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Evaluation of tumor angiogenesis through VEGF modulation in ovarian cancer *in vivo* using RNA interference

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Abstract

The aim of the study was to identify specific correlation between VEGF inhibition by RNA interference and several functional genes involved in in vivo ovarian cancer evolution in vivo. We used RT-PCR evaluation for VEGFA multiple isoforms and PCR Array for 84 angiogenesis genes, of which we have found 11 overexpressed and 13 downregulated genes. siVEGF has demonstrated a good response in ovarian cancer cell lines HeLa at 24 h and a 70% performant inhibition of its expression. RNA interference tool could be used in cancer therapy, if it is targeted on several genes covering multiple activation pathways for angiogenic mechanisms.

Keywords: angiogenesis, molecular profiling, PCR Array, ovarian cancer, RNA interference

Introduction

Angiogenesis represents an important mechanism in normal cyclical ovarian function. Follicular growth and the development of the corpus luteum (CL) depends of the proliferation of new blood vessels (Philips H.S. et al 1990). Previous studies have shown that the VEGF-A mRNA expression is temporally and spatially related to the proliferation of blood vessels in the ovary (Ravindranath, N. et al 1992). VEGF seems to be the principal regulator of ovarian angiogenesis.

The cells developing a solid tumor such as the ***ovarian*** type have the capability to proliferate in two distinct ways, capabilities not possessed by normal cells from which they derive. Of all the tumor cells abnormal features, the ability to induce and develop ***angiogenesis*** (a fundamental step in malignant transformation) is the most important ***in vivo*** environment component (Farzan Rastinejad et al., 2002). To become a clinical relevant tumor, the cellular architecture needs neovascularisation, otherwise the invasion and metastasis potential is not expressed. Several studies using cDNA microarray technology and RT-PCR were able to demonstrate that tumor cells in order to develop the angiogenesis network have to balance the proangiogenic factors and to inhibit the antiangiogenic ones involved in normal cell proliferation (Elbashir S.M. et al 2001). Angiogenesis is a complex multi-step process including the activation, proliferation, and migration of endothelial cells, degradation of vascular basement membranes, with development of new capillaries from pre-existing blood vessels. Vascular endothelial growth factor (VEGF) is a **key molecule** involved in tumor development by promoting angiogenesis (Zhang L. et al. 2003). VEGF binds to its tyrosine-

kinase receptors (VEGF-R1, VEGFR-2, VEGFR-3 and neuropilin) and mediates many components of angiogenesis including endothelial cell proliferation, invasion, migration, vessel permeability and cell survival. VEGF is secreted by most tumors cells including tumors of the lung, gastrointestinal tract, kidney, ovary, and cervix (Andrew V. Benest et al. 2008). The VEGF level of expression is correlated with tumor progression and invasion [3], subsequently being considered one of the most important angiogenic markers in cancer biology. Beside VEGF, which can be find in different forms that bind to specific corresponding receptors, some other well characterized proangiogenic proteins such as platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), tumor necrosis factor-α (TNF-alpha), and fibroblast growth factor 2 (FGF2) have been identified as stimulators of angiogenesis in various cancers (Darren W. Davis et al. 2008).

Ovarian cancer represents a major cause of death in women being considered one of the most aggressive types of cancer. An analysis of angiogenic factors in ovarian carcinoma revealed that VEGF is overexpressed in ovarian cancer cells and is associated with increased tumor growth, ascites fluid accumulation, metastases, poor prognosis, and shorter survival periods. On this basis, inhibition of VEGF function would be a logical approach to inhibit ovarian cancer. Current treatments for ovarian cancer consist in surgery and chemotherapy but the outcomes remain poor, explaining the need for new therapies. ***RNA interference*** (Fire A. et al. 1998, Bartel D.P. et al. 2004)is a form of posttranscriptional gene silencing (PTGS) in which double-stranded RNA (ds), in tandem with protein complexes catalyzes the degradation of complementary mRNA targets. Double-stranded RNAs are processed by Dicer, a cellular RNase, in order to generate duplexes of about 21nt with 3'-overhang small interfering RNA (siRNA), which mediates sequence-specific mRNA degradation. This technology can be directed against cancer cells using different strategies such as the inhibition of overexpressed oncogenes, the promotion of apoptosis and antiangiogenesis or the efficacy enhancement of chemotherapy and radiotherapy.

