Endothelial $p21^{WAF1/Cip1}$ Cell Cycle Inhibitor is Down-regulated in Breast Cancer

T. VREKOUSSIS$^1$, E.N. STATIPOULOS$^2$, M. KAFOUSI$^2$, Z. SARIDAKI$^1$, E. SANIDAS$^3$ and O. ZORAS$^1$

$^1$Department of General Surgery, $^2$Department of Pathology and $^3$Department of Surgical Oncology, University Hospital and Medical School University of Crete, Heraklion, Greece

Abstract. Background: Tumor angiogenesis is considered a multi-pathway process, while $p21^{WAF1/Cip1}$ is a CDK inhibitor involved in cell division and survival. Herein the tumor environment effect on endothelial $p21^{WAF1/Cip1}$ expression is examined. Materials and Methods: The EA.hy 926 endothelial cell line and tumor-conditioned medium (TCM) from the MDA-MB-468 breast cancer cell line were used. Endothelial cells grown alone and in TCM were immunostained for $p21^{WAF1/Cip1}$ and analyzed by RT-PCR. Forty-four cases of breast cancer and forty-three cases of normal breast tissue were immunostained for $p21^{WAF1/Cip1}$. Results: Endothelial $p21^{WAF1/Cip1}$ is transcriptionally down-regulated under the influence of TCM. Moreover, it seems that breast cancer tumor endothelium does not express $p21^{WAF1/Cip1}$. Conclusion: $p21^{WAF1/Cip1}$ plays a major role in angiogenesis, since tumor cells seem to down-regulate endothelial $p21^{WAF1/Cip1}$, compared to endothelial cells grown in serum-free medium. The verification of the tissue culture experiment results by immunohistochemistry on tissue sections indicates $p21^{WAF1/Cip1}$ as a target of modern molecular therapy.

Tumor angiogenesis is a multi-step process required for a primary tumor to exceed a certain size and spread (1). It has been shown that, under the influence of tumor-secreted factors, endothelial cells belonging to vessels adjacent to the tumor start to proliferate to form capillaries, thus supplying the primary tumor with oxygen and nutrients that are important for its growth (2). At the same time, capillary formation favors tumor cell escape via the blood stream and is the initial step in metastatic spreading (3).

Endothelial proliferation, regulated positively or negatively by several factors (4), is a prerequisite for angiogenesis (new capillary formation) and thus has been considered a therapeutic target. For example, anti-VEGF antibodies bind VEGF, blocking its linkage to its respective receptors (5), while tyrosine kinase inhibitors interfere with signal transduction in endothelial cells caused by tumor-secreted angiogenic factors (6, 7). No matter what the factors and their regulatory effect on the endothelial cells are, proliferation is the outcome of cell cycle acceleration.

The cell cycle is regulated via several pathways and modulators acting as inducers and/or inhibitors (8, 9). One of the main modulators of the cell cycle is $p21^{WAF1/Cip1}$, a 21kDa protein considered to act primarily as a cell cycle inhibitor, by interfering with Cyclin-Cdk complexes (10).

In this study, the expression of $p21^{WAF1/Cip1}$ in an endothelial cell line cultured in tumor-conditioned medium was investigated. Possible alterations in $p21^{WAF1/Cip1}$ transcription were also examined. After establishing tumor-conditioned medium-induced $p21^{WAF1/Cip1}$ down-regulation, $p21^{WAF1/Cip1}$ expression was investigated in formalin-fixed paraffin-embedded human normal breast tissues and breast cancer specimens. We report that endothelial $p21^{WAF1/Cip1}$ was also down-regulated in breast cancer, implying a possible role for $p21^{WAF1/Cip1}$ in tumor angiogenesis.

Materials and Methods

Cell lines. The EA.hy 926 endothelial cell line (11) was grown in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose concentration (4500 mg/l) supplemented with Glutamax-I, 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin. The MDA-MB-468 breast cancer cell line was cultured in DMEM with low glucose concentration (1500 mg/l) supplemented with Glutamax-I, 10% FBS and penicillin/streptomycin. Both cell lines were maintained in a 5% CO$_2$ atmosphere, in a humidified incubator. All tissue culture media were purchased from Invitrogen, Scotland, UK and the tissue culture plasticware were from Costar, USA.

