Preclinical studies for gene therapy of head and neck cancers using the HSV-tk/GCV strategy

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The present study has used the suicide gene Herpes Simplex Virus–thymidine kinase (HSV-tk) and ganciclovir (GCV) strategy. A 2 kb sequence of HSV-tk was subcloned into the retroviral vector resulting in a recombinant vector, LTKSN, which was transfected into a packaging cell-line, PA317 and selected on G418. The highest expressing clone, PTK-16 was used for in vitro experiments. Intra-tumoural injections of PTK-16 followed by GCV treatment, in a HNSCC xenograft model in nude mice, showed a significant reduction in the viable tumour volume (p=0.009). These results will form the basis for future clinical trials in HNSCC.

Keywords: gene therapy, HSV-tk, xenograft, nude mice, ganciclovir, head and neck cancer

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Introduction

Squamous cell cancer of the head and neck (HNSCC) is the fifth most frequent cancer of the world¹ and the dominant cancer among the male population in India². The current therapies provide a 2-year survival of 30% in patients with advanced stage III and IV disease³. Gene therapy may provide an alternate approach to the treatment of this cancer.

Suicide gene therapy is an experimental form of cancer therapy that is being evaluated in human trials⁴. This approach involves intratumoral delivery of genes encoding enzymes that convert non-toxic prodrugs into toxic metabolites bringing about a selective kill of dividing cancer cells expressing the suicide gene. This is emerging as a preferred method for cancer gene therapy as 100% of the cancer cells do not have to be transduced with the transgene to get a meaningful cell kill. This is because of the phenomenon called the ‘bystander’ effect, whereby the genetically modified cells in the presence of the prodrug are able to transfer the cytotoxic effect to neighbouring cells lacking the suicide gene⁵,⁶.

Numerous studies have transferred the suicide gene, Herpes Simplex Virus thymidine kinase (HSV-tk) into tumour cells followed by treatment with prodrug Ganciclovir (GCV). Cell kill has been demonstrated by the use of this strategy, in many tumour types both in vitro and in vivo. Several groups have reported efficient transduction of tumour cells by intratumoral injection of retrovirus packaging cells producing HSV-tk bearing recombinant retroviruses⁷-¹⁰. This strategy has resulted in impressive tumour regressions in several animal models, prompting Phase I clinical trials in patients with advanced cancers⁷,¹⁰,¹¹. The authors report in this report that retroviral mediated HSV-tk gene transfer leads to GCV dependent cytotoxicities in a human head and neck cancer cell line (NT8e) and leads to a considerable regression of NT8 tumours in a xenograft model in nude mice injected with Virus Producing Cells (VPC) PTK-16 and treated with GCV.

Materials and Methods

Cell Cultures

Cell lines PA317, a mouse amphotropic packaging cell line and NIH3T3 were maintained in DMEM with 10% fetal calf serum. NT8e, a cell line established from a human head and neck cancer in our laboratory was also maintained in the same medium. NT8, a transplantable tumour was maintained in nude mice¹².

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Retroviral Vector

The vector LTKSN was constructed by subcloning a 2 kb HSV-tk BamH1 fragment from a plasmid TK-26 (gift from S Rhode, Eppley Cancer Institute, USA) into the multiple cloning site of LXSN, a retroviral vector obtained from A Dusty Miller. LTKSN was transfected using the calcium phosphate method into PA317 packaging cells. The transfected cells were selected on 800µg/ml G 418 for 14 days and isolated clones were picked and grown in culture. Viral titres from the supernatants were determined using NIH3T3 as target cells.

Transduction

To infect the target cells, NIH3T3 were plated at $5 \times 10^4$ in 60 mm Petri plates. One ml of the medium, from a confluent plate of VPC, was filtered through 22 µm filter and then serially diluted up to $10^{-6}$. The different dilutions were applied to the target cells along with 8µg/ml polybrene. After 4 hrs, cells were incubated in fresh medium for 24 hrs and then placed in G 418 containing selection medium. Culture plates were stained with 0.5% methylene blue to view the G 418 resistant colonies, 10 to 14 days later.

