Endostatin Effects on Tumor Cells and Vascular Network of Human Renal Cell Carcinoma Implanted on Chick Embryo Chorioallantoic Membrane

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Abstract. *Rare and inconsistent data are reported for chorioallantoic tumor models of renal cell carcinoma and none of them has used endostatin as an inhibitory agent of tumor development. We aimed to perform a comparative analysis of tumor cells and blood vessels from renal cell carcinoma on endostatin-treated and non-treated chorioallantoic membrane (CAM) implants by the assessment of endoglin, vascular endothelial growth factor (VEGF) and smooth muscle actin expression. Endostatin triple action on tumor, endothelial and perivascular cells was observed in the present study. Differential impact of endostatin treatment on intratumor and peritumor blood vessels was noticed on the VEGF expression and behaviour of tumor cells between clear cell and papillary components of RCC. Based on our findings, a high tumor heterogenity response to endostatin has been highlighted. Interplay between VEGF, endoglin and endostatin in RCC could support a combined targeted therapy to improve prognosis of patients with RCC and reduce therapy resistance often developed by monotherapy.*

Renal cell carcinoma (RCC) accounts for approximately 2% of all malignancies and is the most common malignancy in the kidney (1). The incidence of kidney cancer has increased in recent decades, and is reported to be 2-4% of all cancers per year. Targeted therapies of advanced renal cell tumors are promising but, for certain patients, therapeutic resistance develops even from the begining of these treatments. Most

patients develop resistance in about 6-11 months after starting therapy and complete response is very rare (2). Approximately 30% of patients with localized RCC will develop metastases after curative surgery. Several strategies have been developed to target angiogenesis as a method for treating metastatic RCC.

Endostatin is a C fragment of collagen XVIII that has anti-angiogenic activity (3). Use of endostatin as an adjuvant therapy for renal cancer has been less studied. Endostatin serum level is high in patients with clear cell renal cell carcinoma and is correlated with a higher stage and grade (4). Retroviral endostatin gene transfer led to secretion of functional endostatin sufficiently active to inhibit tumor angiogenesis and tumor growth (5).

Several experimental models performed in mice have shown that endostatin has an effective anti-angiogenic potential against RCC tumors and possess anti-metastatic effects (6). Most studies used endostatin associated with other anti-angiogenic or anti-tumor agents (6-8). Mice models are useful experimental tools for the evaluation of tumor response to different therapies but, unfortunately, they have several limitations that mainly arise from the impossibility to observe first stages of tumor response to the applied therapies.

The chick embryo CAM is a cheap, easy-to-perform, reproducible model for testing tumor behavior and response to various drugs (9). The development of chick embryo chorioallantoic model for renal cancer assessement by using human renal cell carcinoma fragments grafted on chick CAM is a relatively new experimental model, established for the first time by Fergelot *et al*. (7). Prior to the present study, all implants on chick CAM were performed by preferential use of renal cell carcinoma cell lines and to a lesser extent by human tumor specimen implantation. The use of renal cell carcinoma cell lines excluded tumor stroma. It is well-known that the tumor microenvironment plays an important role for tumor development and, moreover has a crucial role in tumor response to therapy. For these reasons, the present study used human renal cell carcinoma fragments (including tumor cells

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and stroma) implanted on chick embryo CAM in order to evaluate the effects of endostatin on tumor cells as well as the vascular network inside and around tumor implants.

Materials and Methods

Tumor specimens. Specimens were collected from patients with renal tumor masses detected by ultrasonografic methods and treated by surgery. Informed consent was obtained from each patient before the beginning of the experimental model. Tumor biopsies used as implants on CAM were washed with isotonic saline solution and implanted on CAM no later than 30 min after their removal from patients' bodies. A correspondent fragment from each tumor was fixed in buffered formalin for 24 h and then parraffin embedded.