Tumor-conditioned medium (TCM) preparation. The MDA-MB-468 cell line was cultured in the medium mentioned above. After confluence had been reached, the cells were washed with Phosphate Buffer Saline (PBS), and the medium was replaced with FBS-free DMEM with a high glucose concentration. The culture...
was maintained for 48 hours. The TCM was then collected, cleared by centrifugation and stored in aliquots at –80°C. Each aliquot was thawed once prior to use.

Effect of TCM on endothelial growth rate. The EA.hy 926 endothelial cells were seeded in a 24-well plate at an initial density of 30,000 cells/well and incubated overnight. The following day, the medium was removed, the cells were washed once in PBS and then TCM was added. Cells cultured in FBS-free DMEM were used as controls. After 2 days in culture, the cells were collected and counted using the trypan-blue exclusion assay. The experiment was done in triplicate.

Immunocytochemistry. The EA.hy 926 cells were grown on SuperFrost Plus negatively-charged slides (O. Kindler GmbH & Co, Freiburg, Germany) as previously described (12). Cells were allowed to grow in FBS-enriched DMEM until confluence was reached. After a PBS wash, the medium was replaced with TCM and the culture was maintained for 24 hours. Cells cultured in FBS-free DMEM served as control. The cells were washed twice in PBS and fixed in 4% paraformaldehyde in PBS for 10 minutes. The slides were stored immersed in 70% ethanol at 4°C until stained. Immediately before they were immunostained, cells cultured on slides were warmed to room temperature, washed twice (2 min each washing) with distilled water, immersed in 0.01 M citrate buffer pH 6.2 and heated in a microwave oven at 350 Watts twice. After cooling at room temperature for 30 minutes, washing with distilled water and immersing in TBS, blocking of cross-reactivity with normal rabbit serum (DAKO-X0902, Denmark) diluted 1/20 in TBS, for 30 minutes. After rinsing with TBS, the cells were stained by enhanced APAAP (procedure detailed in Table I). Finally, the slides were exposed to chromogen (Fast Red Substrate System, DAKO-K0699) for 25 minutes, washed with deionized water, counterstained for 30 seconds with Papanicolaou 1a Harris hematoxylin (Merck KGaA 64271, Germany), and cover-slipped with Glycergel (USA). Between the main steps of the procedure, TBS was used as a rinsing buffer. BSA (Albumin Bovine Serum Standard Sol. 30%) was purchased from Eurobio Biotechnology. Positive and negative controls were used in every run.

RNA extraction, reverse transcription and polymerase chain reaction amplification. The EA.hy 926 endothelial cells were cultured until confluence in 25-cm² tissue culture-treated flasks. After confluence had been reached, the cells were washed with PBS and TCM was added for 24 hours. Confluent cell layers were maintained in FBS-free DMEM as control. The cells were then collected by trypsinization and total RNA extraction was performed, using Trizol reagent (Gibco BRL, USA) according to the manufacturer's protocol. Complementary DNA synthesis was performed using 1 μg of total RNA, 5 μl dNTPs mix 2mM (Pharmacia, USA), 3 μl random examer primers (Promega, USA), 1 μl M – MLV reverse transcriptase (Gibco BRL), 10 μl RT-Buffer 5X (Gibco BRL) and 0.5 μl RNase inhibitors (Promega). The reaction was performed in a final volume of 50 μl adding RNase-free water. Primer annealing was achieved by incubation at 65°C for 5 minutes followed by 10- minute incubation at room temperature, prior to reverse transcriptase and RNase inhibitors addition. Reverse transcription was achieved by incubation at 37°C for 2 hours. The synthesized cDNA was stored at –20°C. Polymerase Chain Reaction was performed using 2 μl of the cDNA template, 8 μl dNTPs mix, 0.5 μl Taq DNA polymerase (Promega), 5 μl 10X Taq DNA polymerase Buffer (Promega), 3 μl MgCl₂ 25 mM (Promega), 5 μl forward primer 10 μM, 5 μl reverse primer 10 μM and 21.5 μl DNase-free water. The primers used to amplify p21WAF1/Cip1 and the ARPPO cDNA are shown in Table II. Amplification (denaturing at 94°C for 3 minutes and 30 cycles of denaturing at 94°C for 1 minute, annealing at 57°C for 40 seconds and extension at 72°C for 1 minute) was performed in a MJ cycler.

was maintained for 48 hours. The TCM was then collected, cleared by centrifugation and stored in aliquots at –80°C. Each aliquot was thawed once prior to use.

