ASPECTS OF IMMUNO- AND SUICIDE GENE THERAPIES FOR CANCER - A COMBINATION WITH RADIATION THERAPY

Ph.D. thesis

Gergely Huszty, M.D.

Semmelweis University
School of Ph.D. Studies, Pathological Sciences
Chairman: Prof. László Kopper, M.D., Ph.D., D.Sc.

Tutor: Géza Sáfrány, M.D., Ph.D., D.Sc.,

Official reviewers: Veronika Karcagi, M.D, Ph.D.
László Kóbori, M.D, Ph.D.

PhD final examination board:
president: Prof. Anna Tompa, M.D, Ph.D., D.Sc.
members: András Lászik, M.D, Ph.D.
Péter Várady, M.D, Ph.D.
Zoltán Marcsek, M.D, Ph.D.

Budapest
2009

Introduction

Despite of recent advances in oncology and oncologic surgery, some tumors (e.g. pancreatic cancer and malignant gliomas) remained incurable. Having very different mechanism of action and range of side effects compared to standard treatments, gene therapy may be tolerably combined with them as an adjuvant. In our work, we focused on two major approaches to anticancer gene therapies: immuno-gene- and gene-directed enzyme-prodrug therapies (GDEPT). These gene therapies are especially promising: their effect may be influenced at lower extent by the still unsolved problem of ineffective *in vivo* gene transfer. We also examined the expression of transferrin receptor (TFRC) in human pancreatic tumors – a cell surface transporter with potential in vector targeting.

I. Intratumoral Flt3-ligand immuno-gene therapy

Growing tumors evolve various mechanisms to escape from the immune attack; immunotherapies

aim to restore and/or enhance this altered immune answer. Non-specific immun-activating therapies, adoptive immune therapies or therapeutic vaccinations with ex vivo modified cells have been tried - with limited rate of objective tumor regressions in clinical studies. Local modification of the tumor milieu by direct intratumoral transduction of immune- activating genetic material may be a safe, cheap, easy and reproducible method; however, most immunostimulatory cytokine- coding vectors have still performed poorly in humans.

Fms-like tyrosine kinase 3-ligand (Flt3-ligand) is a recently discovered haematopoietic growth factor. Its receptor is highly expressed on common lymphoid and myeloid progenitors, monocytes and steady-state dendritic cells (DCs). Systemic administration of Flt3-ligand results in expansion of DCs in an extent not comparable to any other cytokines or combinations in vivo. Intravenous Flt3-ligand therapy had antitumoral effect based on enhanced antigen presenting capacity and activation of specific cellular immune responses in animal

models, but not in humans. Little is known about the potential of local (intratumoral) application of Flt3-ligand (or genetic material coding for it), an approach with potential benefits. There is no data available concerning Flt3-ligand -based immunotherapies of pancreatic cancer at all.

II. dCK-gemcitabine GDEPT

The current treatment for malignant gliomas is surgery followed by irradiation; however, malignant gliomas are among tumors considered relatively radioresistant. One possible way to overcome tumor radioresistance is to use a gene directed enzyme prodrug therapy (GDEPT) approach, where the prodrug is a radiosensitizing chemotherapeutic agent.

The aim in GDEPT is to overexpress an enzyme within tumors, which activates a systemically delivered pro-drug. This has been the most frequently applied gene therapy approach by now. The relative success of the technique - despite of

ineffective gene transfer - is thought to be attributable to the bystander effect.

Gemcitabine is a pyrimidine analogue of deoxycytidine. In cells, gemcitabine is phosphorylated by the deoxycytidine kinase (dCK) and then by other cellular kinases to yield the active metabolites gemcitabine mono- di- and triphosphate. The first phosphorylation reaction constitutes the rate-limiting step in the activation of gemcitabine.

Gemcitabine has been shown both in laboratory and in clinical studies to be a potent radiation sensitizer. This drug became the standard chemotherapeutic agent for pancreatic cancer both in adjuvant and palliative setting; its value is currently being evaluated in combination with radiotherapy for the treatment of malignant gliomas and various brain metastases in several clinical trials.

