Targeting the DNA damage response in cervical cancer
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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The chick embryo chorioallantoic membrane model as a platform to study chemoradiotherapy responses in cervical cancer

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Abstract

Introduction: Treatment modalities used in locally-advanced cervical cancer include platin-based chemotherapy and radiotherapy. However, treatment resistance often develops. Novel treatment approaches are therefore needed. Treatment outcome might be improved by sensitizing cervical cancer cells for chemo-/radiotherapy using molecularly targeted agents. To efficiently screen drug candidates, we aimed to investigate the chick embryo chorioallantoic membrane (CAM) assay as an in vivo platform to identify chemo-radiotherapy sensitizing compounds using a 3-dimensional (3D) in ovo cervical cancer model.

Methods: HeLa cervical cancer cells were inoculated on the CAM of fertilized DeKalb chicken eggs at day 6 of embryonic development to establish cervical cancer tumors. Tumors were treated with cisplatin and/or ionizing radiation: on day 10 of embryonic development. Hematoxylin and eosin staining and immunohistochemical analysis of Ki-67, gamma-H2AX (γ-H2AX) and phosphorylated replication protein A 32 (RPA32) were performed to evaluate tumor grafting and therapy delivery.

Results: Viable 3D cervical cancer tumors were established using the CAM-assay. Tumor reduction was observed after a single dose of cisplatin (10 μg: \( P = 0.03 \); 50 μg: \( P = 0.05 \)) or ionizing radiation (4, 6 and 8 Gy; all \( P<0.01 \)) as well as with combined treatment (1 μg cisplatin with either 2 or 4Gy: \( P = 0.02 \) and \( P<0.01 \) respectively).

Conclusion: We have established a 3D-cervical cancer in vivo model by using the CAM assay where single or combined treatment with cisplatin or radiation resulted in tumor reduction. Hereby we provide a reliable, cost-effective and fast screening tool to evaluate chemoradiotherapy-sensitizing effects of novel therapeutic compounds using an in ovo cervical cancer xenograft model.
6. The chick embryo chorioallantoic membrane model as a platform to study chemoradiotherapy responses in cervical cancer

Introduction

Cervical cancer is a common female malignancy which accounts for approximately 8% of all the cancer-related deaths of women worldwide [1]. When cervical cancer is locally-advanced, cure is primarily achieved by use of platin-based chemoradiotherapy [2-4]. Nonetheless, therapy success is not complete; even in stage IIB the 5 year overall survival is 65.8% [5].

In the last decades, multiple molecularly targeted therapies have been developed with the aim to potentiate chemotherapeutics or radiotherapy. In the context of cervical cancer, many of these compounds have thus far only been tested preclinically [6, 7]. Clinical translation of candidate drugs requires careful choices given the large number of drug candidates as well as the fact that the pharmacodynamic properties of many of these compounds preclude in vivo testing [7]. In preclinical studies novel therapeutic modalities are typically only tested as monotherapy or in combination with a single DNA damaging agent [7,8]. Notably, novel compounds are typically not tested in the context of chemoradiotherapy, which is the current standard of care in locally advanced cervical cancer. An easy and cost-effective platform to test novel compounds for their chemo- and/or radiosensitizing properties, is therefore warranted to make a pre-selection for further in vivo testing.

Cell lines xenografted in immune-deficient mice are frequently used as an intermediate step to validate in vitro observations into the in vivo situation. However, testing of series of compounds in mice remains expensive, time consuming and goes at the expense of many animals [9]. An interesting alternative to efficiently select the most promising agents for further testing is the use of eggs to perform the chicken embryo chorioallantoic membrane (CAM) assay.

The CAM assay is an established model to study angiogenesis, which has been successfully employed to investigate novel anti-angiogenetic drug in the context of tumor growth and metastasis [10-13]. Many different cell lines, including cervical cancer cell lines, were shown to engraft efficiently onto the CAM [14-17]. The extensive vascularization and natural immunodeficiency probably contribute to the high grafting rate of the CAM model [18, 19]. This model provides important additional advantages compared to in vitro models. Specifically, engrafted tumors grow 3-dimensionally and are vascularized with bloodvessels originating from the CAM [15, 20, 21]. This enables drug delivery via a vascular system and with the CAM model being a closed system, the required amounts of the tested agents are limited [22]. Another advantage of the CAM assay versus a cell line model is the presence of a microenvironment [22, 23]. During tumor inoculation, the ectoderm is mechanically damaged, which results in a breached epithelial and basal membrane layer [24, 25]. Tumor cells therefore proliferate while being surrounded by epithelial cells and extracellular matrix proteins, originating from the mesoderm [23, 26]. Furthermore, the easy handling of the CAM assay and the low cost make it a promising preclinical tool for efficient selection of drug candidates.

