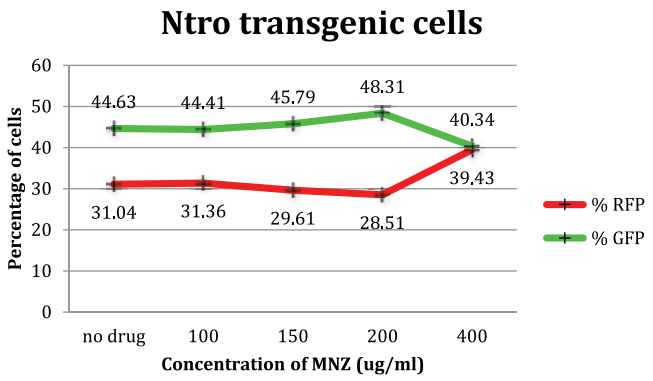

Figure 4: The percentage of RFP positive Ntro transgenic cells non-dividing and GFP positive cells dividing seven days after treatment with various concentrations of MNZ. The ratio of green dividing cells and red non-dividing cells is largely preserved throughout the different MNZ concentrations.

when the highest concentration of MNZ was given to TK transgenic cells. The 400 ug/ml concentration of MNZ substantially decreased the number of TK cells (Fig.5).

Ntro/MNZ kills less efficiently than the TK/GCV

Seven days after initiation of drug treatment, the numbers of remaining live cells were determined via flow cytometry. We show that the number of Ntro transgenic cells generally decreases when MNZ is given. There was a slight increase of live Ntro cells between 100 ug/ml and 200 ug/ml concentration of MNZ. However, the general trend from the no drug control to the 400 ug/ml concentration of MNZ was decreasing, best described with an exponentially decaying curve with a coefficient of determination (r2) of 0.81129. The exponential curve of best fit is modeled by the function f(x)=21775e-0.151x. There were approximately 1.3 times less cells with 100 ug/ml through 200 ug/ml concentrations of MNZ, and 2 times less cells using 400 ug/ml concentration of MNZ in comparison to the no drug control. The highest concentration of GCV resulted in the least amount of Ntro transgenic cells, approximately 2 times less than the no drug control (Fig.6).

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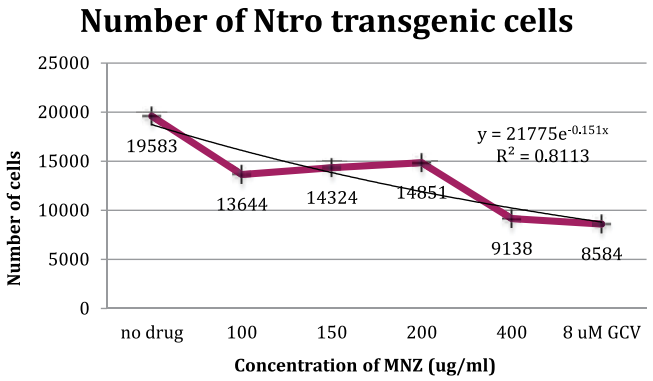

Figure 6: The total number of Ntro transgenic cells seven days after MNZ treatments of differing concentrations. There is roughly an exponential decrease in the number of cells after administration of MNZ.

DISCUSSION

Recent studies using Ntro/MNZ at a concentration of 170 ug/ml to eliminate human T-cells[8] suggest that Ntro and MNZ are a compatible pairing for another suicide gene system. The aim of this project was to characterize where in the cell cycle the Ntro/MNZ system is active and to investigate if this could be a potential alternative to the already existing TK/GCV suicide gene system. It appears that MNZ kills Ntro transgenic cells, regardless of what phase of the cell cycle they are in, as the ratio of non-dividing to dividing cells remains very well preserved in any of the tested concentrations of MNZ. However, the Ntro/MNZ suicide system was not as efficient as TK/GCV (Fig.7) (Fig.8). 2 uM of GCV killed 300 times the TK cells as compared to the no-drug control, whereas 100 ug/ml of MNZ only killed 1.3 times the Ntro cells as compared to the no-drug control. The TK/GCV system is mainly exclusive for proliferating cells, while the Ntro/MNZ system is more versatile and targets all cells without discriminating based on cell cycle phase, regardless if they are dividing or non-dividing. The highest concentration of MNZ treating TK transgenic cells resulted in 83.4% cell death, in comparison to the no drug control. While this is less toxic than any of the tested concentrations of GCV, where 99.6% of the cells resulted in death, theoretically MNZ or other pro-drugs should not have an effect on TK transgenic cells, as MNZ does not phosphorylate the TK gene, therefore not resulting in apoptosis. Similarly, the highest concentration of GCV treating Ntro transgenic cells resulted in 50% cell death, in comparison to the no drug control. Unlike the relationship with MNZ and TK transgenic cells, 8 uM of GCV had a higher killing rate than any of the tested concentrations of MNZ on Ntro transgenic cells.

GCV was toxic to proliferating TK transgenic cells at a concentration of 2 uM, which is comparable to the concentration used in literature[8]. MNZ was toxic to both proliferating and non-proliferating cells at a concentration of 100 ug/ml. The Ntro/MNZ system acts on all cells – dividing and non-dividing, but there is still an active area of investigation identifying the mechanisms behind the killing. More experiments should be performed, with a FACS analysis done on day 0 of treatment, for a benchmark of the number of cells and cell cycle phase for later comparison at further experiment days.

Based on our results, Ntro/MNZ hold potential to be used for safeguarding any form of cell therapy that requires the complete removal of all cells once healing has been accomplished. An example of such therapies is treatment of spinal cord injury. The transplantation of neural progenitor Ntro transgenic cells into the spinal cord may be used to repair the damage. Once the repair has been accomplished, the transplanted cells may no longer be needed. However, there is always a risk that in vitro cultured cells may give rise to malignancies. Ntro/TK could in such cases be used, completely removing grafted cells by administration of MNZ. This allows for the complete control of transplanted cells with a prodrug, which is essential for ensuring and testing the success of future cell therapies.

FUTURE DIRECTIONS

Before the Ntro/MNZ suicide system can move forward to clinical trials like TK/GCV, a wider range of concentrations need to be tested to maximize the efficiency of the killing. This was an in vitro experiment, and it would need to be applied to animal models, such as the C57BL/6 strain of mice that the cells in this experiment were derived from. In vivo experimentation could involve transplanting the Ntro-transfected cells into the mice and monitoring the growth and death of these cells with administration of MNZ. The efficiency of different transplantation locations on the mice could also be observed. Knowing the versatility of this new system is a significant advance in both suicide gene research and cell therapy; the next steps would be to learn about

the mechanisms behind the killing, and investigate ways to make the system more efficient.

ABBREVIATIONS

TK – BHerpes simplex virus thymidine kinase 007 derivative

Ntro – Nitroreductase derivative

GCV – Ganciclovir

MNZ – Metronidazole

PCR – Polymerase chain reaction

PB transposon system – PiggyBac transposon system

FACS – Fluorescent Activated Cell Sorting

FUCCI – Fluorescent Ubiquitination-based Cell Cycle Indicator

LacZ – One gene of the lactose operon, which allows for selection with beta-galactosidase

TetO – Tetracycline-controlled transcriptional activation on system. Activated in presence of tetracycline (and subsequently doxycycline)

ESC – Embryonic stem cells

AttB sites – Attachment site in bacterial and phage genomes

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Andras Nagy = PI

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