The aim of our second study was to test the applicability of a new potential GDEPT system with potential radiosensitizing properties based on dCK and gemcitabine.

III. TFRC expression of human pancreatic tumors

Transferrin receptor may be upregulated in malignancies and be a potential tool in the diagnosis and targeting of cancer. The expression of TFRC on pancreatic cancers has not yet been investigated systematically in detail.

Aims

I. Intratumoral Flt3-ligand immuno-gene therapy

In our first experiment we aimed to

(1) describe and clone the unknown rat Flt3-ligand cDNA and create an Flt3-ligand producing plasmid vector,

(2) to test a cationic liposome - based gene delivery system for intratumoral transfection in the rat DSL6A pancreatic cancer model, and

(3) to use therapeutic intratumoral Flt3-ligand-gene delivery in the same model, follow the tumor growth and monitor immune parameters during and after therapy.

II. dCK-gemcitabine GDEPT

In our second experiment we aimed to

(4) quantify the basal dCK activity and gemcitabine sensitivity in human U373 and rat glioma C6 cell lines,

(5) to create a dCK-coding adenovirus-vector (Ad-dCK) and measure its capability to elevate the cellular dCK activity of the cell lines,

(6) to describe the effect of chemo-irradiation on the transduced cells in vitro, and

(7) to investigate the effect of chemo-irradiation on orthotopically growing C6 gliomas with increased dCK activity in vivo.

III. TFRC expression of human pancreatic tumors

In our third study, we aimed to

(8) investigate the expression of TFRC on a large series of various human pancreatic tumors, and

(9) on a large series of human pancreatic cancer cell lines.

Methods

I. Intratumoral Flt3-ligand immuno-gene therapy

The rat Flt3-ligand cDNA was amplified using the "3'-rapid amplification of cDNA ends" method from splenic mRNA extract, and than sequenced and cloned into the pcDNA3.1 plasmid. Transfectional efficiency of subcutaneously growing rat duct-like pancreatic cancers (DSL6A) with a commercially available DOTAP/cholesterol-liposome system was tested using a lacZ-pcDNA3.1 construct. Flt3-ligand production of Flt3-ligand-pcDNA3.1 transfected tumors was measured by ELISA. In the therapeutic model, tumor induction was achieved in 20 Lewis rats by s.c. inoculation of DSL6A cells. Animals were allocated into 3 groups: untreated control (n=6), liposome-pcDNA3.1 treatment (n=6), treatment with liposome-Flt3-ligand-pcDNA3.1 (n=8). Therapy was started at 5 mm tumor diameter. Intratumoral injections (10μg DNA/20 μl) were performed 6 times during 2 weeks. Tumor growth was followed for 6 weeks.

Lymphocyte infiltration, tumor proliferation rate and microvessel density were quantified by immunohistochemistry. Peripheral blood leukocytes (during therapy) and splenic leukocytes (after the 6^{th} week) were characterized by surface markers (NKR-P1A, CD4-CD25, CD8-CD28, CD18-CD62L, CD11b/c-CD40, CD80, CD86) with flow cytometry. Plasma TNF-α and INF-γ levels were measured during therapy by ELISA.

II. dCK-gemcitabine GDEPT

A dCK encoding recombinant adenovirus vector (Ad-dCK) was generated. Gemcitabine sensitivity of rat C6 and human U373 glioma cell lines was measured by survival analysis in vitro. Transfectional efficiency of the adenovirus vector was determined using Ad-LacZ at different MOI (multiplicity of infection). Cells were transduced with Ad-HudCK at different MOI, and the resulting dCK activity was measured by an enzymatic assay; potential toxicity was measured by survival analysis. To determine the combined effects of Ad-dCK

transduction, gemcitabine treatment and irradiation in vitro, C6 and U373 cells were transduced at MOIs that resulted in similar dCK activities (~8 nmol/hour/mg protein) at identical transfectional rates (~70%) in both cell lines prior to chemo-radiotherapy. Equally toxic sublethal doses of gemcitabine (25 nM for C6 and 250 nM for U373) and irradiation (4Gy) were applied. The enhancement rate of combinational therapies was calculated.