Aim of the study

The aim of the study was to evaluate the extent of VEGF modulation as a consequence of RNA interference tool in ovarian cancer cell lines and to define some other angiogenic factors as functional genes in tumor development. In respect to this purpose, we transfected ovarian cancer cell lines with the small interfering RNA (si RNA) that targets human VEGF, aiming to elucidate the effect of this treatment on the VEGF expression, and implicitly in tumor progression.

MATERIALS AND METHODS

Cell culture

Human ovarian cancer cells HeLa were kept in DMEM medium with 4500 mg/l glucose, supplemented with 10% fetal bovine serum, 2mM L-glutamine and 1% gentamicin in a 7% CO2 incubator at 37°C.

Transfection protocol

$5x10^5$ cells per well in 6-well plates were reverse transfected in 6- well plates with siPort NeoFx Transfection Agent kit (Ambion Inc.) and siRNA for VEGF (Ambion Inc. ID # 4638) with a final concentration of 30 nM in Opti-Mem medium. The siVEGF was chosen in order to target all 7 isoforms of VEGFA.

Healthy, growing and adherent cells were prepared for transfection and were first tripsinised. Trypsin was inactivated by resuspending the cells in normal growth medium to reach a concentration of $1x\ 10^5$cells/ml. All cells have been kept at 37° C while preparing the

transfection complexes. siRNA and transfection agent has been stored at room temperature for 10 min. The tests were done for 24, 48 and 72 hours, respectively.
In order to establish the appropriate amount of transfection agent, several reagent quantities are added per well for optimization. After determining the optimal volume of transfection agent for target knockdown, we maximized cell viability by adjusting the periods of time cells were exposed to transfection complexes (data not shown). We used a common siRNA for all isoforms of VEGF, evaluation of the inhibition being done by RT-qPCR.

RNA Isolation and cDNA

Total RNA was isolated with TriReagent and further analyzed for quantity and quality properties with Agilent Bioanalyzer (data not shown). 500 ng of the total RNA were reverse transcribed in a 20 µl reaction system using FirstStrand cDNA Synthesis Kit (Roche) (under conditions described by the supplier) and than analyzed by RT- PCR.

RT-qPCR protocol, primers and probes for VEGF

The mRNA expression level was quantified by RT-qPCR (reverse transcription quantitative PCR) using the LightCycler 2.0 Detection System (Roche). The PCR reaction was performed using LightCycler Taqman Master Kit (Roche) according to the manufacturer's instructions. The PCR amplification of the housekeeping gene (18S) was performed as an internal control in order to eliminate well to well variation. The relative expression in each sample was calculated in comparison with untreated cells.
VEGFA isoforms covered by siVEGF (Ambion Inc.) in cell line transfection study were the following: $VEGF_{121}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{165b}VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$.
The following primers and probes were used in RT-qPCR evaluation of VEGFA isoforms: F primer1079 5' ctacctccaccatgccaagt 3' 1098; R primer 1133 3' ccacttcgtgatgattctgc 5' 1152; probe: ggcagaag; 18S: F primer 1766 5' gcaattattccccatgaacg 3' 1785; R primer 1718 3' gggacttaatcaacgcaagc 5'1737; probe tccagtgt

PCR Array

1000 ng of the isolated RNA after 24h of transfection were reverse transcribed with First strand synthesis kit (superarray) and amplified in a 98-well plate (Angiogenesis RT^2 Profiler PCR Array) and detected by RT2 Real-Timer SyBR Green purchased from SuperArray Bioscience Corporation (Frederick, MD). The Human Angiogenesis RT^2 PCR Array was performed on LightCycler 480 Detector System (Hofman LaRoche). The internal control was GAPDH gene. For data analysis we used the ΔΔCt method; for each gene fold-changes were calculated as difference in gene expression between the treated (siRNA for VEGF) and untreated (control) cells. The control report value was 1.00. A value higher than 1.00 indicates gene up-regulation and a value lower than 1.00 indicates gene down-regulation.

