Role of Renin-Angiotensin-System in Human Breast Cancer Cells: Is There a Difference in Regulation of Angiogenesis between Hormone-Receptor Positive and Negative Breast Cancer Cells?

Bedeutung des Renin-Angiotensin-Systems in humanen Mammakarzinomzellen: Gibt es einen Unterschied zwischen hormonrezeptorpositiven und -negativen Mammakarzinomzellen bei der Regulation von Angiogenese?

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ABSTRACT
Objective This study examined the role of the RAS in human breast cancer cells to question if there are differences between HR-positive and HR-negative cells with regard to regulation of VEGF.

Methods Expression of different RAS components in hormone receptor (HR)-positive and HR-negative breast cancer cells was investigated using RT-PCR. Different stimulation protocols with different RAS inhibitors were used to investigate the effect on VEGF expression. Angiotensin II-dependent expression of VEGF was quantified by real time PCR. In addition, the effect of intrinsic RAS was studied performing siRNA knockdown of angiotensinogen (AGT). Statistical analysis were calculated using IBM SPSS Statistics Version 21.

Results Expression of AT₁R, AT₂R, AGT and ACE was shown in HR-positive and HR-negative breast cancer cell lines. Extrinsic stimulation with angiotensin II increased VEGF significantly. After treatment with captopril or AT₁R-inhibitor candesartan, VEGF-expression decreased significantly in HR-positive and HR-negative cell lines. However, inhibition of AT₂R using PD 123,319 did not show any significant changes of VEGF. After prevention of intrinsic angiotensin II, extrinsic angiotensin II as well as the combination with inhibitors of the receptors caused a significant reduction of VEGF. Surprisingly, the overall effect of the RAS after knockdown of AGT revealed a significant increase of VEGF in HR-positive cells at any time while a significant decrease was observed in HR-negative cells after 144 hours incubation.

Conclusion The RAS-dependent regulation of VEGF between HR-positive and HR-negative breast cancer cells seems to be different. These findings provide evidence for a possible future therapeutic strategy.
Introduction

Growth and metastasis of malignant tumors depend on angiogenesis in order to link the growing cancer tissue to blood supply. The safekeeping of nourishment is thereby controlled by self-regulation of renal homeostasis and the vascular tone in the cardiovascular system [12,13]. One of those systems is the renin-angiotensin-system (RAS), which is responsible for the regulation of renal homeostasis and the vascular tone in the cardiovascular system [12,13].

Angiotensinogen (AGT) becomes converted via catalytic activity of renin to angiotensin I, and angiotensin I via angiotensin-converting enzyme (ACE) to angiotensin II, which is the main effector of the RAS. It mediates its effects by binding to four different angiotensin II-receptors. The most important ones are angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) [14]. Both are G-protein-coupled receptors, whereas the activating effects are mainly mediated via AT1R and antagonised via AT2R [15–18]. Besides the above mentioned pathway of angiotensin II synthesis, an alternative way has been described via angiotensin 1-12, which is expressed independently from renin and which becomes also converted to angiotensin II by enzymatic activity of ACE and chymase mainly in the local tissue [19–21]. In addition, there is a further cascade with antagonistic activity in the RAS, since angiotensin-converting enzyme type 2 (ACE 2) converts angiotensin I into angiotensin 1-9, which on this part becomes converted to angiotensin 1-7 via ACE [22] (Fig. 1). ACE 2 is also able to mediate the conversion from angiotensin II to angiotensin 1-7. Angiotensin 1-7 activates the mas-receptor [23, 24] and thereby mediates mainly antagonistic effects as compared to angiotensin II. This effect results in vasodilatation and anti-angiogenic activity [25]. Moreover, both players antagonise each other, since angiotensin II inhibits ACE 2- and angiotensin 1-7 increases ACE 2-expression [26] (Fig. 1). Finally, the local RAS contains of two different axes of angiotensin, and it has to be assumed that influencing the systemic RAS via ACE-inhibitors and AT1R-inhibitors might also affect the balance of local RAS with regard to potential anti-angiogenic therapeutic strategies.