Intracranial C6 tumors were established in Wistar rats by stereotaxic implantation using wild-type or Ad-dCK pre-transduced glioma cells (70% transfection rate, 4-5 animals/group, experiments were repeated 3 times). 3 days after implantation animals were subjected to gemcitabine (60 mg/kg) and 24h thereafter to irradiation (4Gy). Survival was followed for 100 days.

III. TFRC expression of human pancreatic tumors

51 specimens of human ductal pancreatic cancer (39 primary and 12 metastasis) and 14 samples of

pancreatic neuroendocrine tumors were obtained by surgery. 8 specimens of healthy pancreatic tissue were sampled far from resected tumors. The expression of TFRC, transferrin and cytokeratin were studied by immunohistochemistry. TFRC expression of 9 human ductal pancreatic cancer cell lines was investigated by flow cytometry.

Results

I. Intratumoral Flt3-ligand immuno-gene therapy

(1) The rat Flt3-ligand cDNA consists of 693 base pairs; the calculated peptide sequence shows 72,92% and 89.65% identity compared to human and mouse peptids, respectively.

(2) 10% intratumoral transfection rate was achieved cationic liposomes in the DSL6A model; transduced tumors produced Flt3-ligand.

(3) Control tumors grew exponentially. Durable reduction in tumor volume (response) was found only in the Flt3-ligand-pcDNA3.1 treated group. Tumor volume reduction remained significant for three weeks only. Total tumor regression was

observed in one and tumor size stabilization in a second case. Other responders began to grow exponentially shortly after cessation of treatment.

There were no differences in plasma TNF-α or INF-γ levels and peripheral blood leukocyte markers during therapy. Prominently elevated level of CD80 expression was found on splenic dendritic cells of some Flt3-ligand-pcDNA3.1 treated animals, but the splenic DC number remained unaltered. Splenic NK-cell number was significantly elevated in therapy responders. The level of tumor infiltrating lymphocytes was low in every case, proliferation rate and tumor blood vessel density remained unaffected.

II. dCK-gemcitabine GDEPT

(4) Basal dCK activity was relatively low in both glioma cell lines.

(5) Higher transduction efficiency with Ad-dCK was detected in U373 cells than in C6 cells. Cellular dCK activity reached a plateau at around 8

nmol/hour/mg protein in both cell lines (~70% transduction rate).

(6) In the absence of dCK overexpression, the combination of gemcitabine treatment and irradiation had only additive effects *in vitro*. Increased dCK activity had no radiosensitizing effect. dCK overexpression enhanced the toxicity of gemcitabine (2 and 3.4-fold enhancement in C6 and U373 cells, respectively), and also strongly improved the radiosensitizing effect of gemcitabine (2.3 and 3.6-fold in C6 and U373 cells, respectively).

(7) Overexpression of the dCK gene had no effect on *in vivo* tumor growth, but had a mild radiosensitizing effect. Gemcitabine treatment had minor effect on tumor growth, which was not improved by dCK overexpression. Chemoradiotherapy of untransduced tumors led to 16% survival. Significantly improved ($P<0.0162$) survival rate (67%) and animal life span (41%) was detected when gemcitabine treatment and irradiation was combined with dCK overexpression.

III. TFRC expression of human pancreatic tumors

(8) 90% of ductal pancreatic tumors showed strongly positive or heterogeneous TFRC expression. Only malignant epithelial (cytokeratin positive) cells expressed TFRC. Primary tumors and metastases showed similar frequency of expression. Three out of four neuroendocrine carcinomas showed positive expressional pattern. TFRC was not stained on benign neuroendocrine tumors and on normal pancreatic tissue.

(9) Human pancreatic cancer cell lines were characterized by low TFRC expression (mean±SD = 8.9±5.6%) in vitro.

Conclusions

I. Intratumoral Flt3-ligand immuno-gene therapy

The sequence data of the rat Flt3-ligand published here may broaden the field of research with Flt3-ligand-based immunotherapeutic models by allowing the use of the species specific sequence.

Cationic liposomes may transfect DSL6A pancreatic cancers very effectively *in vivo*.