Experimental model design. Two groups of 10 fertilized eggs each, were organized for the present study. Chick embryo chorioallantoic membrane preparation was performed following protocols described in our previous similar studies (10, 11). The study started on day 7 of incubation by placing on the surface of the chorioallantoic membrane, in a silicon ring, 0.2/0.2 cm renal tumors fragments. Tumor fragment was implanted in a blood vessel free area, close to bifurcation of big vessels of the CAM.. The addition of endostatin started on day 7 also, for the treated group. For each treated egg, we used 2 μl of endostatin at a concentration of 500 μg/ml. The control group was treated with 2 μl of distillated water/egg. During the experiments, all implants were carefully monitored by stereomicroscopy regarding tumor viability and blood vessels aquuisition. The experiment was stopped on day 14 of incubation (day 7 post-implantation of the tumor) and collected tumor implants were fixed in bufferred formalin, followed by parraffin embeeding.

Histopathological evaluation of specimens. We performed 3-μm serial sections from each paraffin block (both from tumor biopsies and chick CAM implants specimens). Histopathological diagnosis and morphological assessement included haematoxylin and eosin staining followed by microscopic analysis. Based on these procedures, we selected slides for immunohistochemistry.

Immunohistochemistry. Immunohistochemical workflow was fully automated, and it was performed with BOND MAX System (Leica Microsystems, Linford Wood, Milton Kenyes, UK). Evaluation of angiogenic growth factors and vascular network included immunohistochemical procedures which specifically highlighted endothelial cells from tumor blood vessels and also, comparative expression of growth factors in tumor cells from treated and non treated RCC implants. Two types of endothelial markers specific for human endothelial cells were used: a panendothelial marker CD34 (clone QBEnd10, ready to use, Novocastra, Newcastle, UK) and endoglin (CD105, clone SN6h, Dako, Carpinteria, USA). Primary antibody for VEGF assessement was of mouse anti-human monoclonal type (clone VG1, dilution 1:50, Dako Carpinteria USA). Fully standardized Polymer Refine Detection System (Leica Microsystems, Newcastle, UK) was then applied for visualisation of the blood neovessels and VEGF expression in tumor cells.

RNAscope technique assessed celular RNA content as single molecule visualisation in individual cells on formalin fixed paraffin embeeded (FFPE) specimens through use of a novel probe design strategy and a hybridization-based signal amplification system to simultaneously amplify signals and suppress background. A HRP conjugated double-Z probes for VEGF, complementary for the target RNA were designed by Advanced Cell Diagnostics (Hayward, CA, USA). We used RNAscope 2.0 High Definition Kit (Advanced Cell Diagnostics) for signal amplification followed by visualisation of amplified signals with diaminobenzidine as chromogen.

Briefly, to prepare FFPE specimens for RNAscope procedure, the tissues were fixed and permeabilized to allow for target probe access. A 2-h hybridisation step performed at 40°C was followed by multistep signal amplification performed by applying reagents followed by RNA visualisation as brown dotted spots with diaminobenzidine. The working protocol was similar with those described by Wang *et al*. (12). The detection sensitivity of RNAscope method was proved by using positive (POLR2A) and negative (probes against the bacterial gene dapB) controls assessed by using the same protocol as for pterygium specimens.

Interpretation of VEGF mRNA amplification. The procedure was manually performed in a semi-quantitative manner following scoring guidelines provided by the manufacturer. The presence of *VEGF* mRNA amplification was scored into five grades as 0 (no staining or less than 1 dot to every 10 cells, ×40 magnification), 1 (1-3 dots/cell visible at magnification ×20-40), 2 (4-10 dots/cell, very few dot clusters visible at magnification ×20-40), 3 (>10 dots/cell, less than 10% positive cells have dot clusters visible at magnification ×20) and 4 (>10 dots/cell, more than 10% positive cells have dot clusters visible at magnification ×20).

Microscopic analysis and image aquisition was performed with AxioZoom A2 research microscope (Zeiss, Jena, Germany). Also, for dynamic assessement of tumor implants we used AxioZoom Stereomicroscope from the same manufacturer. Counting of blood vessels was manually performed by using a protocol previously described by Weidner *et al*. (14). VEGF expression in tumor cells was noted with a score between 0 (no presence) to 3 (intense expression). Immunohistochemical expression was confirmed by *in situ* hybridisation RNA scope method.

