Adenovirus-mediated Thymidine Kinase Gene Therapy and Coxsackie Adenovirus Receptor Expression in Ovarian Cancer Cells

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Abstract. Coxsackie adenovirus receptor (CAR) expression is the main mechanism of adenovirus entry into target cells. It is unclear whether CAR expression itself is influenced by transduction with the adenovirus-Rous sarcoma virus-thymidine kinase (ADV-RSV-TK) gene therapy construct or by the subsequent intracellular accumulation of the TK gene product. Antibody generation and characterization, immunocytochemistry, Western blotting and 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay were performed to investigate the relationship of gene transfer and CAR expression as well as differences in therapeutic susceptibility of MDAH-2774 and OVCAR-3 cell lines to ADV-RSV-TK gene therapy. CAR expression was observed on the membranes but intracellular translocation of CAR also took place dependent on cellular growth patterns. TK gene expression was dependent on multiplicity of infection (MOI) and thus on vector dose in a linear fashion. Neither TK expression nor ADV transduction influenced CAR expression, or ADV-RSV-TK transduction. Differential susceptibility of different cell lines to TK-induced cell killing by acyclovir metabolites was observed. CAR expression appears not to be influenced by adenoviral transduction or by the accumulation of the TK gene product. Differences in therapeutic sensitivity are most likely mediated by intracellular mechanisms and not by modulation of CAR expression.

Adenovirus (ADV)-mediated transduction of tumor cells with therapeutic genes, e.g. the thymidine kinase gene, is believed to be mediated chiefly by the Coxsackie adenovirus receptor (CAR) expressed by cells on their surface. Expression of CAR by ovarian cancer cells has been described in vitro and in vivo (1).

Adenoviral gene therapy with the thymidine kinase gene (TK) under control of the Rous sarcoma virus (RSV) promoter followed by the administration of acyclovir leads to replication errors in transcription and to cell death. This concept has been established in vitro for the treatment of ovarian cancer cells (1) and has been used as the basis for intraperitoneal phase I clinical trials also in combination with chemotherapy (2). In these investigations, one cycle of therapy was tested and was found to be well tolerated.

It remains unclear whether CAR expression itself is influenced by transduction with the ADV-RSV-TK construct or by the subsequent intracellular accumulation of the TK gene product. The answer to this question is important when considering the therapeutic potential of repeated dosing of adenovirus-mediated gene therapy.

Materials and Methods

The human epithelial ovarian cancer cell line MDAH-2774 and OVCAR-3 were grown to 80% confluency following standard published protocols [(1), cell lines graciously provided by L.A. Jones, M.D. Anderson Cancer Center, Houston, TX, USA]. Anti-TK-antibody was generated in rabbits by EUROGENTEC against HSV-TK peptides 1 and 3. Preimmune serum, 4-week serum and 8-week serum were collected. The analysis of antibody specificity is shown in Figures 1 and 2. Anti-CAR antibody staining was performed. Western Blot analysis was used for semiquantitative analysis of CAR protein production. Analysis was performed at baseline, 8, 16, 24, 32 and 48 hours after transduction.

In order to separate vector-induced effects from transgene-induced changes, adenoviral constructs containing TK, lactoferrin Z and green fluorescent protein were used in parallel and CAR protein expression was analysed by parallel Western blotting. Prolonged analysis beyond 48 hours was not performed, as cell growth would obscure potential gene therapy effects on CAR expression.

The 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) based assay was used to quantify cell viability during treatment with HSV-RSV-TK gene therapy (3, 4). Ab 1397 was

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used for immunostaining and Western blot analysis of TK expression in the transduced ovarian cancer cells. Transgene expression was analysed using different multiplicities of infection (MOI). Susceptibility to TK activity-induced cell killing by the addition of acyclovir was compared at MOI of 0-83. All assays were run in duplicate.

**Results**

CAR expression was observed in MDAH-2774 and OVCAR-3 cells (Figure 3). It is normally limited to the cell surface. However, in both cell lines, partial cytoplasmatic translocation of the receptor was observed. Intracytoplasmatic translocation of CAR appeared to be more prominent in single cells than in cell clusters, indicating a possible role of cell-cell interaction in this process (Figure 4).

The method of fixation was found to interfere profoundly with the sensitivity of anti-TK immunocytochemistry. Methanol was shown to provide far superior results (Figure 5). The antibody performed well up to a dilution of 1:500 (Figure 6).

