Safety and Efficacy of Suicide Gene Therapy with Adenosine Deaminase 5-Fluorocytosine Simultaneously in in Vitro Cultures of Melanoma and Retinal Cell Lines

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Abstract

Local treatment as a treatment modality is gaining increased general acceptance over time. Novel drugs and methodologies of local administration are being investigated in an effort to achieve disease local control. Suicide gene therapy is a method that has been investigated as a local treatment with simultaneously distant disease control. In our current experiment we purchased HTB-70 (melanoma cell line, derived from metastatic axillary node) and CRL-2302 (human retinal epithelium) were from ATCC LGC Standards and Ancotil®, 2.5 g/250 ml (1 g/00ml) (5-Flucytosine) MEDA; Pharmaceuticals Ltd. UK. Adenosine Cytosine Deaminase (Ad.CD) was also used in order to convert the pro-drug 5-Flucytosine to the active 5-Fluoracil. Three different concentrations of 5-Flucytosine (5-FC) were administered (0.2ml, 0.8ml and 1.2ml). At indicated time-points (4h, 8h and 24h) cell viability and apoptosis were measured. Our concept was to investigate whether suicide gene therapy with Ad. CD-5-FC could be used with safety and efficiency as a future local treatment for melanoma located in the eye cavity. Indeed, our results indicated that in every 5-FC administration had mild cytotoxicity for the retinal cells, while increased apoptosis was observed for the melanoma cell line.

Key words: suicide gene therapy, 5-fluorocytosine, melanoma, retinal.

Introduction

Melanoma is a malignant tumor of melanocytes.1 Melanocytes produce the dark pigment, melanin, which is responsible for the color of skin. These cells can be found in skin, but also in other parts of the body, including the bowel and the eye. Uveal melanoma is also an entity that has been reported.2,3 Melanoma can originate in any part of the body that contains melanocytes. Melanoma is less common than
other skin cancers, however, it is much more aggressive if it is not treated early. It causes the majority (75%) of deaths related to skin cancer. The treatment of choice if it is discovered early is surgical removal of the tumor.\textsuperscript{4} Surgical treatment is successful while it is still small and thin, and if it is completely removed, then the chance of cure is high. The likelihood of relapse or spreading depends on how deeply it has gone into the layers of the skin. For melanoma that relapses or spreads, additional treatments include chemother- and immuno-therapy, or radiation therapy.\textsuperscript{5,8} Chemotherapy and radiotherapy have adverse effects and in several situations the patients’ treatment has to be postponed.\textsuperscript{9} Moreover, severe bone marrow suppression in many cases requires hospitalization with additional costs for the national health system.\textsuperscript{10,11} Novel routes of drug administration are being investigated in an effort to reduce the adverse effects in many types of cancer with different strategies, with the main concept being the local treatment.\textsuperscript{12-22} Additional investigation of cancer pathways revealed underling mechanisms that could be utilized to block chemotherapy resistance and sensitize tumors to chemotherapy and radiotherapy.\textsuperscript{23} A major breakthrough has been achieved with so called: “suicide gene therapy” modality. The introduction of a therapeutic gene encoding enzyme capable of transforming a nontoxic pro-drug into a cell toxin enhances the cytotoxic effect for cancer cells and protects the healthy cells.\textsuperscript{15-17} Suicide gene therapy, utilizing the cytosine deaminase/5-fluorocytosine (CD/5-FC) system, is an efficient methodology for targeted therapy in cancer research with favorable results in previously published studies.\textsuperscript{24-28} In specific the cytosine deaminase (CD) enzyme converts the anti-fungal agent 5-Fluorocytosine (5-FC) into its antimetabolite 5-Fluorouracil (5-FU), thereby killing tumor cells. Most suicide genes under investigation mediate sensitivity by encoding viral or bacterial enzymes that convert inactive forms of a drug, into toxic metabolites capable of inhibiting nucleic acid synthesis.\textsuperscript{29,30} The second suicide gene therapy methodology that has been extensively investigated is the herpes simplex virus thymidine kinase gene (HSV-tk), which converts ganciclovir kinase gene (GCV) to ganciclovir monophosphate and consequense inside the cancer cell the enzymes covert it to ganciclovir triphosphate.