Characterisation of the G418 Resistant Clones

Total cellular RNA was extracted from the G418 resistant clones by method of Chomczynski and Sacchi13. 10 µg of RNA was electrophoresed in a 1.2% agarose formaldehyde gel. The RNA was transferred to Hybond-C membrane and probed with a TK probe labeled with 32P á-dCTP using Megaprime labeling kit (Amersham).

GCV Sensitivity

PTK-16, the highest expresser of TK from the G 418 resistant VPC was used for further experiments. $5 \times 10^3$ PTK-16 cells/well were plated in a 96 well plate and treated with different dilutions of GCV ranging from 10 ng/ml to 50 µg/ml. After 3 days of treatment, 0.5 µCi of $^3$H-thymidine was added per well, incubated overnight and the plate harvested on GFC filters the following day. Percent $^3$H-thymidine incorporation was determined by taking the counts in the control wells as 100%.

Polymerase Chain Reaction

100 ng of genomic DNA from—(1) LTKSN transfected PA317 clones; (2) nude mouse tumours injected with PTK-16 VPC; and (3) various organs from the nude mice, were amplified using 10pM of HSV-tk primers (P1 5’ CGGTCTTACGGGATGGGAAAAACCAACACC AC 3’/P2 5’ GCAGGCCGTCCCCGACGCGCGCCGGCGATTGG 3’) to obtain a 860 bp fragment of HSV-tk. (35 cycles: 1min at 94°C, 1 min at 62°C and 3 min at 72°C, final extension at 72°C for 10 min). Glyceraldehyde 3 phosphate dehydrogenase (G3PDH) was used as the housekeeping gene and was amplified using 10 pM of G3PDH specific primers (P1 5’ TGAAGTCGGAGTCAACGGATTGTTG3’/P2 5’ CATGTGGGCCATGAGGTCACCAC3’) to obtain a 983 bp fragment of G3PDH (35 cycles: 45 sec at 94°C, 45 sec at 55°C and 1 min at 72°C, final extension at 72°C for 10 min).

In vivo Studies

NT8 cells ($10^6$/100µl) were injected subcutaneously in the flank of nude mice (nu/nu). On formation of tumours, ~5mm³ in size, $10^6$ PTK16 cells in 100 µl HBSS were injected intratumorally. From the 6th day GCV (100mg/kg body weight) was injected intraperitoneally twice daily for 14 days only in the treated group (n=13). The tumour was dissected carefully along with the surrounding stroma and quick frozen in liquid nitrogen. 3 mm thick slices were cut and fixed in neutral buffered formalin. Each slice was processed, embedded in paraffin, and 5µm sections cut. Atleast 3 sections were put on each slide and stained with hematoxylin and eosin. The viable, differentiated and necrotic regions in sections from each slice were then marked and corresponding areas calculated as described earlier14. In the first set of experiments, the treated and the control tumours were on either of the flanks of the same mouse (n=9) and the animals were sacrificed after 14 days of GCV treatment (data not shown). In the second set of experiments, separate mice were used for control (n=5) and treated (n=13) tumours and the animals were sacrificed only when they were weak.

Statistical Analysis

SPSS software, version 10.0 was used for statistical analysis. Statistical significance was defined as p value = 0.05. One way Anova test was used for comparing the differentiated, necrotic, viable and total volumes of tumour specimens amongst different groups (controls: mice administered NT8+GCV and NT8+VPCs and no GCV & treated: mice administered NT8+VPCs and GCV). Differences between individual groups were studied by the Scheffe’s test.
Results

Characterization of Transfected Clones

Several G418 resistant clones were obtained on transfecting LTKSN DNA into PA317 packaging cells. Presence of HSV-tk was confirmed by PCR and the expected 860 bp product was seen in the investigated clones (Fig. 1). Clone PTK16, the highest expresser of HSV-tk by Northern blot analysis (Fig. 2) was selected for further studies. Ability of the PTK16 cell supernatant to transduce the tk gene into different cell lines indicated that it was a virus producing cell line making infective viral particles. Northern blot analysis of the PTK16 supernatant infected, G418 resistant NIH3T3 clones indicated that these cells had been transduced by the HSV-tk gene (Fig. 2). Viral titres of PTK16 as determined using NIH3T3 cells was $4 \times 10^4$ cfu/ml.

