ELSEVIER

Urologic Oncology: Seminars and Original Investigations ▮ (2017) ▮▮▮–▮▮▮

UROLOGIC ONCOLOGY

Original article

Applying the chicken embryo chorioallantoic membrane assay to study treatment approaches in urothelial carcinoma

Margaretha A. Skowron, M.Sc[a], Anuja Sathe, M.D., Ph.D.[b], Andrea Romano, Ph.D.[c], Michèle J. Hoffmann, Ph.D.[a], Wolfgang A. Schulz, Ph.D.[a], Gommert A. van Koeveringe, M.D.[d], Peter Albers, M.D.[a], Roman Nawroth, Ph.D.[b], Günter Niegisch, M.D.[a,*]

[a] *Department of Urology, Medical Faculty, Heinrich Heine University Duesseldorf, Düsseldorf, Germany*
[b] *Department of Urology, Klinikum rechts der Isar der Technischen Universität Muenchen, Munich, Germany*
[c] *Department of Obstetrics and Gynaecology, GROW—School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands*
[d] *Department of Urology, Maastricht University Medical Centre, Maastricht, The Netherlands*

Received 14 February 2017; received in revised form 27 April 2017; accepted 8 May 2017

Abstract

Background: Rapid development of novel treatment options demands valid preclinical screening models for urothelial carcinoma (UC). The translational value of high-throughput drug testing using 2-dimensional (2D) cultures is limited while for xenograft models handling efforts and costs often become prohibitive for larger-scale drug testing. Therefore, we investigated to which extent the chicken chorioallantoic membrane (CAM) assay might provide an alternative model to study antineoplastic treatment approaches for UC.

Methods: The ability of 8 human UC cell lines (UCCs) to form tumors after implantation on CAMs was investigated. Epithelial-like RT-112 and mesenchymal-like T-24 UCCs in cell culture or as CAM tumors were treated with cisplatin alone or combined with histone deacetylase inhibitors (HDACi) romidepsin and suberanilohydroxamic acid. Tumor weight, size, and bioluminescence activity were monitored; tumor specimens were analyzed by histology and immunohistochemistry. Western blotting and quantitative real time polymerase chain reaction were used to measure protein and mRNA expression.

Results: UCCs were reliably implantable on the CAM, but tumor development varied among cell lines. Expression of differentiation markers (E-cadherin, vimentin, CK5, CK18, and CK20) was similar in CAM tumors and 2D cultures. Cellular phenotypes also remained stable after recultivation of CAM tumors in 2D cultures. Bioluminescence images correlated with tumor weight.

Cisplatin and HDACi decreased weight and growth of CAM tumors in a dose-dependent manner, but HDACi treatment acted less efficiently as in 2D cultures, especially on its typically associated molecular markers. Synergistic effects of HDACi and subsequent cisplatin treatment on UCCs were neither detected in 2D cultures nor detected in CAM tumors.

Conclusion: Our results demonstrate that the CAM assay is a useful tool for studying tumor growth and response to conventional anticancer drugs under 3D conditions, especially cytotoxic drugs as cisplatin. With some limitations, it might serve as a cost- and time-effective preclinical screening assay for novel therapeutic approaches before further assessment in expensive and cumbersome animal models.

Keywords: Urothelial carcinoma; Chicken chorioallantoic membrane (CAM) model; HDAC inhibitors; Cisplatin; Romidepsin; Vorinostat

1. Background

Novel antineoplastic drugs are usually first assessed in 2-dimensional (2D) cell cultures. For urothelial carcinoma (UC), a broad range of established cell culture models are available reflecting the different subtypes and heterogeneity of UC [1–3]. Cell culture models are usually simple, easy to

This study was supported by a fellowship of the Duesseldorf School of Oncology (funded by the Comprehensive Cancer Centre Dusseldorf/ Deutsche Krebshilfe and the Medical Faculty of the Heinrich-Heine University Duesseldorf) to M.A.S. and by the Strategischer Forschungsförderungs-Fond of the Heinrich-Heine University.

* Corresponding author. Tel.: +49-221-811-8110; fax: +49-221-811-8646.