The bystander effect, through which the Ad.CD system applies, has to do with the fact that a pro-drug is converted into an antineoplastic agent in only a percentage of the target cells expressing the drug activating enzyme. These cells are killed as a result of this expression, thus releasing the newly formed anticancer agent to the tumor microenvironment killing also adjacent cells.\textsuperscript{29-38} The suicide gene therapy has been investigated in several cancer types; a) colon\textsuperscript{16,39,40} b) lung\textsuperscript{41,42} c) liver\textsuperscript{17,43,44} d) medulloblastomas\textsuperscript{45} e) spinal cord tumors\textsuperscript{46} f) neuroendocrine\textsuperscript{47} g) prostate\textsuperscript{48} h) breast\textsuperscript{49,50} i) bladder\textsuperscript{51} j) head and neck\textsuperscript{52} k) brain\textsuperscript{53} l) gliomas\textsuperscript{54-56} m) sarcomas\textsuperscript{57} and n) melanoma (HSV-tk- GCV)\textsuperscript{58}. The suicide gene modality has also been investigated as; a) anti-vascular endothelial treatment\textsuperscript{59,60} b) interleukin-12 (IL-12)\textsuperscript{61} and c) immune stimulation with interleukin-7 (IL-7)\textsuperscript{62} (Table 1.) Moreover, suicide gene therapy has been proven to be efficient in chemotherapy resistant cancer cell lines\textsuperscript{63} and to enhance radiotherapy treatment modality.\textsuperscript{64} Additional control of micrometastasis has also been observed in suicide gene therapy studies.\textsuperscript{65} In the current in vitro study we investigated the safety and efficiency of ad.CD-5-FC in melanoma and uveal cell lines and proposed a future method of local administration for primary or metastatic uveal melanoma treatment methodology.

| Author | Cells lines | Design | Result | Transport | Ref |
|--------|-------------|--------|--------|----------|----|
| Michaelsen S. R. et al. | GLC-14, GLC-16, GLC-19, NCI-H69, H69-VP, H69-CPR, H69-DALI, H69-BCNU | \textit{In Vitro} \textit{In Vivo} | Effective both in chemosensitive and chemoresistant cell lines | INSM1 promoter-driven SG | 63 |
| Mader R.M. et al. | CCL22(7 with low and Intermediate phenotypes) | \textit{In Vitro} \textit{In Vivo} | Effective with 100% Activation | Adenoviral cosmids | 16 |
| Bondanza A. et al. | Leukemia (mouse) | \textit{In Vitro} \textit{In Vivo} | Effective with IL-7 receptor expression (HA-1, H-Y) | Herpes simplex virus | 62 |
| Xu Y. et al. | Lewis Lung Cancer A549 | \textit{In Vitro} \textit{In Vivo} | Combination IL-12 and suicide gene therapy enhances the antitumor effect as a factor modifying the tumor microenvironment | AdCMV(-), AdhTERTHRP, AdCMVMIL-12 | 61 |
| Sia KC. et al. | HCC 26-1004 | \textit{In Vitro} \textit{In Vivo} | Effective HSV-1 amplicon viral vector and 5-FU administration | HSV-1 amplicon viral | 17 |
| Li S. et al. | C17.2 NSC line | \textit{In Vitro} \textit{In Vivo} | ATRA enhanced the HSV-tk/GCV | HSVtk/GCV | 45 |
| Finocchiaro | sarcoma | \textit{In Vivo} | Effective | Lipid-complexed plasmid | 57 |