Although the characteristics of the chicken embryo CAM model have been previously described extensively [12, 14, 15, 27, 28], the combined use of radiotherapy and chemotherapy in this model has not been reported thus far. In this study, we have therefore tested the combined treatment with cisplatin and ionizing radiation of HeLa cervical cancer cells engrafted on the chicken CAM. Our data show that the chicken embryo CAM assay is an efficient model to study the effects of radiotherapy and chemotherapy in cervical cancer cells. This allows the investigation
and selection of novel molecularly targeted compounds in a combined chemoradiotherapy setting in a fast, and cost-effective way.

**Results**

**HeLa cells engraft in ovo and form viable 3D-tumors**

To establish a 3-dimensional cervical cancer model for studying anti-cancer therapeutic interventions, we first assessed whether HeLa cervical cancer cells can be engrafted on the chicken embryo CAM [16, 17]. Our procedure is described in detail in the materials and methods paragraph and graphically represented in Figure 1A. In summary, 5x10^6 HeLa cells were inoculated onto the CAM on day 6, and the chicken embryos were terminated on day 17. Tumor morphology, viability as well as proliferation were evaluated by H&E staining and Ki-67 immunostaining respectively. As illustrated in Figure 1B, we observed that HeLa tumors consistently showed a highly homogenous morphology and were extensively vascularized in ovo. These tumors were viable, based on the observed gradual increase in tumor size and the high percentage of Ki-67-positive cells throughout the entire tumor on day 17. Around 75% of the obtained eggs could be used for inoculation, as the remaining part was not fertilized or showed idiopathic embryonic mortality between day 0 and 4. Upon inoculation, 100% of chicken embryos survived until day 17. Notably, we did not observe macroscopic metastases of HeLa cells into the chicken embryo, a phenomenon on that was observed using other tumor cell lines [11, 29, 30].

**Figure 1.** HeLa cervical cancer cells in ovo. **A.** General experimental design of cervical cancer xenografts in ovo. **B.** Immunohistochemical representation for H&E and Ki-67 staining in HeLa xenograft tumors. Indicated with the black arrow is an example of intra-tumor blood vessel formation.
Cisplatin treatment reduces growth of HeLa xenografts \textit{in ovo}

Cisplatin is commonly administered as concomitant chemotherapy in advanced cervical cancer patients [2]. To examine whether the effects of cisplatin treatment can be measured \textit{in ovo}, we first examined whether cisplatin was taken up by the CAM and subsequently delivered to the tumor. Cervical cancer xenografts were established on the CAM and left untreated until day 10. At this time point, we applied 5 μg cisplatin on the CAM and harvested the tumor 6 hours after cisplatin administration. To measure effective cisplatin uptake, we assessed the phosphorylation of replication protein A-32 (RPA32) on serine-33 (Ser-33), which is an established substrate of the ATR kinase, which is activated upon cisplatin treatment (Fig. 2A). Immunohistochemical analysis showed increased phosphorylation of RPA32 after treatment with cisplatin, indicating that cisplatin systemically arrived in the HeLa xenograft (Fig. 2A). To subsequently assess whether cisplatin treatment resulted in tumor weight reduction, we applied different concentrations of cisplatin, as previously used in literature (1 μg, 10 μg, 50 μg and 100 μg) [31]. Per condition, 9-10 fertilized eggs were inoculated, but only 3-4 could be included for analysis per treatment group due to lack of engraftment of the tumor cells in eggs, or excess (>25%) CAM tissue in the harvested tumors. Significant tumor weight reduction was observed after treatment with a single dose of 10 μg cisplatin ($P=0.03$) and 50 μg cisplatin ($P=0.05$) (Fig. 2B). Not unexpectedly, cisplatin treatment also affected viability of the embryo, and we observed a complete embryo mortality at 17 days after treatment initiation with 100 μg cisplatin [31, 32].