This experiment was the first attempt on Flt3-ligand based immunotherapy in a pancreatic cancer model, and one of the first reports trying to utilize intratumoral transfection with Flt3-ligand coding vectors instead of systemic delivery. Our results that liposome-based Flt3-ligand gene delivery may lead to total or partial tumor regression and is accompanied by detectable changes in some systemic immunparameters (splenic NK cell number, splenic CD80+ dendritic cells) is promising. However, the effect of local Flt3-ligand gene therapy was limited both in time and extent in most cases; the approach needs further refinement. The fact that the antitumoral effect of Flt3-ligand may disappear after cessation of therapy is frequently seen after systemic Flt3-ligand administration. Studying the possible underlying mechanism of our therapeutic failure would need further experiments. DC recruitment was possibly not satisfactory to the tumor site, or they may have remained locally immature. Reaching higher level of intratumoral Flt3-ligand expression or exposing

DCs to signals improving maturation may be beneficial.

II. dCK-gemcitabine GDEPT

We present the first experimental attempt to enhance the radiosensitizing effect of gemcitabine by means of gene-directed enzyme pro-drug therapy (GDEPT). Both the cytotoxic and radiosensitizing effect of gemcitabine could be significantly improved by adenovirus mediated overexpression of the dCK enzyme in murine C6 and human U373 glioma cell lines. dCK overexpression in pre-transduced C6 gliomas significantly improved the survival rate of tumor bearing rats in response to chemoradiotherapy by enhancing the radiosensitizing effect of gemcitabine. After further in vivo studies in a therapeutic setting (local transfection), the dCK/gemcitabine GDEPT system might be a candidate of adjuvant gene-therapeutical protocols against tumors, where gemcitabine and radiation is already in clinical use - such as pancreatic cancer and gliomas.

III. TFRC expression of human pancreatic tumors

We found, that in contrast to healthy pancreatic tissue and benign pancreatic neuroendocrine tumors, ductal pancreatic cancer and neuroendocrine carcinoma strongly expresses TFRC. In contrast to in vitro conditions, TFRC may be upregulated because of iron depletion in consequence of increased metabolic activity and tumor hypoxia, and thus may be an indirect marker of malignancy. This observation in pancreatic tumors may have both diagnostic and therapeutic value: the transferrin-TFRC system has already been applied for the targeted delivery of various compounds including genetic material into tumors.

Publications

Original publications related to the thesis:

1. Ryschich E, **Huszty G**, Wentzensen N, Schmidt E, Knaebel HP, Märten A, Büchler MW, Schmidt J: Effect of Flt3-ligand-gene transfer in experimental pancreatic cancer. International Journal of Colorectal Disease. 2007 Feb;22(2):215-23. **(RE&HG contributed equally to the work)**

2. Szatmári T, **Huszty G**, Désaknai S, Spasokoukotskaja T, Sasvári-Székely M, Staub M, Esik O, Sáfrány G, Lumniczky K.: Adenoviral vector transduction of the human deoxycytidine kinase gene enhances the cytotoxic and radiosensitizing effect of gemcitabine on experimental gliomas. Cancer Gene Therapy. 2008 Mar;15(3):154-64. **(RE&HG contributed equally to the work)**.

3. Ryschich E, **Huszty G**, Knaebel HP, Hartel M, Büchler MW, Schmidt J: Transferrin receptor is marker of malignant phenotype in human pancreatic cancer and in neuroendocrine carcinoma of the

pancreas. European Journal of Cancer. 2004 Jun; 40(9): 1418-22.

Other original publications:

4. **Huszty G**, Mogami K, Sawada T, Seki H, Sakusabe M, Ohuchi S, Kotanagi H: Preoperative evaluation of irreversible bowel ischemia in obturator hernia. Hepatogastroenterology. 2007 Apr-May;54(75):775-9.

5. Hortobagyi L, Kis B, Hrabak A, Horvath B, **Huszty G**, Schweer H, Benyo B, Sandor P, Busija DW, Benyo Z: Adaptation of the hypothalamic blood flow to chronic nitric oxide deficiency is independent of vasodilator prostanoids. Brain Research 2007 Feb 2;1131(1):129-37.