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Products were separated with gel electrophoresis using 1% agarose gel, stained with ethidium bromide, visualized by UV light and photographed.

Patients and immunohistochemistry. Forty-four cases of breast cancer and forty-three cases of normal breast tissue were randomly chosen from the archives of the Laboratory of Pathology of the University Hospital of Heraklion, Crete, Greece. All tissues had been fixed in 4% buffered formalin and embedded in paraffin according to the normal schedule used in the laboratory. From each block, 3-μm-thick sections were cut, mounted on SuperFrost Plus slides and dried at 50°C for 1 h. The sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol to distilled water. After immersion in 0.01 M citrate buffer pH 6.2 and heating (twice) in a microwave oven at 350 Watts for 5 minutes, immuno-staining with anti-p21WAF1/Cip-1, as mentioned above, followed. Immuno-stained tissue sections were examined under a Nikon Microphot FXA light microscope equipped with a Nikon Coolpix E995 digital camera. A section was considered positive when even one endothelial cell showed nuclear or cytoplasmic reactivity. Positive nuclear reactivity of epithelial or tumor cells served as internal control.

Image analysis. To clarify fine differences in positivity, image analysis was performed using Adobe Photoshop 8.0. Red channel intensity was calculated as the number of red pixels of the image area under analysis, divided by the total pixels of the analyzed image area.

Statistical analysis. To evaluate the significance of the TCM-effect on the endothelial growth rate, one-way analysis of variance (ANOVA) was used. Depending on the results of the immunohistochemical study, Chi-square test, with or without Yates' correction for continuity, was used.

Results

Tumor-conditioned medium increases endothelial cell growth rate. Tumor-conditioned medium-induced angiogenesis has been described previously (13). To verify that in the coculture system used in our experiments breast cancer cells induce endothelial proliferation and consequently angiogenesis, endothelial cells were grown in TCM. As shown (Figure 1), after 2 days in culture, endothelial cells grown in TCM have a significantly increased growth rate (F=237.5, p<0.001) compared to endothelial cells maintained in FBS-free medium.

Tumor-conditioned medium down-regulates endothelial p21WAF1/Cip1. In order to specify whether endothelial p21WAF1/Cip1 expression is affected by the breast cancer cell line MDA-MB 468, EA.hy 926 endothelial cells grown under the influence of TCM were stained with anti-p21WAF1/Cip1 using the above-described APAAP technique. Endothelial cells grown in FBS-free DMEM were positively stained for p21WAF1/Cip1, showing intense cytoplasmic and a weaker nuclear reactivity (Figure 2A-2Fa). On the contrary, endothelial cells grown in TCM were completely negative (Figure 2B-2Fb).

Endothelial p21WAF1/Cip1 down-regulation is transcriptional. Since p21WAF1/Cip1 is reduced at the protein level, it was necessary to evaluate whether this reduction was due to transcriptional down-regulation or to post-transcriptional modifications (increased p21WAF1/Cip1 protein instability, cleavage). Subsequently, cDNA prepared from endothelial cells grown in TCM was amplified with primers specific for p21WAF1/Cip1. The results in Figure 2E indicate a significant decrease in p21WAF1/Cip1 mRNA, implying that factors in the TCM interfere directly or indirectly with p21WAF1/Cip1 transcription.

Endothelial p21WAF1/Cip-1 expression is also down-regulated in breast cancer. Following the in vitro experiments, whether endothelial p21WAF1/Cip-1 was affected by breast cancer cells was examined. Tissue sections of 44 breast cancers and normal breast tissue sections from 43 cases of excisional biopsies (performed for lesions other than cancer) were studied. As shown in Table III, in 27 out of 43 normal breast tissue samples (62.79%) endothelial cells presented nuclear and cytoplasmic reactivity. On the contrary, only 1 breast cancer sample out of 44 (2.27%) was positive for p21WAF1/Cip-1. Chi-square statistical analysis revealed that this difference in expression is significant (Yates' corrected \( \chi^2 = 33.772 \), degrees of freedom = 1, \( p < 0.001 \)).
Discussion

Angiogenesis is the combined end-effect of multiple inducing or inhibiting factors (14), leading eventually to increased endothelial proliferative activity, a feature well-described in tumors (15). Indeed this study, based on a TCM-endothelium cell culture model, was designed to simulate the whole tumor effect on the endothelium. $p21^{WAF1/Cip1}$ was selected as the specific target because of the critical role that it seemed to play in endothelial cell cycle regulation.