![Figure 2: In ovo cisplatin delivery and tumor weight reduction.](image)

\textbf{Figure 2. In ovo cisplatin delivery and tumor weight reduction.} \textbf{A.} phospho-RPA32 (Ser-33) expression in HeLa xenografts \textit{in ovo} on day 10, at 6 hours after cisplatin (5 μg) administration. \textbf{B.} Tumor weight reduction by single-dose treatment of cisplatin (0, 1, 10, 50 and 100 μg) measured on day 17; * $P<0.03$; ** $P=0.05$; § = embryonic lethal dose (100 μg).

Ionizing radiation reduces weight of HeLa xenografts \textit{in ovo}

Ionizing radiation (IR) is the backbone modality in the treatment of advanced cervical cancer [2, 33]. To study whether IR could be efficiently delivered in HeLa tumors \textit{in ovo}, we performed...
whole egg irradiation (10 Gy) on day 10. At 6 hours after irradiation, tumors were harvested and processed for immunohistochemical analysis of DNA breaks, using γH2AX staining. We observed increased nuclear γH2AX expression after IR (10 Gy) throughout the tumor (Fig. 3A). Subsequently, we tested whether IR treatment reduced tumor weight in ovo. We used increasing doses of IR (0, 1, 2, 4, 6 and 8 Gy; n=7-15 per condition). Compared to our non-irradiated controls, a significant tumor weight reduction (P<0.05) was observed after treatment with 2, 4 and 6 Gy (Fig. 3B). Treatment with a dose of 8 Gy resulted in 100% embryonic lethality on day 17, and precluded analysis of the xenografted tumor cells (Fig. 3B).

Figure 3. In ovo IR delivery and tumor weight reduction by IR. A. γH2AX (Ser-139) expression in HeLa in ovo xenografts. Treatment with 5 Gy resulted in increased nuclear γH2AX expression throughout the tumor. B. Tumor weight reduction by single-dose IR (0, 1, 2, 4, 6 and 8 Gy) treatment measured on day 17; * P<0.05; § = embryonic lethal dose (8 Gy).

Combined therapy with cisplatin and ionizing radiation reduces tumor weight in a HeLa in ovo xenograft

Finally, we combined irradiation and cisplatin treatment, as this is the standard of care for advanced stage cervical cancer patients, and novel therapeutics should ideally be tested in this context. To this end, we measured whether a fixed dose of cisplatin (1 μg), either alone or in combination with 2 or 4 Gy IR resulted in reduced tumorweight in ovo (n=15 per condition; Fig. 4A). As a result of embryonic death before our endpoint on day 17, differences exist in the final numbers of included replicates per condition, which are shown in Figure 4A. Importantly, a tumor weight reduction was observed with cisplatin combined with either 2 Gy (P= 0.02) or 4 Gy (P<0.01) when compared to the control-treated CAMs (Fig. 4A).
Figure 4. In ovo tumor weight reduction by chemoradiotherapy and a model for potential experimental set-ups A. Tumor weight reduction by combined chemoradiotherapy (* P=0.02; ** P<0.01) in a HeLa CAM xenograft measured on day 17. B. Model that illustrated potential opportunities of the CAM model.

Discussion
To our knowledge, this is first report in which combined chemoradiotherapy is examined in ovo. We showed that both irradiation and cisplatin effectively induce DNA damage in HeLa cells in ovo. Treatment-induced DNA damage was assessed by immunohistochemical analysis of phospho-RPA32 (phospho-Ser-33) and γH2AX(phospho-Ser-139) for cisplatin and radiotherapy respectively. Importantly, both treatments individually as well as in combination resulted in a reduction of tumor weight (Fig. 2B, 3B, 4A). We thus propose the CAM HeLa xenograft as a platform to study novel therapeutics, such as small molecule DNA repair inhibitors, in combination with chemoradiotherapy (Fig. 4B). This model not only allows studying effects on tumor growth, but also other tumor properties, such as metabolic processes, metastasis and angiogenesis.