Results

All cells were cultivated in similar conditions and exposed to transfection by optimal amounts of siRNA for VEGF. The treated cells (siRNA for VEGF) were evaluated by RT-qPCR using primers and probes as nominated for all 7 transcripts of VEGF at different time moments: 24, 48 and 72 hours, respectively. The maximum inhibition of VEGF in ovarian cancer cell line HeLa was found (*figure 1*) at 24 h, the inhibition representing 70% of protein expression. The results obtained at 48 h and 72 hours were lower in VEGF inhibition and had the tendency to restore the initial cellular potential.

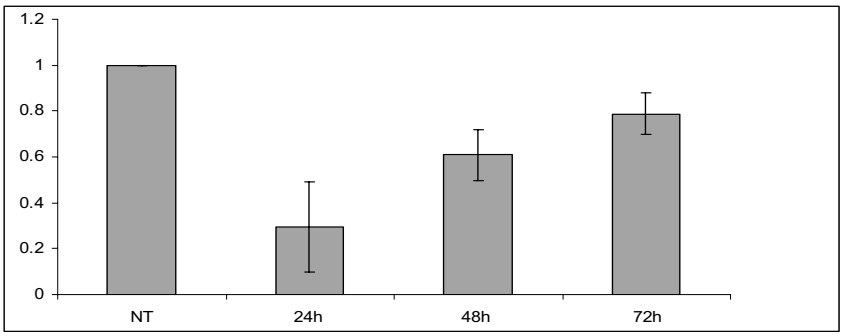

Fig. 1. VEGF A inhibition by siRNA for VEGF at different time spells 24, 48 and 72 hours evaluated by RT-PCR

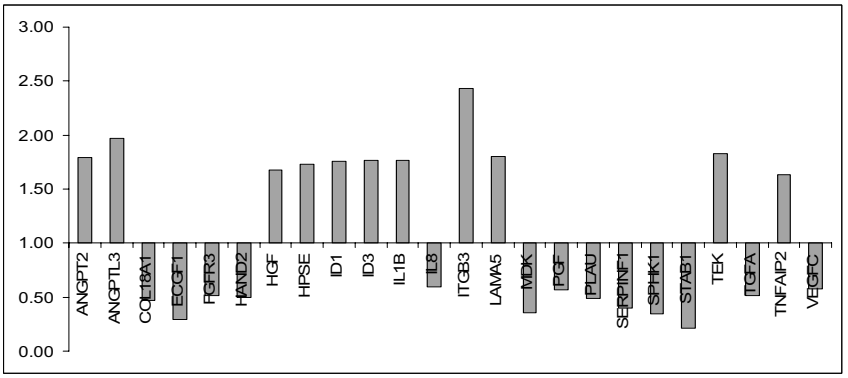

Fig. 2. Functional gene groups involved in angiogenesis of ovarian cancer expressed by PCR array.

The Human Angiogenesis RT^2 PCR Array analysis was done for the 24 h treatment of cells with siVEGF, regarding to RT-qPCR results. The kit design provides the expression of 84 key genes involved in modulating the biological process of angiogenesis. The array includes growth factors and their receptors, chemokines and cytokines, matrix and adhesion molecules, proteases and their inhibitors, as well as transcription factors, all involved in the development of new blood vessels. Human Angiogenesis RT^2 PCR Array revealed 24 genes (figure 2) from the total number of 84 angiogenesis genes, of which 11 were up-regulated and 13 down-regulated, as indicated in *Table 1* and *Table 2*.

Table 1. Effects of RNA interference in ovarian cancer cell lines HeLa for siVEGF upon different functional gene groups involved in angiogenesis. Functional genes overexpressed in the experiment.

Functional gene	**Level of expression reported to control(untreated cells)**
ANGPT2(growth factor) proangiogenic	1.79
ANGPTL3 (adhesion molecule) proangiogenic	1.97
HGF (growth factor) proangiogenic	1.68
HPSE (matrix protein) proangiogenic	1.73
ID1 (transcription factor) proangiogenic	1.75
ID3 (transcription factor) proangiogenic	1.77
IL1B (cytokine) proangiogenic	1.77
ITGB3 (adhesion molecule) proangiogenic	2.43
LAMA5 (growth factor) proangiogenic	1.80
TEK (growth factor)proangiogenic	1.83
TNFAIP2 (matrix protein)proangiogenic	1.64

Table 2. Effects of RNA interference in ovarian cancer cell lines HeLa for siVEGF upon different functional gene groups involved in angiogenesis. Functional genes downregulated in the experiment.