In vitro Studies

GCV sensitivity of the PTK-16 was tested at different doses, ranging from 100 ng/ml to 50 µg/ml. It was observed from in vitro studies that 1 µg/ml GCV was the optimum dose for cell kill with minimal toxicity to the parental non-transduced cells (Fig. 3). This dose was thus used for all further studies. In the colony formation assay, when PTK16 cells were treated with 1 µg/ml GCV for 24 hrs, there were no colonies seen (data not shown).

In vivo Studies

In the first set of animals (n=9), it was observed that, there was no significant difference in the total, viable, differentiated or necrotic tumour volume in the treated and control tumours (data not shown). In the second set (n=13 treated and 5 control), the animals were allowed to survive until weak. Tumours in the GCV treated group (NT8+VPC+GCV) had significantly lower volume of viable tumour (median: 117 sq. mm.; range: 0-915 sq. mm.) as compared to the controls (median: 825 sq. mm.; range: 120-1596 sq. mm.) (p=0.009) (Fig. 4).
However, the total volume of treated tumours (median: 350 mm$^3$; range: 15-2949 mm$^3$) as compared to that of control tumours (median: 1341 mm$^3$, range: 576-1995 mm$^3$) was not significant (p=0.262). Volume of the differentiated and the necrotic components did not differ among the treated and the control groups (Table 1).

The bio-distribution of HSV-tk of genomic DNA extracted from different organs of the tumour bearing nude mice was studied by PCR. In the mice treated with GCV, none of the organs including the tumour showed a band specific for HSV-tk (Fig. 5a). In animals that were not treated with GCV (controls), only the tumour showed the presence of an HSV-tk specific PCR product (Fig. 5b). This indicated that all the HSV-tk expressing cells and possibly the cells in the immediate vicinity (‘bystander’ effect) were destroyed on treatment with GCV. We can also infer that the viral infection does not spread beyond the gross confines of the tumour.

**Discussion**

The transduction of HSV-tk gene into tumour cells is currently being investigated in Phase I and II trials as a therapeutic approach in the treatment of brain, ovarian, prostate, lung, head and neck and liver tumours\textsuperscript{10}. In some of these studies, replication deficient adenoviral vectors are being used\textsuperscript{11}. Adenoviruses can transduce non-dividing cells and are not inactivated by the complement, however, they induce a specific host immune response that restricts their repeated usage. In order to circumvent these problems, the authors have used a retroviral vector.

<table>
<thead>
<tr>
<th>Total volume</th>
<th>Mean ± SE</th>
<th>Median</th>
<th>Range</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT8 / NT8+VPC</td>
<td>1366.8 ± 266.6</td>
<td>1341</td>
<td>576-1995</td>
<td>N.S.</td>
</tr>
<tr>
<td>NT8 + VPC + GCV</td>
<td>612.9 ± 220.5</td>
<td>350</td>
<td>15-2949</td>
<td>N.S.</td>
</tr>
<tr>
<td>Viable volume</td>
<td></td>
<td></td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>NT8 / NT8+VPC</td>
<td>811.8 ± 242.6</td>
<td>825</td>
<td>120-1596</td>
<td>N.S.</td>
</tr>
<tr>
<td>NT8 + VPC + GCV</td>
<td>169.5 ± 66</td>
<td>117</td>
<td>0-915</td>
<td>N.S.</td>
</tr>
<tr>
<td>Differentiated volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT8 / NT8+VPC</td>
<td>460.2 ± 162</td>
<td>384</td>
<td>81-1058</td>
<td>N.S.</td>
</tr>
<tr>
<td>NT8 + VPC + GCV</td>
<td>360.3 ± 181.6</td>
<td>243</td>
<td>0-2448</td>
<td>N.S.</td>
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<tr>
<td>Necrotic volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT8 / NT8+VPC</td>
<td>94.8 ± 36.6</td>
<td>72</td>
<td>0-222</td>
<td>N.S.</td>
</tr>
<tr>
<td>NT8 + VPC + GCV</td>
<td>83 ± 39.8</td>
<td>36</td>
<td>0-450</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S. Not Significant