$P21^{WAF1/Cip1}$ is a molecule believed to act in cell cycle and survival. It is described as a cell cycle inhibitor (16), while at the same time it protects a cell from inducible apoptosis (17). Although members of the CDK-inhibitors' family have been proven to have different functions depending on intracellular localization – nuclear or cytoplasmic – (18), in breast cancer a $p21^{WAF1/Cip1}$ cytoplasmic localization has been recently described (19). In our study, endothelial $p21^{WAF1/Cip1}$ expression was revealed in cells cultured in the absence (but not in the presence) of TCM. This could be
attributed to a shortage of growth factors supplied to the culture by the serum added, as previously reported (20). Endothelial p21WAF1/Cip1 was localized both in the cytoplasm and the nucleus. The expression of p21WAF1/Cip1 by endothelial cells, deprived of growth factors, can be perceived as a mechanism aimed at inhibiting the endothelial cell cycle (signified by nuclear p21WAF1/Cip1 localization) and at protecting against inducible apoptosis (signified by cytoplasmic p21WAF1/Cip1 localization) (21). On the contrary, when endothelial cells are cultured in TCM, simulating tumor angiogenesis, they under-express p21WAF1/Cip1. Our findings are compatible with those of previous studies reporting that VEGF – produced by breast carcinoma cells – may lead to endothelial p21WAF1/Cip1 down-regulation (22).

Moreover our experiments produced evidence that p21WAF1/Cip1 expression was transcriptionally-regulated. However, proof of a possible interaction between p21WAF1/Cip1 promoter and angiogenic factors in the TCM is still pending and needs further study.

CDK inhibitors have already been studied in breast cancer using immunohistochemistry (23-25). Several features such as spread to lymph nodes and survival were analyzed showing both positive and negative correlation (26, 27). However, all the studies using an immunohistochemical approach focused only on the breast cancer cell. Our work is the first, to our knowledge, to focus on p21WAF1/Cip1 expression alterations in the tumor vasculature. The immunohistochemical study performed (Figure 2G, 2H), verified the results of the tissue culture experiments. Endothelial p21WAF1/Cip1 was significantly down-regulated in the tumor vasculature when compared to the normal tissue vasculature (p<0.001). Furthermore, in some cases, endothelial cells of microvasculature located in the normal tissue adjacent to the tumor were found to express p21WAF1/Cip1. This can be attributed either to an inherent abnormality of the tumor neovasculature or possibly to hypoxia-induced VEGF expression (22, 28).

P21WAF1/Cip1 down-regulation in the tumor vasculature could be of great importance to modern therapeutics. An effort to inhibit p21WAF1/Cip1 down-regulation or to restore p21WAF1/Cip1 intracellular concentration to a level comparable to that of normal vessels could decelerate the increased tumor-induced angiogenic rate, and thus could prevent disease progression. P21WAF1/Cip1 restoration could be achieved either by gene therapy interventions, inserting the p21WAF1/Cip1 coding sequence into the tumor endothelium (29), or by applying protein transduction technology (30). Another pertinent issue is the effect of radiation on cell survival when p21WAF1/Cip1 expression is reduced. It has been shown (31) that cells, which do not express p21WAF1/Cip1, are resistant to radiotherapy. Thus, tumor vasculature is expected to be resistant to radiotherapy. By inhibiting the tumor effect on the endothelium, the newly-formed capillaries could become vulnerable to radiation. This could be beneficial in maximizing the radiation contribution to local recurrence prevention. From all the above, it becomes clear that p21WAF1/Cip1 is a molecule that could influence the efficacy of therapeutic interventions.

In conclusion, it has been demonstrated in vitro that breast cancer cells seem to down-regulate endothelial p21WAF1/Cip1 transcription and expression. This down-regulation leads to endothelial proliferation and, thus, angiogenesis. From our experiments in tissue sections, this phenomenon seems to happen in vivo as well, since a substantial percentage of breast cancer tumors do not express tumor-endothelial p21WAF1/Cip1. Our findings support the thesis of p21WAF1/Cip1 involvement in angiogenesis. Still further experimentation and larger series of samples are needed in order to elucidate the precise mechanism, as well as the outcome of p21WAF1/Cip1 interference in the angiogenic process.

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References