Results

Morphological assessement of intratumor and peritumor vascular network and tumor cells. The RCC tumor implants from both groups (control and endostatin-treated group) were alive after 7 days of implantation. Two main aspects were considered regarding the evaluation of RCC implants on CAM. The first one referred to the tumor parameters evaluation (size, tumor cells viability, presence of necrosis, growth factor expression by tumor cells) and the second one was focused on peritumor and intratumor vascular network differences observed between control and treated groups.

The implanted tumors from the control group gradually increased in size inside the silicon ring and acquired blood vessels from the host CAM which were radially organized around tumor implants. By contrast, endostatin-treated tumors decreased in size despite the fact that they retained a well-visible vascular network on the surface of the implants. These macroscopic observations were microscopically confirmed by the presence of blood vessels containing

nucleated red blood cells for both treated and non treated tumors. Presence of nucleated red blood cells (specific for chicken) inside blood vessels from the tumors core proved the inter-connection between tumor implants and the host by aquisition of new blood vessels by the tumor and its perfusion establishment.

The intratumoral blood vessels remained viable despite of endostatin treatment. The evidence for this is that, on day 7 post-implantation, we were able to highlight intratumor persistent blood vessels with nucleated red blood cells inside. This proves that after 7 days of treatment with endostatin, the intratumor blood vessels persist and moreover are still functional by the presence of blood flow with nucleated elements from the host.

Despite the persistence of intratumor blood vessels inside endostatin-treated tumors, they had a different morphology compared to correspondent blood vessels from the non-treated group. Persistent intratumor blood vessels were small with a well-defined perfused lumen but whitout adjacent collaterals, compared to those from non-treated tumors that were large and highly branched. No significant decrease of microvessel density was observed between the intratumoral area of treated and non-treated implants in a comparative study of CD34 and CD105 immunohistochemical stainings. Also, for both types of stainings, several vascular-like channels without blood content inside but lining of CD34- and CD105-positive structures has been identified.

The acquisition of new blood vessels was more obvious in the peritumoral area, around the silicon ring containing tumor implants. The peritumoral vascular network of non-treated tumor implants had a radial arrangement around the silicon ring in a „spoke wheel" like fashion. Density of radially-arranged blood vessels was significantly higher compared to normal chick CAM. Peritumor vessels morphology were highly suggestive for an intense angiogenic process induced by non-treated tumor implant. The blood vessels had a small caliber in cross-section and appeared highly branched and already perfused. At a higher magnification, the assessement of peritumor blood vessels showed a discountinous wall with a frequent tip endothelial cells emmerging from their contour or blood vessels with splitted lumen by pillars of endothelial cells. Also, the alternation of lumen and cord-like structures along the same vascular structures was highly suggestive for an intense angiogenic process induced by RCC non-treated implants.

But the most obvious differences were found between treated and non-treated implants regarding the morphology and density of peritumor blood vessels. The peritumor vessels surrounding non-treated implants were small, with a well-defined perfused lumen. The tumor implant invasion by these vessels was evident, numerous small blood vessels penetrating the tumor implant.

For the endostatin-treated group, density of peritumor blood vessels was lower compared to the non-treated group, being similar with that of normal CAM. The blood vessels did not have a branched morphology and no other morphological sign of active angiogenesis was detected. No particular distribution, as a spoke wheel-like arrangement was observed. Regarding the endostatin influence on tumor cells from treated implants, we observed that the papillary component showed extensive areas of cellular degeneration with loss of the papillary morphology and extensive necrosis. The clear cell component showed no significant changes in tumor cell morphology that was similar with that from the untreated specimens. We noticed the absence of inflammatory infiltrate in the implant treated with endostatin compared to the untreated implants.

Comparative evaluation of CD34-microvessel density (MVD) and CD105-MVD in non-treated and endostatin-treated specimens. The initial tumor specimens from which a fragment for implantation was taken, had a microvessel density ranged between 80 to 100 vessels per field ×400.