Table 1. Suicide Gene Therapy Studies.
| Authors          | Assay/Tumor Type                                                                 | Comments/Experimental Details                                                                 |
|------------------|--------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| M.E.L. et al.    | Human colon carcinoma (Lovo) cell line                                         | In Vivo Microenvironment Control and Distant metastasis Bearing IFN-β and suicide genes co-administered with ganciclovir (ISG) 5-FC, CPNP-shVEGF-CDTK |
| Leng A. et al.   | SGC7901 human gastric Cancer cell line                                         | In Vivo Anti-VEGF-A- Suicide gene therapy Anti-VEGF- Suicide gene therapy 5-FC, triple gene vector Expressing VEGF-shRNA and fusion suicide gene yCDglyTK delivered by CPNP's HSVGk-GCV  |
| Liu T. et al.    | Human HT29 and murine DHDK12 pro-b                                             | In Vivo MTX, aphidicolin and ara-C. The rate of apoptosis increased two-fold in MTX-treated DHDK12 cells after treatment with GCV. |
| Finzi et al.     | VX2 liver cancer                                                               | In Vivo Effective with lipiodol embolism and WTP53 TK/CD plus intraperitoneal Injection of GCV at 100mg/(kg.d) and 5-FC at 500mg/(kg.d) |
| Niu H. et al.    | HCC                                                                           | In Vivo Effective Mac-1, CD4, CD8a-positive and TNF increase -HSV-tk/GCV and MCP-1 -rAd harboring human MCP-1 and the membrane-spanning domain of the tumor cell surface |
| Marukawa Y. et al.| 9L rat glioma cells and 293 cells                                              | In vitro MSC-EGFP or MSC-CD-5-FC resulted in significant prolongation of survival AdexCAEGFP AdexCAD |
| Kosaka H. et al. | Head and Neck squamous carcinoma cell line FADU                                 | In Vivo Effective with deletion Mutant of ETA as a Target gene Gene Switch System |
| Schmidt M. et al.| Glioblastoma                                                                  | In Vivo Effective against Cx43 cytoplasmic localization Lentiviral delivery of HSV-tk/GCV |
| Cottin S. et al. | R3327-AT rat prostate Carcinoma cells                                          | In Vivo Effective against hypoxic cells Bifunctional cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT) with 5-FC and radiotherapy |
| Kinoki K. et al. | C6 glioma cells                                                                | In Vivo Safety evaluation of the Stem cell therapy in brain tissue Rat MSCtk/GCV |
| Zhao Y. et al.   | U87 glioma and H4 cells                                                        | In Vivo Effective as cellular Vehicle for targeted suicide gene therapy Tumor-tropic neural stem cells, HSV-tk/GCV |
| Amano S. et al.  | NCI-H460-GFP cells                                                            | In Vivo Effective brain metastasis treatment Effective both in extrinsic and intrisinc papoptosis pathways NSC line expressing CD and TK BI-HSV-tk/GCV |
| Wang C. et al.   | Bladder cancer with N-methyl-nitrosourea perfusion                            | In Vivo Effective both in extrinsic and intrisinc papoptosis pathways NSC line expressing CD and TK BI-HSV-tk/GCV |
| Yin X. et al.    | SCLC: GLC16, DMS53 and NCI-H69 and NSCLC cancer lines: H1299 and A549          | In Vivo Improved plasmid nuclear delivery NFnB-DTS in an YCD-YUPRT (SCD) |
| Cramer F. et al. | R3327-AT rat prostate Carcinoma cells                                          | In Vivo DMP Delivered Survivin-T34A gene DMP/S-T34A which induced apoptosis DOTAP and MPEG-PCL hybrid micelles (DMP) |
| Duan X. et al.   | C-26                                                                           | In Vivo Survival and malignant pleural effusion control with higher efficiency observed for SCLC. Ad.CD+5-FC |
| Zarogoulidis P. et al. | Lewis lung cancer, SCLC, NSCLC patients                                      | In Vivo Animals Humans Survival and malignant pleural effusion control with higher efficiency observed for SCLC. Ad.CD+5-FC |
| Yi B. et al.     | A549, 16HBE, SPC-A-1 And NCI-H520                                               | In Vivo Review Specific CA-positive Target gene expression Review CEA promoter and double suicide genes TK and CD. pCEA-TK/CD rPOA/HSV-tk/GCV |
| Qiu Y. et al.    | C6, U87, F98 and 9L                                                          | In Vivo Tumor growth Suppression and locomotor function maintenance INSM1 promoter, HSV-tk to generate Ad-K5 virus |
| Akerstrom V. et al. | Neuroendocrine tumors: NCI-H69, NCI-H1155, NCI-H727, DMS53, U87MG, IMR-32, N-S-H, SK-N-BE(2), Y79, WERI-Rbl, HeLa, ANC-1, BEAS, RIN, D283 Med, HerP2 | In Vivo Enhanced antitumor activity over the RSV control |