The results from the control (NT8e / NT8e+VPC) group (n=5) and treated (NT8e+VPC+GCV) group (n=13) depicted in the table.
Co-culture experiments to study the bystander effect using NIH3T3 as target cells indicated 1:1 was the best effector-target ratio. This is lower than values reported in literature and could be due to low viral titres of the PTK16. Virionis et al.\(^{15}\) have shown in co-culture experiments, viral titres greater than \(10^5\) cfu/ml are necessary for transduction of HSV-tk to neighbouring cells to provide a significant addition to bystander effect.

In the current in vivo studies, there was a marked reduction in the viable tumour volume and a near significant reduction in the total tumour volume. In vivo gene therapy experiments using HSV-tk/GCV have shown variable but highly promising reduction in tumour volume. Tumour cells stably transfected with HSV-tk and injected into syngeneic mice showed complete regression of the tumours on GCV treatment\(^{16,17}\). This was not surprising as 100% of the cells expressed HSV-tk in these studies. However, Culver et al.\(^{6}\) demonstrated complete eradication of tumours even when only 10% of the tumour cells were transduced with HSV-tk gene. This was attributed to the ‘bystander effect’\(^7\). In studies using nude mice the response has been relatively lower. While immunocompetent animals have shown a 90-100% reduction in tumour volume, the reduction was not statistically significant in nude mice suggesting that anti-tumour effect may be partly immune mediated\(^{18}\).

Rats with peritoneal carcinomatosis when administered a single i.p. dose of HSV-tk producing cells showed a weak expression of the tk transgene in liver, spleen, lungs and bowels\(^{19}\). The authors have concluded that a small proportion of cells from these tissues had captured the transgene. O’Malley\(^{20}\) injected Ad/RSV-tk directly into tumours and reported some leakage of the virus into the surrounding muscle, salivary gland and subcutaneous tissues. In our study where mice were injected intratumorally, failure to detect HSV-tk transgene by PCR in liver, spleen, lungs, kidney, intestine, brain and ovary except the tumour could indicate absence of such a leakage of viruses from the NT8 tumours.

Partial tumour kill seen in the present study may be due to the injection of VPC at a single site within the tumour. Thereby, only a proportion of tumour cells could have been transduced with HSV-tk. The other reason for a reduced kill could be that not all the tumour cells were cycling. Since we did not see any difference in the differentiated tumour volume between treated and control tumours, we can infer that the strategy has been more effective on the proliferative compartment of the tumour as against the differentiated compartment.

Tumours are heterogeneous and have a multitude of genetic lesions, with every cell having a different alteration. Gene therapy strategies that target specific genetic lesions will have to target multiple lesions. The HSV-tk/GCV system affects cells irrespective of their genetic lesions and all cells expressing the viral-tk show a cytotoxic effect. The additional kill of the neighbouring cells by the bystander effect makes this strategy even more attractive. Furthermore, as the endogenous-tk has a low affinity for GCV, the prodrug is virtually harmless to normal host cells. The HSV-tk/GCV gene therapy strategy does not target any particular molecular pathway altered in specific cancers and thus provides the advantage of using one common strategy for many different cancers.
Squamous cell carcinoma of the head and neck is among the most morbid of human cancers. Presently available therapeutic modalities include surgery, which can be deforming, coupled with radiotherapy and/or chemotherapy, which have associated side effects. Gene therapy of HNSCC using the HSV-tk/GCV strategy may offer an alternate treatment modality.

Acknowledgement
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