CAR expression over time remained unchanged by the integration of adenoviral vector particles and by the induced expression of the TK transgene. There was a clear cell line dependency in the subcellular localization of CAR expression, while absolute amounts of CAR protein and the corresponding staining intensity were comparable (Figures 7-9).

Transgene expression was dependent on the MOI in a linear dose-dependent manner and persisted after 48 hours (Figures 10-12).

MDAH-2774 and OVCAR-3 exhibited significantly different sensitivities to acyclovir induced TK-mediated cell killing, as shown by the MTT assay (Figure 13).

Adenovirus-mediated transduction of ovarian cancer cells by the TK gene and its subsequent intracellular expression did not interfere with CAR expression on the cell surface.

**Discussion**

The presented results are preliminary. Cell-cell interaction may be a defining factor in the subcellular localization of CAR in ovarian cancer cells. Ovarian cancer cell lines

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Figure 1. Thymidine kinase antibody (Ab) specificity against TK peptide 1 in rabbit sera obtained 4 and 8 weeks after inoculation. MDAH-2774 cells were transduced with ADV-RSV-TK (MOI=50) and protein lysates were made 48 hours after transduction. Negative controls are MDAH-2774 without transduction. The Western blot was performed with 6 μg protein/lane.

Figure 2. Thymidine kinase antibody (Ab) specificity against TK peptide 3 in rabbit sera obtained 4 and 8 weeks after inoculation. MDAH-2774 cells were transduced with ADV-RSV-TK (MOI=50) and protein lysates were made 48 hours after transduction. Negative controls are MDAH-2774 without transduction. The Western blot was performed with 6 μg protein/lane.
Figure 3. Immunocytochemistry of the Coxsackie adenovirus receptor in MDAH-2774 and OVCAR-3 ovarian cancer cell lines.

Figure 4. Immunocytochemistry showing different Coxsackie adenovirus receptor expression patterns in OVCAR-3 cells.

Figure 5. Influence of fixation methods on TK staining in MDAH-2774 cells transduced with AD-RSV-TK.
Figure 6. Titration of the thymidine kinase antibody (1397) (immunofluorescence staining in methanol-fixed MDAH-2774 cells).

Figure 7. Time (minutes)-dependent CAR expression in OVCAR-3 ovarian cancer cells after transduction with ADV-RSV-TK.
OVCAR-3 and MDAH-2774, while different in their growth characteristics and in their sensitivity to TK gene therapy, expressed very similar amounts of CAR receptor, indicating that intracellular metabolic mechanisms are responsible for differences in TK gene therapy effects, while the ability of adenoviral particles to enter the cancer cell and express the transgene appears to be quite uniform and comparable. Internalization is directly proportional to the MOI.

Thus, the internalization of ADV gene therapy constructs and the transcription of the transgene appear not to be the limiting steps in the ADV-RSV-TK gene therapy of ovarian cancer cells. Whether differences in subcellular CAR localization play a role in treatment susceptibility remains uncertain at this time.

Molecular therapies using small molecules have been successful in targeting well-defined cellular mechanisms ranging from immunogenicity to angiogenesis inhibition.

Figure 8. Time (minutes)-dependent CAR expression in MDAH-2774 ovarian cancer cells after transduction with ADV-RSV-TK.

Figure 9. CAR protein expression after induction with different adenovirus constructs in ovarian cancer cell lines.
However, mono-drug intrinsic activity of such agents against tumors so far has been mostly very limited. By contrast, the intraperitoneal TK gene therapy of ovarian cancer provides a suitable model for a dose-dependent biological tumoricidal treatment concept with an additional potentially synergistic effect with chemotherapeutic agents (5). Persistent expression of CAR after ADV-mediated tumor cell transduction with the therapeutic gene provides a realistic basis for the development of multi-cycle ADV-mediated gene therapy approaches in the treatment of this disease. Using the techniques and reagents developed in the context of the presented experiments, important parameters of gene therapy biology can be monitored during such clinical evaluations.

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**References**

Figure 12. Thymidine kinase expression in OVCAR-3 ovarian cancer cells 48 hours after transduction with different MOI (0-83) of ADV-RSV-TK.

Figure 13. Cell killing in MDAH-2774 and OVCAR-3 ovarian cancer cells after acyclovir treatment in cells induced with different MOI (0-83) of ADV-RSV-TK.


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