http://www.jcancer.org
Lu M. et al. | Prostate | Human | Initiated and recruiting at the time of publication | Replication-Competent Adenovirus-mediated suicide gene therapy  
| MCF-7 and MDA-MB-231 Breast cell lines | In Vitro | Effective antitumor control | Drosophila melanogaster (Dm-dNK)  
| | In Vivo | Continuous complete remission | TK.007 novel suicide gene  
| Preuss E. et al. | G62 human glioblastoma cell line, A549 human lung Carcinoma, SW620 human Colorectal adenocarcinoma Cell line and IPC298 Human Melanoma cell line | In Vitro | Effective combination Suicide immunity therapy | shRNA-lentivirus and Ad5.CMV.HSV.tk  
| Ahn Y. et al. | CT26 murine colon adenocarcinoma cells and A549 human gastric adenocarcinoma cells | In Vitro | Efficient transfection of RDEB SCC | SLO-PTM  
| Luo X. et al. | SGC7901 human gastric Cancer cell lines | In Vitro | Higher efficiency with double suicide gene therapy CD/TK | Double suicide gene therapy  
| Freytag S. O. et al. | Prostate cancer | Human | Transgene expression up to 3 weeks, PSA decline, Acute urinary and gastrointestinal toxicities | Cytosine deaminasa(CD)/herpes simplex virus thymidine kinase (HSV-1 TK) and 3D-CRT  
| Pandha H. S. et al. | Breast cancer | Human | Efficient selectivity against erb-2 | Therapeutic cassette that contains the Escherichia coli cytosine deaminase gene driven by the tumor-specific erb-2 promoter  
| Li N. et al. | HCC cancer | Human | Recurrence free survival | Adjvant ADV-TK  
| Voges J. et al. | Glioblastoma | Human | Inhomogeneity of tissue formulation distribution | HSV-1 tk liposomal vector  
| Nasu Y. et al. | Prostate | Human | No serum cytokine changes after treatment, decreased PSA values, increased CD8+/HLA-DR+ | Ad.HSV-tk/GCV  
| Rainov N.G. et al. | Glioblastoma | Human | Surgical resection and Radiotherapy or standard therapy plus adjuvant adeno-gene therapy during surgery. Progression-free median survival in the gene group was 180 days compared with 83 days of control group | RV-HSV-tk/GCV  
| Xu F. et al. | Head and Neck | Human | Local response | Intratumoral RV-HSV-tk/GCV  
| Nemunaitis J. et al. | Refractory cancer patients | Human | Salmonella bacterium can be utilized as a delivery vehicle of the cytosine deaminase gene to malignant tissue with low dose 3 x 10^7 CFU/m²effciently. | TAPET-CD  
| Freytag S. O. et al. | Pancreas | Human | Augments radiotherapy treatment of pancreatic cancer without excessive toxicity | Ad5-yCD/mutTKasxor-ADP  

**Notes:**  
- INS1: insulinoma-associated 1 gene, IFN-β: Interferon-β, GCV: ganciclovir, CEA: Carcinomaembryonic antigen, ELISA: Enzyme-linked immuno sorbent assay, IL-7: Interleukin-7, wtCPE: wild type C. perfringens enterotoxin, optCPE: translation-optimised C. perfringens enterotoxin, IL-12: interleukin-12, HSV-4: herpes simplex virus-1, 5-FU:5-Fluorouracil, VEGF: vascular endothelial growth factor, CPNP: bioreosorbable calcium phosphate nanoparticles, HSV-tk: herpes simplex virus-thymidine kinase, yCDglyTK: fusion gene therapy of cytosine deaminase and thymidine kinase, Mac-1: Macrophage-1 antigen, CD4+; CD8+; T lymphocytes, referring to those that carry the CD4 antigen, CD8+; T lymphocytes, referring to those that carry the CD8 antigen, CC2: chemokine (C-C motif) ligand 2 [Homo sapiens (human)], rAd: recombinant adenovirus, MCP-1: Monocyte chemotactrant protein-1, ETA: ETA receptors, WtP53; wild type p53, CX43; integral membrane protein of the connexin family, alpha-type (group II) subfamily, MSC: mesenchymal stem cells, AdexCAEGFP: MSC-adenovirus carrying either enhanced green fluorescent protein gene, AdexCADC: MSC-cytosine deaminase gene, UPRF: uracil phosphoribosyltransferase, NSC6: neural stem cells, hTERT: human telomerase reverse transcriptase, HRP: expressing horseradich peroxidase, IAA: iodole-3-acetic acid, CARG: Smooth muscle alpha-actin CARG elements, BL: B. subtilis subtilin, VSV: vesicular stomatitis virus, NFF8: nuclear factor B, DOTAP: N-[1-[2,3-dioleoyloxy]propyl]-N,N,N-trimethylammonium methyl sulfate, MPEG-PCL: monomethoxy poly(ethylene glycol)-poly(3-caprolactone), DMP: DOTAP-MPEG-PCL, KDR: kinase insert domain receptor, AFP: α-fetoprotein, pPOA: poly (oligo-D-arginine), DM: Drosophila melanogaster, PET: positron emission tomography, Ad5.CMV.HSV.tk: adenoviruses 5 harboring the herpes simplex virus thymidine kinase gene, SLO-PTM; toxin Streptolydin O-3 trans-splicing molecules, RDEB-SCC; recessive dystrophic epidermolysis bullosa squamous cell carcinoma, PTM screen; pre-trans-splicing molecules, PSA: prostate specific antigen, GDEPT; gene-directed enzyme pro-drug therapy, TAPET-CD; pCD4242-mut8-FNP2009
Results