In the CAM tumor implant on day 7 post-implantation we noticed the presence of CD34-positive blood vessels with different densities at the periphery of the tumor and its center. If at the periphery of the implant the positive CD34 vessel density was 55 vessels per field ×400, inside the tumor, MVD did not exceed 15 vessels per field ×400.

By the use of endoglin on both specimens to identify activated vessels, we identified an average of 30 vessels per field x400 in the original tumor and the persistence of CD105-positive vessels in the untreated chorioallantoic membrane tumor implant with a density of 10 positive CD105 vessels per field ×400 at the periphery of the implant and 5 positive vessels per field ×400 inside the implant.

CD105-positive vessels at the periphery of the implant were mostly cord-like structures, while in the implant we noticed both vessels with visible lumen positive to CD105 and also isolated CD105-positive cells.

VEGF expression in tumor specimens and chick CAM implants. Inside the tumors that were used to obtain fragments for chick CAM implantation, VEGF was identified by immunohistochemistry in tumor cells, with a moderate-to-high intensity and heterogeneous distribution (its overexpression being confirmed by *RNAscope in situ* hybridization), and also in the endothelium of the tumor vessels where its expression was constantly observed. Untreated tumor implants retained the VEGF expression, with the highest intensity especially in the papillary component but moderate in the clear cell component.