As in other models, use of chemicals in the CAM assay is restricted by dose-limiting embryonic toxicities. Growth of the tumor xenograft will only be maintained when the chicken embryo
is vital. Single intra-amniotic injection of cisplatin in a range of 0.3-15.0 μg on embryonic day 5 caused teratogenic or lethal effects in chicken embryos [32]. However, administration of cisplatin in a later development stage causes less chicken embryo lethality [34]. In literature, intravenous injection of 4.7 μg cisplatin on embryonic development day 9, caused 48h after cisplatin administration no chicken embryo mortality [34]. We observed no chicken embryo lethality with a single dose cisplatin of 1, 10 or 50 μg, when applied on to the CAM. However, it has been shown that intravenous injection of 47 μg of cisplatin results in complete mortality 48h after administration [34]. This discrepancy between our findings and the reported embryo lethality at lower concentrations is likely related to the different route of administration. Specifically, our route of cisplatin administration directly onto the CAM, likely results in lower cisplatin exposure to the embryo, when compared to intravenous injection. Thus, our experiments show that a single local administration of 1, 10 or 50 μg cisplatin onto the CAM can be used to study tumor growth effects, without causing embryo lethality. Analogously, our findings suggest an embryonic safe use of ionizing radiation administered in a single dose of up to 6 Gy, with doses of 8 Gy and higher causing embryo lethality. Importantly, not teratogenic effects nor additional loss of embryo weight was observed when IR (2 Gy or 4 Gy) was used in combination with cisplatin.

Specific considerations should be made when using xenografted cell lines on the CAM. Importantly, chicken embryo movement and hatching will affect the ability to accurately measure tumor weight on the CAM. The embryo will increasingly occupy the intra-ovo cavity during the early stages of embryonic development. Also embryonic movements occur with more frequency in later stages of development. Both these factors may influence the position of the tumor xenograft and influence tumor xenograft physiology and consequently may shorten the time window when tumor growth can be reliably assessed. In addition, after approximately two weeks, the chicken immune system starts to develop, with T and B cells being found around day 11 and 12 respectively [35]. So although the chicken embryo is considered immune-deficient, chicken embryos eventually become immunocompetent on day 18, which may result in immune responses directed against the xenografted tumor cells [36].

Not withstanding these limitations, the CAM model has numerous advantages. As the CAM model is a closed system, only small amounts of candidate drugs are required. Together with the low costs of fertilized eggs, and the ability to maintain fertilized eggs in atmospheric incubators, results in low overall costs. Additionally, the relatively short duration of the CAM assay allows for efficient screening of panels of drug candidates and combinations of such drugs with currently used radiochemotherapy. Finally, this model has the potential to include the analysis of important parameters, including hypoxia, and allows to study metastasis and angiogenesis in the context of radiochemotherapy (Fig. 4B).

Materials and methods
Chemicals and cell culture
The HeLa cervical cancer cell line was cultured in Dulbecco’s modified Eagle’s medium (DMEM): Ham’s F12 (1:1) culture media, supplemented with 10% fetal calf serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Short tandem repeats (STR)-profiling was used to confirm cell
line identity (BaseClear, The Netherlands). Upon indicated treatment regimes, HeLa xenografts were treated with cisplatin (Tocris Biosciences) and/or exposed to a cesium\(^{137}\) source (0.01083 Gy/s; CIS International/IBL 637).

**CAM-assay procedure**

Previously described protocols were adapted and optimized for our lab [13, 16, 18, 20, 22]. In summary, fertilized Dekalb chicken eggs (from 26-50 weeks old chickens) were rinsed with 70% ethanol and incubated while rotating in a 37°C and 60% humidified atmosphere on day 1. On day 3, rotation was discontinued and an airpocket was created via puncture of the shell with a 26 gauge needle, to allow the CAM to dissociate from the shell. The punctured hole was covered with tape, and eggs were returned to the incubator. On day 6, embryo viability was confirmed based on visual inspection of blood vessels establishment using a lamp. If vital, the opening in the shell was widened to create a greater window. Subsequently, the CAM was gently mechanically damaged to stimulate re-vascularization, and the CAM was then inoculated with 5x10^6 HeLa cells in 50 μl of ice-cold culture media mixed 1:1 with Matrigel (Corning, #356230). The window was again covered with tape and returned to the incubator. Conform literature, treatment intervention has started at 96 hours after inoculation (at day 10), when tumor cells were engrafted and vascularized (see Figure1A for timeline) [21]. Treatment consisted of cisplatin and radiotherapy. If indicated, 3 hours prior to cisplatin treatment, an elastic latex ring (Dentsply International) was applied onto the CAM to ensure administration of cisplatin locally at a distant site from the tumor, and to ensure that cisplatin uptake in the tumor was through blood vessels. If indicated, whole egg irradiation was used with indicated doses (CIS International/IBL 637, equipped with a cesium\(^{137}\) source, 0.01083 Gy/s). Chicken embryos were terminated on day 17 and tumors were excised from the CAM. Tumors were weighted prior to fixation in 4% formaldehyde and were subsequently paraffin-embedded.