Results of cell viability and apoptosis analysis

Flow cytometry showed that ad.CD-5-FC + ancolit treatment induced apoptosis in both cell lines after 4h and 8h as determined by 7-AAD and Annexin V staining (Table 3). At these time-points, sensitivity to 0.2, 0.8 and 1.2mg ancolit was similar in both cell lines. However, the 24h measurement for cell cytometry for all the doses revealed that cell viability was increased for retinal cell line, whereas ad.CD-5-FC + ancolit treatment continued to induce apoptosis for melanoma cell line. The results of 7-AAD and Annexin V staining were also confirmed by trypan blue assay. (Table 2.) Comparison among the time-points revealed that 1.2 ml of ancolit increased the number of viable cells by 87% after 8h to 95% after 24h in retinal cell line, whereas in melanoma cell line viable cells were decreased by 78% after 8h to 75% after 24h. Similar observations were revealed for the other doses of ancolit.

Table 2: cell viability by trypan blue counting.

| Concentrations/Time points | 4h  | 8h  | 24h |
|---------------------------|-----|-----|-----|
| Melanoma cell line        |     |     |     |
| 0.2 ml ancolit            | 80% | 80% | 78% |
| 0.8 ml ancolit            | 75% | 75% | 75% |
| 1.2 ml ancolit            | 78% | 78% | 75% |
| Retinal cell line         |     |     |     |
| 0.2 ml ancolit            | 82% | 85% | 93% |
| 0.8 ml ancolit            | 80% | 85% | 90% |
| 1.2 ml ancolit            | 87% | 95% | 95% |

Table 3. Cell viability with 7-AAD and Annexin V/PI.

| 7-AAD | 4H | 8H | 24H |
|-------|----|----|-----|
| CELLS%| VIABILITY% | CELLS% | VIABILITY% | CELLS% | VIABILITY% |
| R CELLS | 36.7 | 78.9 | 50.7 | 74.7 | 85.1 | 89.8 |
| 0.2 | 20 | 71.4 | 56.4 | 70.1 | 65.4 | 82.1 |
| 1.2 | 31.1 | 69.3 | 55 | 77.9 | 82.5 | 86.1 |
| M CELLS | 53.7 | 82.6 | 81.3 | 79.5 | 76.6 | 89.3 |
| 0.8 | 56.3 | 80.1 | 79.5 | 85.7 | 83 | 85.9 |
| 1.2 | 55.5 | 79.5 | 79.2 | 83.9 | 75.9 | 83.9 |
| ANNEXIN V/PI |
| 4H | 8H | 24H |
| CELLS% | VIABILITY% | CELLS% | VIABILITY% | CELLS% | VIABILITY% |
| R CELLS | 20.7 | 58.2 | 43.7 | 33 | 57.5 | 65.4 | 20.3 | 72.7 |
| 0.2 | 30.1 | 48.7 | 28.7 | 43.9 | 41.8 | 42.3 | 36.8 | 44.6 |
| 1.2 | 38.5 | 46 | 36.4 | 38.2 | 50.2 | 57 | 28.3 | 62.9 |
| M CELLS | 5.1 | 66.3 | 83.4 | 85.2 | 0.7 | 75.7 | 1.6 | 80.7 |
| 0.8 | 3.3 | 65 | 59.6 | 3 | 78.5 | 74.4 | 0.9 | 82.2 |
| 1.2 | 3.7 | 72.8 | 55.5 | 2.9 | 73.3 | 73.9 | 1.9 | 75.1 |