After 7 days of treatment with endostatin VEGF expression was maintained in the papillary component, being absent in the clear-cell component, an aspect also assessed by immunohistochemistry and confirmed by RNAscope method.
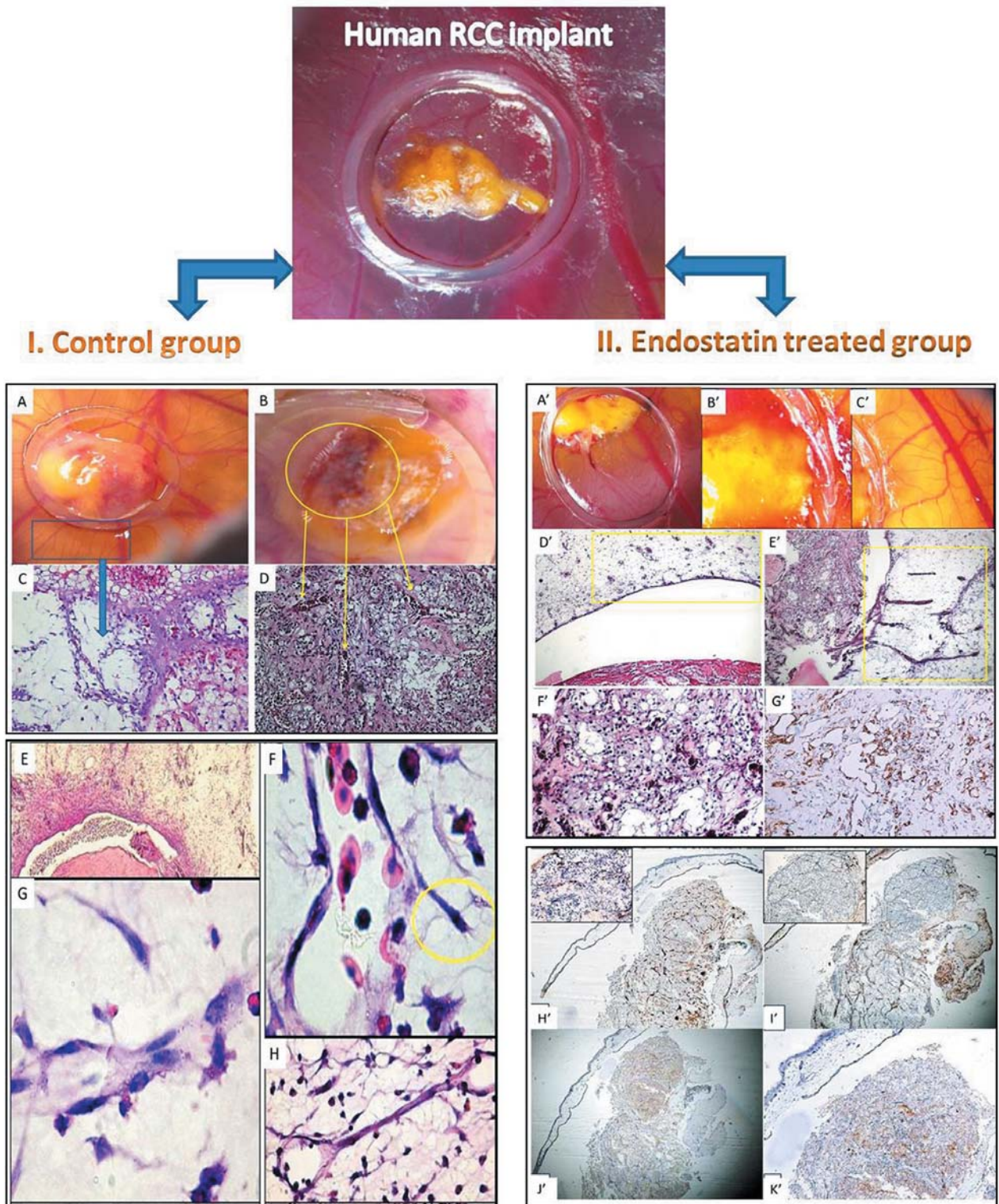
Figure 1. *Comparative overview of non-treated and endostatin-treated renal cell carcinoma specimens implanted on chick embryo chorioallantoic membrane. Note the tumor mass decreased size in endostatin-treated specimens (A'-C') compared to non-treated specimens (A-C). Blood vessels morphology and distribution around tumor implant and inside it, showing different and heterogeneous patterns in non-treated (C,D, E) versus treated specimens (D', E', F'). Active angiogenesis in non-treated group was microscopically assessed by direct observation of morphologic specific features of peritumor vessels (F, G, H). CD34 and CD105 tumor blood vessels assessement in treated specimens (G', H', I')and persistence of VEGF in treated specimens (J', K').*
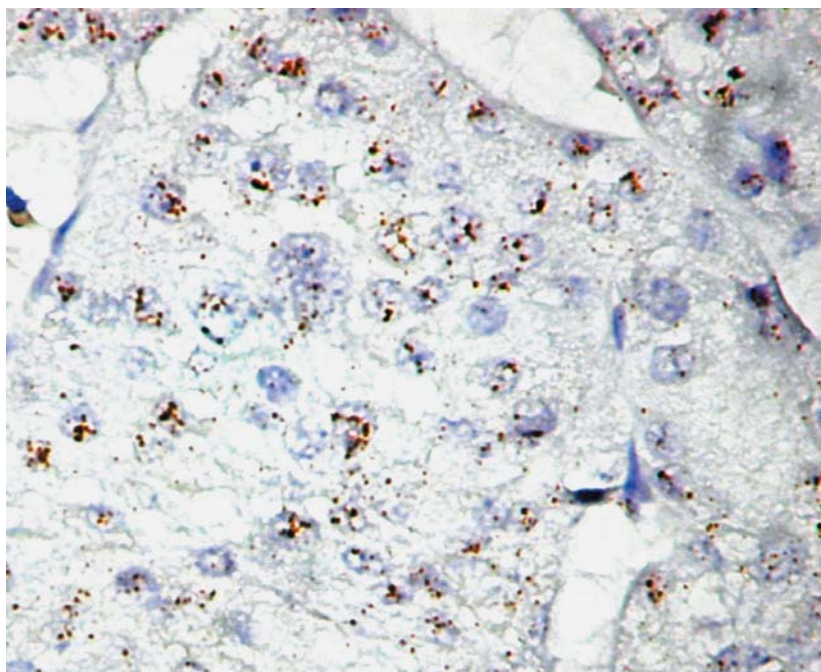
Figure 2. *VEGF mRNA expression in treated specimens, verifying the immunohistochemical findings.*

In the endothelium of the peritumor vessels VEGF expression was absent, unlike the intratumoral vessels where it was present, unsteady, particularly in cord-like structures and in isolated cells.