E-mail addresses: Guenter.Niegisch@med.uni-duesseldorf.de, guenter.niegisch@hhu.de (G. Niegisch).

http://dx.doi.org/10.1016/j.urolonc.2017.05.003

handle, yielding results quickly, and at low cost. However, cell culture models differ substantially from the original tumor, lacking the natural tumor environment including stromal cells and vascularization [1]. Xenogeneic models using patient-derived xenografts or established UC cell lines (UCCs) overcome some of these limitations [4]. However, these models are expensive, time-consuming, and occasionally difficult to establish. Furthermore, they are associated with a high administrative effort and ethical and legal concerns.

The chicken chorioallantoic membrane (CAM) model constitutes an alternative approach in preclinical drug development. Recently, interest in this model has widened owing to its simplicity, rapidity, versatility, naturally immunodeficient, and low cost. Implantation of various carcinoma cell lines has been investigated (Table S1) [5,6]. Following seeding on top of the extraembryonic CAM, cells grow as a vascularized 3D tumor surrounded by a mesenchymal matrix. Detailed overviews on CAM physiological features are available [5–10].

As little data on the applicability of the CAM model to UCCs is so far available (Table S2), the aim of our study was to systematically investigate CAM model applicability in UC preclinical research. We thus investigated the potential of commonly used UCCs to form tumors on CAMs, compared these to the corresponding 2D cell cultures, and ascertained their molecular phenotype. Further, we investigated whether antineoplastic drugs for UC, specifically cisplatin and histone deacetylase inhibitors (HDACi) and their combination, can be reliably tested in the CAM model [11–13].

2. Material and methods

2.1. Cell culture and transfection

UCCs RT-112, VM-CUB-1, 5637, HT-1376, UM-UC-3, T-24, 639 V, and J82 were grown in DMEM GlutaMAX-I (Gibco) containing 10% FCS (Merck Millipore) as described [13]. Immortalized human urothelial HBLAK cells were cultured in CnT-Prime Epithelial Culture Medium [14]. Cell lines were authenticated by standard DNA fingerprint analysis [12]. RT-112 cells were stably transduced with a lentivirus expressing luciferase (RT-112 Luc).

2.2. CAM assay

The assay was performed as described and detailed in supplemented methods [15]. Shortly, fertilized Leghorn chicken eggs were incubated at 37°C and opened on embryonic day 4 (E4). On E8, cell pellets were seeded onto the CAM. Tumors were topically treated with HDACi or cisplatin between E11 and E14, and they were harvested on E15 for RNA, protein extraction, paraffin embedding, or 2D reculturing. HDACi or cisplatin dosage was adapted to the estimated blood volumes of corresponding embryonic days [16,17].

2.3. Molecular analyses

RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed as described [18] using self-designed primers (Table S4). For normalization, the housekeeping gene *SDHA* was used. Determination of protein concentrations and western blot analysis of whole-cell extracts were performed as described [12].

2.4. Detection of luciferase activity

About 94.2 mM D-luciferin solution was topically applied onto the CAM tumor on E15, and bioluminescence images (BLI) were acquired after 10 minutes. In 2D cultures, 0.47 mM D-luciferin was applied for 10 minutes.

2.5. Immunohistochemistry

Endogenous peroxidases were deactivated, antigens retrieved, and nonspecific antibody binding blocked by incubation in 0.5% H_2O_2 in methanol, TE buffer, and 5% goat-serum, respectively. Tissues were incubated with antibodies overnight at 4°C (Table S5) and counterstained with hematoxylin.

2.6. Statistical analysis

SPSS Statistics software version 21 (IBM) was used for statistical analysis. Differences between groups were analyzed using Student's *t*-test after checking for normal distribution of results. Bonferroni's post hoc test was used to correct for multiple comparisons. Correlations were calculated by Pearson's test. All results were confirmed by independent experiments. Statistically significant differences are highlighted by asterisks ($^{*}P < 0.05$ or $^{**}P < 0.01$).

3. Results

3.1. Most UCCs form CAM tumors

UCCs HT-1376, RT-112, VM-CUB-1, and 5637 cells (epithelial phenotype) as well as J82, UM-UC-3, T-24, and 639 V, (mesenchymal phenotype) and benign HBLAK were tested for their ability to form CAM tumors [14,18]. Except for 5637 cells, implantation was successful for all UCCs and for HBLAK. Notably, tumor weights and sizes differed for each cell line and did not always correlate (Fig. 1, Table S6).

Macroscopic growth patterns of RT-112 and T-24 cells were further compared. Take rate (RT-112: 89.2%; T-24: 69.3%), tumor weight, and size were higher for RT-112 than T-24, and RT-112 tumors appeared to be more