Discussion

Currently there is need for more systems activating more pro-drugs. Therefore the thymidine-active mutant of dCK, dCK.DM.S74E was created which activates multiple pro-drugs such as; BVdU, LdUNAs and LdT. This system has the ability to sensitize and re-sensitize tumors to chemotherapeutic agents. Moreover, it can simutaneously activate more than one drug and prevents multi drug resistance. Previous studies have investigated suicide gene therapy as a local treatment to the tumor site without any remarkable histological adverse effects in lung cancer patients, and in glioma cancer cell lines. Recently suicide gene therapy was applied for melanoma with (HSV-tk), which converts ganciclovir (GCV). However, loco-regional administration is not always possible and therefore the “Trojan horse” approach has been investigated. In the study by Zhao Y. et. al. (2012) the tumor-tropic neural stem cells (NSCs) derived from HES1 human embryonic stem cell line had the ability to migrate from the injection site (vein systemic administration) or intracranial to the intracranial glioma xenografts. A baculovirus vector was used to insert the HSV-tk suicide gene into the cells. A concentration of ganciclovir was also administered in order for an amount of the drug to be present locally for the suicide system to act. A prolonged transgene expression was observed for three weeks. This study presented where a sustain release system of suicide gene therapy could be used as a future concept. The same concept has been also applied with MSC in a hepatocellular (HCC) model. Additionally, Wang C. et al. investigated NSCs (F3)
as dual suicide gene therapy with cytosine deaminase (CD) and Thymidine Kinase (TK) creating the NSC-F3.CD-TK. Enhanced antitumor activity was observed against lung cancer metastasis in comparison to single suicide gene therapy. Dual suicide gene therapy was also used in lung cancer cell lines with a carcinoembryonic antigen (CEA) promoter with TK and CD constructing the pCEA-TK/CD. Dual suicide gene therapy was also investigated with surviving promoter with Ad-survivin/GFP and Ad-survivin/CD/TK. A very important parameter that has to be presented is the fact that the pro-drug has to be already diffused within the target tissue prior the administration of the adenovirus in order for the therapy to be efficient. Further investigation of transporting vehicles has led to the development of nanoparticles. In the study by Duan X. et al. the novel gene transfection cationic self-assembled DOTAP and MPEG-PCL hybrid micelles (DMP) was investigated. Less toxicity was observed when compared to the polymer Polyethyleneimine (PEI) with 25kDa. The DMP delivered efficiently the urvivin-T34 25kDa. The DMP delivered efficiently the urvivin-T34 gene (S-T34A) to treat C-26 colon cancer cell lines.

Currently there are very few clinical studies in patients with suicide gene therapy and therefore every effort is welcomed. Recently the first clinical trial design for early prostate cancer was published and another one with extensive stage has already been initiated. Interestingly, novel suicide genes such as the TK007, have already been introduced and demonstrated efficiency in several cancer cell lines (G62 human glioblastoma cell line, A549 human lung carcinoma, SW620 human colorectal adenocarcinoma cell line and IPC298 human melanoma cell line) In the study by Gruber C. et al. the efficiency of 3' pre-trans-splicing molecules (PTM) was investigated and high efficiency was observed against highly malignant tumors. In the study by Di Stasi et al. the inducible caspase 9 (iCasp9) gene was investigated. It was applied to children who developed graft-vs.-host disease (GVHD) by donor lymphocytes; it was observed that the process was reversed with the novel suicide gene therapy.

Using promoters as a method to target specific overexpressed pathways has been also used for; a) carcino-embryonic antigen (CEA) b) EGFR c) prostate specific antigen (PSA) d) transferrin receptor (TfR) e) cyclooxygenase (Cox) f) Telomerase-hTERT and g) Cytokeratin 18 and 19. We suggest that a future method of application could be made with local injections or instillation with eye droplets.

Conclusions

Suicide gene therapy with ad.CD-5-FC could be used as a local treatment for primary or metastatic melanoma. We observed safety for the therapeutic dosages of 5-FC from 0.2 mg up to 1.2 mg for the normal retinal cells lines while the same dosages were lethal for the human melanoma cell lines. Future studies in animals and clinical trials remain to elicit the in vivo safety and efficiency of this therapeutic application.