**Immunohistochemistry**

Tumors were sectioned into 4 μm slices and mounted on amino-propyl-ethoxy-silan-coated glass slides. Before staining, xylene was used to deparaffinize tissue slides. Blocking of endogenous peroxidase was performed by incubation for 30 minutes with 0.3% hydrogen peroxidase. Slides were stained with hematoxylin and eosin (H&E) for histopathological analysis. On serial slides, DAB staining was used to visualize antibody staining and hematoxylin was used for counterstaining. Slides were either stained for phosphorylated replication protein A 32 (pRPA32) on serine-33 (ser-33), gamma-H2AX (γ-H2AX) on serine 139 (ser-139) or Ki-67. Further details of antigen retrieval, primary antibodies and detection methods are summarized in Supplemental Table 1. Representative control immunostainings of used antibodies are demonstrated in Supplemental Figures 1 and 2.

**Statistical analysis**

Data were expressed as the means ± standard deviations (SD). The differences between two groups were analyzed using the one-way Mann-Whitney U-test. Eggs were included for statistical analysis when 1) tumor cells showed successful engraftment as judged by proliferation, tumor...
architecture and tumor integrity), and 2) >75% of the tissue with the H&E staining consisted of tumor cells. Therapy effects were measured by tumor weight. Statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad software). Statistical differences were considered significant if $P<0.05$.

**Acknowledgments**

We thank Marloes Peters and other members of the Medical Oncology and Gynecological Oncology Departments for helpful discussions. This work is supported by grants from the Dutch Cancer Society (RUG/NKB 2010-4833) and from the Netherlands Organization for Scientific Research to M.A.T.M.v.V. (NWO-VIDI 016.136.334). H.W.W. is supported by a fellowship of the Junior Scientific Master class (JSM) of the University of Groningen.
6. The chick embryo chorioallantoic membrane model as a platform to study chemoradiotherapy responses in cervical cancer

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Supplementals

Supplemental Table 1. Details of antibodies used for immunohistochemical analysis.

<table>
<thead>
<tr>
<th>Target</th>
<th>Brand</th>
<th>Catalog no.</th>
<th>Antigen retrieval</th>
<th>Dilution</th>
<th>Incubation time</th>
<th>Detection method</th>
</tr>
</thead>
<tbody>
<tr>
<td>γH2AX</td>
<td>Millipore</td>
<td>05-636</td>
<td>Citrate (pH 6.0)</td>
<td>1:300</td>
<td>60 minutes RT</td>
<td>RAMpo-GARpo</td>
</tr>
<tr>
<td>pRPA32</td>
<td>Bethyl</td>
<td>A300-246A</td>
<td>TRIS/HCL (pH 9.0)</td>
<td>1:1500</td>
<td>60 minutes RT</td>
<td>Avidin-biotin</td>
</tr>
<tr>
<td>Ki67</td>
<td>Abcam</td>
<td>Ab15580</td>
<td>Citrate (pH 6.0)</td>
<td>1:500</td>
<td>60 minutes RT</td>
<td>GARpo-RAGpo</td>
</tr>
</tbody>
</table>

TRIS = tris(hydroxymethyl)aminomethane; HCL = hydrochloric acid; No. = number; RT = room temperature; RAMpo = Rabbit Anti-Mouse horseradish peroxidase; GARpo = Goat Anti-Rabbit horseradish peroxidase; RAGpo = Rabbit Anti-Goat horseradish peroxidase; o/n = overnight.

Supplemental Figure 1. A, H&E staining in HeLa xenograft tumor as used for Ki-67 staining in Figure 1. B, representative immunostainings using control PBS and IgG1 antibody in human colon tissue and HeLa cells.
Supplemental Figure 2. A, representative immunostainings for IgG antibodies for phospho-RPA32 and γH2AX in para n embedded HeLa cell line pellets. Cells were 6 hours before harvesting treated with 5μM cisplatin. B, representative immunostainings for phospho-RPA32 (S33) and γH2AX (S139) in para n embedded HeLa cell line pellets which were either untreated or treated with cisplatin 6 hours before harvesting. C, representative immunostainings for phospho-RPA32 (S33) and γH2AX (S139) in in ovo HeLa xenograft. Six hours after cisplatin or vehicle treatment, HeLa xenografts were harvested.