The proliferation rate of tumor cells from the original tumor was about 50%.The presence of Ki-67 was noted at the intra- and peritumoral level, especially in the vessels presenting the sprouting phenomenon. Most intratumoral vessels showed non-proliferative endothelial cells, negative to Ki-67.

Discussion

The chick embryo CAM is a cheap and fast model for tumor angiogenesis assessement. The quick development and maturation of chick CAM vessels and the possibility of their direct visualization represent strong advantages of this model compared to other experimental models such as the murine model, where we are not able to directly monitor tumor progression or its vascularization (1).

Less than 25 articles on international scientific databases reported data regarding experimental approaches of renal cell carcinoma by using the chick embryo CAM model. Most of them used different renal cell carincoma cell llines and to a lesser extent fresh tumor samples obtained after surgical resection. Some of them were only recently validated by Fergelot and collaborators as a potential method for quantification of tumor angiogenesis (7).

The tumor model of RCC implanted on chick CAM was recently used to test anti-tumor agents (3), but anti-angiogenic and anti-vascular drugs have not been been studied in such an experimental model for renal cancer. In the present study we chose a well-known endogenous inhibitor of angiogenesis (endostatin), to test its effects on chick CAM tumor model of human RCC implants. The testing of endostatin on chorioallantoic membrane has been reported in no more than 34 articles indexed in Pubmed, and this can be considered a recent event, given that the first article in relation to this model was published in 2004 by Pan *et al*. (9).

The quantification of endostatin effects on renal carcinoma has been, so far, achieved only at the serum level of patients with such tumors, with first evidence on the antitumor effect of endostatin reported by Dhanabal and coworkers in 1999 (13). The influence of endostatin on VEGF-dependent migration of endothelial cells was studied by Yamaguchi *et al*., but in a murine model with subcutaneous implant of human renal cancer cells in nude mice (14). That study suggested that endostatin causes blockage of one or more several steps in migrating endothelial-dependent VEGF. Our results are partially similar with previously reported results. We noticed the

absence of VEGF in the endothelium of peritumor vessels, but the persistence of its expression in the intratumoral endothelium vessels, especially in the cord-like vessels and the isolated endothelial cells, in endostatin treated specimens, suggesting an intratumoral VEGF-dependent endothelial migration, which may be considered discordant compared with the previous results from the literature.

Recent data have shown that endostatin does not act only at the level of VEGF, but also at the level of the intercellular adhesion molecules of endothelial cells of the tumor vessels, leading to their stabilization, most likely through an inflammatory mechanism (14).

Our study highlighted a differential VEGF inhibition between different morphological patterns of implanted specimens. Endostatin inhibited VEGF in the clear cell component of renal cell carcinoma, but had no effect on its expression in the papillary component. This could, in part, explain the heterogeneity of VEGF isoforms secreted by the tumor cells of renal cell carcinoma and also, the lack of efficiency of anti-VEGF therapy. These data are similar with recent reports on the antitumor effect of endostatin gene therapy found in the literature (15, 16).

In our study the endostatin-treated tumors became smaller especially due to the lack of the clear-cell component, but with the persistence of the papillary component, an aspect unreported so far in the literature.

Regarding the relation between renal cell carcinoma implants and inflammation, we noticed the absence of the inflammatory infiltrate in specimens treated with endostatin compared to untreated ones. It seems that endostatin also acts as an anti-inflammatory drug, most probably by inhibiting VEGF secreted by inflammatory cells.

The present study assessed the effects of endostatin on a CAM tumor model of renal cell carcinoma without association of other specific or non-specific inhibitors, currently used in renal cancer. A particular feature of the present study can be considered to be the VEGF comparative assessement between endostatin-treated and untreated groups and also, the assessment of CD105-positive tumor blood vessels (activated) under the influence of endostatin in close correlation with VEGF expression.

Endoglin quantification in renal cancer is less reported in the literature. Data on the use of endoglin as a therapeutic target are still a controversial issue in renal cancer but its involvement as a biomarker has already been accepted for several other cancer types (17-19).