Materials and Methods

Adenosine Cytosine Deaminase

The Ad.CD used in this study was kindly donated by Dr. A.B. Deisseroth (Yale University School of Medicine, New Haven, US). This vector is a replication-incompetent recombinant adenoviral vector that contained the Escherichia coli CD gene in a L-plastine promoter-driven transcription unit. Ad.CD was propagated in 293 cells (ATCC, Teddington, UK) and recovered 36 hours after infection by five cycles of freezing/thawing of the infected cells. All viral preparations were purified by CsCl density centrifugation, dialyzed, and stored in dialysis buffer (10 mM Tris pH 7.8, 150 mM NaCl, 10mM MgCl2, 10% glycerol) at -70°C before use. Titers of the viral stocks were determined by plaque assay using 293 cells by standard method.

Cell cultures and reagents

HTB-70 (melanoma cell line, derived from metastatic axillary node) and CRL-2302 (human retinal epithelium) were purchased from ATCC LGC Standards. HTB-70 cells were isolated from a 24 year old female patient and CRL-2302 cells from a 19 year old male (http://www.lgcstandards-atcc.org). HTB-70 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) culture medium (ATCC-30-2003) supplemented with 10% Fetal Bovine Serum (FBS) (Biochrom S0115). CRL-2302 cells were cultured in DMEM (ATCC-30-2006) supplemented with 10% FBS. Both cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO2. Cell lines were cultured in Comin’s tissue culture flasks (25 and 75 cm2) according to the manufacturer’s protocol. After cultures reached confluence, cells were detached with trypsin (1:250) 2.5 % (Biochrom L2133) and passaged. The indicated cell lines were seeded in 25 cm2 flasks at a seeding density of 0.7 x 106 cells. At confluence, (approximately 2.8 X 106 cells), adenovirus (85μl of Crude viral lysate –CVL, approximately 1-10pfu/cell) was added in both cell lines. The adenovirus vector was provided by Prof. A. Deisseroth, Yale University School of Medicine, and cultured in the research la-
Labortory of the Lung Tumor Research Section of the Pulmonary Dept. Aristotle Univ. Medical School. Cytopathic effect was observed only in melanoma cell lines after 36h and then Ancotil® 2.5 g/250 ml (1 g/00ml) (5-Flucytosine) MEDA; Pharmaceuticals Ltd. UK was added in both cell lines at different concentrations (0.2ml, 0.8ml and 1.2ml). At indicated time-points (4h, 8h and 24h) cell viability and apoptosis was measured.

**Trypan Blue Assay**

Trypan blue assay was applied to measure cell viability. Trypan blue dye can penetrate only porous, permeable membranes of lethally damaged (dead) cells, which is clearly detectable under optical microscopy.99 After adenovirus infection and ancotil treatment, both cell lines were trypsinized and collected, washed with PBS and suspended in complete culture medium. Then, 50μl of this cell suspension were added to 50 μL of 0.04% trypan blue dye (Sigma Aldrich Corp.). This solution was maintained in room temperature for 2 minutes to allow trypan blue penetration and then viable and dead cells were counted in the hemocytometer under an inverted light microscope (Zeiss, West Germany). Cell viability was calculated by deducting the number of nonviable cells from the number of total cells. The number of cells obtained in the counting corresponded to n × 104 cells permilliliter of suspension. (Table 2.) (Figure 1.)

**Flow Cytometry**

Separation of dead and alive cells with 7-AAD staining (7-amino-actinomycin D)

7-Aminoactinomycin D (7-AAD) is a fluorescent chemical compound with a strong affinity for DNA. It is used as a fluorescent marker for DNA in fluorescence microscopy and flow cytometry. 7-AAD staining was purchased from Immunostep company (Spain) and the analysis of the samples was performed using BD FACSCalibur 4 colors, with CELLQUEST software (BECTON-DICKINSON USA). After adenovirus infection and ancotil treatment, both cell lines were trypsinized and collected, washed with PBS and suspended in complete culture medium. 100 μL of cell suspension (concentration 3000 to 5000 cells/ μL) were added to 5 μL of 7AAD staining. This solution was incubated for 10 min in a dark place at room temperature. Then it was diluted with 0.5 ml of PBS and analyzed in the flow cytometer at indicated time-points (after 4h, 8h and 24h). (Table 3.) (Figures 2-10)