Functional gene	**Level of expression reported to control(untreated cells)**
COL18A1 (matrix protein) natural antiangiogenic VEGF A	0.47
ECGF1 (growth factor) proangiogenic	0.30
FGFR3 (growth factor) proangiogenic	0.51
HAND2 (transcription factor) proangiogenic	0.50
IL8 (cytokine) proangiogenic	0.59
MDK (growth factor/ligand/cytokine/chemokine) proangiogenic	0.35
PGF (growth factor) proangiogenic	0.57
PLAU (matrix protein) proangiogenic	0.49
SERPINF1 (matrix protein) proangiogenic	0.40
SPHK1(transcription factor) proangiogenic	0.35
STAB1(growth factor) proangiogenic	0.21
TGFA (growth factor) proangiogenic	0.51
VEGF proangiogenic	***0.58***

Discussion

The results obtained in our study demonstrate that VEGF could be inhibited by this new molecular tool which is RNA interference. Using this type of siVEGF (Ambion Inc.) for all gene isoforms, we assumed that the reaction mix can cover the whole seven transcripts for VEGF, modifying the main mechanism of angiogenesis generated by VEGF. The fact that the major cell inhibition was observed after 24 hours, confirms the clinical aspects of antiangiogenic therapy administered in clinical trials, which has to be done continuously. More, the restoration of the VEGF proangiogenic potential in less than 72 hours helps understanding the multiple pathways governing the acquiring and preservation of the malignant potential. Inhibition of VEGF by RNA interference could be followed by other siRNA for different genes involved in angiogenesis in order to compete to a better reduction of their potential. $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ levels are increased in several human malignancies including breast, lung, brain, pancreatic, ovarian, kidney, and bladder carcinomas. Less frequently expressed are the VEGF-A spliced forms $VEGF_{145}$, $VEGF_{183}$, $VEGF_{162}$, and $VEGF_{165b}$.

At present, very few studies concerning siVEGF blocking of ovarian cancer cell lines development are available (Kiyoshi M. et al. 2008). This is the reason why we decided to use PCR Array technology for the evaluation of the 84 genes involved in angiogenesis. From the total number of genes, twenty-three are proangiogenic and one (COL18A1-a matrix protein encoding endostatin) is antiangiogenic (Darren W. Davis et al. 2008). Surprisingly, 11 proangiogenic genes (*Table 1*) expressed an up-regulated level even if the VEGF was inhibited at 24 h (as seen in *Table 2*). Inhibition of VEGF activated some other important pathways for angiogenic mechanism validation, like the growth factor ANGPT2 and cell adhesion molecule ANGPTL3 involved in stimulation of cell invasion. ID1 and ID3 which are both transcription factors and downregulate the thrombospondin are overexpressed as a consequence of COL18A1 inhibition (Belotti D. et al. 2003). The COX2 enzymes convert the arachidonic acid to prostaglandin and subsequently thromboxane and prostaglandins PGF (Tae-Hee Lee et al. 2007).

PGF expression could by downregulated as a consequence of prostaglandin pathway and its involvement in VEGF synthesis, which shows inhibition in our experiments. On the

other side HPSE (heparanase) is an endo-beta-D-glucuronidase which cleaves the HPSG (heparan sulfate proteoglycans), its expression being associated with tumor metastasis and angiogenesis. FGF/HPSG interaction is inhibited in our study regarding the downregulated value of FGFR3 which does not permit the complex formation and the presentation of FGF to the TK-receptors (Hough CD et al. 2001). Our results confirm the multitude of mechanisms and pathways involving pro and anti angiogenic factors, as well as the necessity to search for several RNA interference molecules to block an important number of these factors and their genes respectively (Xiao-Hong Liu et al. 2008).

Conclusions

Numerous antiangiogenic agents are currently used in clinical trials but the results are not very decisive. In this study we demonstrate that RNA interference is a real positive tool for blocking the VEGF expression in tumor angiogenesis, but we also revealed the fact that one target is not enough even if VEGF is considered to be a major player in validation of malignant phenotype. The consequence of this remark is represented by targeting as many molecules as possible in order to block the angiogenesis process and reestablish the normal behavior of the cell at least for a short period of time.

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