Regarding renal cancer, Saroufim and his team (20) assessed CD105 on renal tumor samples of 102 patients and demonstrated its expression (considered as stem cell markers), in both tumor and endothelial cells. CD105-positive blood vessels from renal cell carcinoma have been negatively correlated with nuclear grade, tumor stage, and the Leibovitch score. By analysis of several variables together with CD105-positive tumor cells it was found that the presence of these cells represents an independent predictor of reduced overall survival.

Endoglin retained its expression inside tumor implant specimens even 7 days post-implantation being restricted to the endothelial cells of intratumor blood vessels of non-treated specimens. Endoglin-positive blood vessels persist in the endostatin-treated specimens, but CD105 MVD was significantly lower in treated versus non-treated specimens implanted on CAM. These findings support the inhibitory effect of endostatin on endoglin-positive tumor blood vessels from renal cell carcinoma by a mechanism not yet described in the literature. A divergent expression of endoglin and endostatin in serum patients with different cancer types was introduced by another article and correlated this expression with nodal and vascular metastasis. In a study published by Landt in 2011, the high serum levels of endostatin and angiogenin associated with low serum endoglin concentration reflected increased tumor malignancy in cervical cancer. Patients with nodal metastases had higher concentrations of endostatin and angiogenin and lower concentrations of endoglin, whereas vessel invasion was not appreciably correlated. Grading and menopausal status were loosely correlated with expression of angiogenic factors, but displayed similar tendencies, with the exception of endoglin, which was inversely correlated with menopausal status (21).

One particular aspect of renal cell carcinoma blood vessels is represented by their ability of a rapid aquisition of smooth muscle perivascular cells and their transformation into mature-type blood vessels, less sensitive to anti-angiogenic and/or anti-vascular therapies. Endoglin involvement in quick tumor blood vessels maturation, by a mechanism mediated through transforming growth factor-β receptor pathways is widely accepted for different tumor types and their correspondent metastasis (22), but is scarcely reported for renal cell carcinoma, mostly by observational studies (23, 24).

In renal cell carcinomas, combination between endoglin positivity in endothelial cells and smooth muscle actin in perivascular cells strongly suggests an early maturation of tumor blood vessels (previous studies of our group, unpublished data).

Based on our findings from the experimental model, correlated with literature data, we can launch a hypothesis regarding early maturation of tumor blood vessels in renal cell carcinoma. Despite previous reports that support high levels of endoglin as a favorable prognostic factor for patients with various malignancies, for renal cell carcinoma, high endoglin expression in endothelial cells from tumor blood vessels can induce their rapid maturation not directly, but most probably through an indirect mechanism through TGFβ family factors

mediated contribution (this factors known to stimulate vessels maturation). This hypothesis is sustained by the fact that, after endostatin treatment of renal cell carcinomas implanted on CAM the number of endoglin-positive tumor blood vessels significantly decreased but did not disseapear with CD105 isolated cells certifying the maintenance of an active angiogenesis after endostatin therapy. The remanent vessels showed an immature phenotype by the detachment of perivascular cells inconstantly observed compared to control group similar blood vessels.

A recently initiated study, still in progress, on 38 patients with solid tumors including renal tumors uses a combination of bevacizumab with IgG1 anti-endogline antibodies (TRC105) (25). It was observed that the combination of bevacizumab with TRC105 was effective in a VEGF inhibitor-refractory population.

Conclusion

The present study underlined the heterogeneity of renal cell carcinoma response to endostatin treatment on a chick embryo CAM tumor model. Endostatin triple action on tumor cells, endothelial and perivascular cells sustain it as a promising therapeutic agent in renal cancer associated with anti-VEGF drugs or other targeted inhibitors. Data obtained by testing the activity of endostatin on VEGF expression and endoglin in renal cancer implanted on CAM support the heterogeneity of renal cancer angiogenic process and suggest the involvement of multiple mechanisms, complex and different, in developing and maintaining the viability of renal cancer tumor blood vessels. This fact is a clear evidence of the use of a combination of antiangiogenic /antivascular factors in renal tumors. These issues can reduce the failure rate of monotherapy (administration of only bevaciziumab or other targeted agents alone) currently registered in clinical trials enrolling patients with such tumors.

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