**Analysis of the apoptotic cells with ANNEXIN V/ PI**

Annexin V staining is used as a probe to detect cells that have expressed phosphatidylserine (PS) on the cell surface, an event found in apoptosis as well as other forms of cell death. Propidium iodide (PI) is used as a DNA stain for both flow cytometry, to evaluate cell viability or DNA content in cell cycle analysis, and microscopy to visualize the nucleus and other DNA containing organelles. It can be used to differentiate necrotic, apoptotic and normal cells. The Annexin V kit used in this study was purchased from Immunostep company (Spain) and the analysis of the samples were performed in BD FACSCalibur 4 colors, with CELLQUEST software (BECTON-DICKINSON USA). After adenovirus infection and ancotil treatment, both cell lines were trypsinized and collected, washed with PBS and suspended in complete culture medium. 100 μl of cell suspension (concentration 3000 to 5000 cells/ μL) were added to 500 μL of Annexin binding buffer. Then 5 μL of Annexin V and 5 μL PI were added to this solution and it was incubated for 15 min in a dark place at room temperature. Then it was analyzed in the flow cytometer at indicated time-points (after 4h, 8h and 24h). (Table 3.) (Figures 2-10)

**5-Florocytosine**

The Ancotil® 2.5 g/250 ml (1 g/00ml) (5-Flucytosine) MEDA; Pharmaceuticals Ltd. UK was purchased and used for the experiment.

![Figure 1. A) Melanoma trypan blue x 400, B) Retinal Trypan Blue x 400, C) Melanoma cells plus adenovirus x 400 (black arrows indicate the Ad.CD), D) Retinal cells plus adenovirus x 400 (black arrows indicate the Ad.CD).](http://www.jcancer.org)
Figure 2. A) Melanoma cells 0.2 mg ancostil and viability at 4 hours with 7-AAD, B) Retinal cells 0.2 mg ancostil and viability at 4 hours with 7-AAD, C) Melanoma cell 0.2mg ancostil and viability at 4 hours with annexin, D) Retinal cells 0.2 mg ancostil and viability at 4 hours with annexin.

Figure 3. A) Melanoma cells 0.8 mg ancostil and viability at 4 hours with 7-AAD, B) Retinal cells 0.8 mg ancostil and viability at 4 hours with 7-AAD, C) Melanoma cell 0.8mg ancostil and viability at 4 hours with annexin, D) Retinal cells 0.8 mg ancostil and viability at 4 hours with annexin.
Figure 4. A) Melanoma cells 1.2 mg ancotil and viability at 4 hours with 7-AAD, B) Retinal cells 1.2 mg ancotil and viability at 4 hours with 7-AAD, C) Melanoma cell 1.2 mg ancotil and viability at 4 hours with annexin, D) Retinal cells 1.2 mg ancotil and viability at 4 hours with annexin.

Figure 5. A) Melanoma cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, C) Melanoma cell 0.2 mg ancotil and viability at 8 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 8 hours with annexin.
Figure 6. A) Melanoma cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, C) Melanoma cell 0.2mg ancotil and viability at 8 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 8 hours with annexin.

Figure 7. A) Melanoma cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, B) Retinal cells 0.2 mg ancotil and viability at 8 hours with 7-AAD, C) Melanoma cell 0.2mg ancotil and viability at 8 hours with annexin, D) Retinal cells 0.2 mg ancotil and viability at 8 hours with annexin.
Figure 8. A) Melanoma cells 0.2 mg anticofil and viability at 24 hours with 7-AAD, B) Retinal cells 0.2 mg anticofil and viability at 24 hours with 7-AAD, C) Melanoma cell 0.2mg anticofil and viability at 24 hours with annexin, D) Retinal cells 0.2 mg anticofil and viability at 24 hours with annexin.

Figure 9. A) Melanoma cells 0.2 mg anticofil and viability at 24 hours with 7-AAD, B) Retinal cells 0.2 mg anticofil and viability at 24 hours with 7-AAD, C) Melanoma cell 0.2mg anticofil and viability at 24 hours with annexin, D) Retinal cells 0.2 mg anticofil and viability at 24 hours with annexin.
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Conflict of Interest

None to declare.

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