Therefore, we addressed the question if there is a role of the RAS in the regulation of angiogenesis in hormone-receptor positive (HR-positive) and hormone-receptor negative (HR-negative) breast cancer cells. We investigated if angiotensin II synthesized locally by cancer cells increases the VEGF-expression and if the VEGF-expression can be influenced by inhibition of ACE, AT1R and AT2R. In addition, suppression of the RAS was performed by

![Fig. 1 Cascade of major components of the RAS and their receptors.](image-url)
knockdown of AGT in order to analyse a potential effect on VEGF with possible differences in the regulation of tumour angiogenesis between HR-positive and HR-negative breast cancer cells with regard to future therapeutic anti-angiogenic strategies in breast cancer.

Material and Methods

Cell cultures for breast cancer cell lines

Overall, 6 different cell lines were cultivated: 3 hormone-receptor positive (MCF-7, ZZR-75-1, MDA-MB 361) and 3 hormone-receptor negative cell lines (MDA-MB 231, MDA-MB 468, MDA-MB 453). MCF-7, ZZR-75-1, MDA-MB 361 were cultivated in RPMI 1640 (PAA Laboratories, Pasching, Austria), MDA-MB 231 und MDA-MB 468 in DMEM with High Glucose (4,5 g/l) and MDA-MB 453 in DMEM/Ham’s F-12 (PAA Laboratories, Pasching, Austria) supplemented by 1% Penicillin/Streptomycin (PAA Laboratories, Pasching, saturated humidity and an atmosphere containing 5% CO2. Media were changed every 48 h.

Stimulation with angiotensin II

Cultivated breast cancer cells were stimulated with 10⁻⁷ mol/l angiotensin II. For determination between the effects of extrinsic and intrinsic angiotensin II, incubation with 10⁻⁶ mol/l ACE-inhibitor captopril was performed in order to inhibit the expression of endogenous angiotensin II. This experiment was done with and without simultaneous stimulation of extrinsic angiotensin II. Furthermore, in order to investigate across which angiotensin II receptor mediates a possible increased VEGF-expression, both receptors have been blocked using specific inhibitors (AT₁R: 10⁻⁶ mol/l candesartan; AT₂R: 10⁻⁶ mol/l PD 123,319). This inhibition was again performed with and without simultaneous treatment of extrinsic angiotensin II.

AGT-knockdown

In order to exclude the influence of intrinsic angiotensin II on the expression of VEGF of the breast cancer cells, a knockdown of AGT was performed by transfection with siRNA: 8 × 10⁵ cells were seeded into primaria cell culture flasks T25 (BD Falcon) and cultured under normal growth conditions (37°C; 5% CO2). After 24 hours, a transfection reagent was added containing 400 µL of culture medium without serum, 6 µL of small interfering RNA (siRNA) of negative control (Qiagen 1027281 20 nmol, Qiagen) or a mixture of AGT-1-siRNA, AGT-4-siRNA and AGT-7-siRNA (Qiagen, Hildesheim, Germany). The transfection medium was incubated at least for 5 minutes to allow the formation of transfection complexes. Cells were seeded under their normal growth conditions with the growth medium and the transfection complexes. After 48 hours and 96 hours transfection was repeated. 48, 72, 96, 120 and 144 hours after the initial transfection, cells with culture medium were removed and the RNA was isolated for confirmation of a successful knockdown.

RNA Isolation and reverse transcription

Total RNA from the cultivated cells was extracted using the RNeasy mini kit (Qiagen, Hildesheim, Germany) according to the manufacturer’s instructions. The amount of RNA was quantified by absorbance at 260 nm (DU640, Beckmann, USA) and 2.5 g of total RNA was reverse transcribed into cDNA using random hexamer primers according to the manufacturer’s instructions (Applied Biosystems, Foster City, USA).

RT-PCR

RT-PCR was used for the detection of the housekeeping gene (GAPDH), as well as AT₁R, AT₂R, AGT, ACE, and VEGF expression. Four microliters of a 1:10 dilution of the transcribed cDNA was used as tem-plate. PCR amplification was carried out using dNTP, forward primer, reverse primer, MgCl₂, Taq polymerase (Qiagen, Hilden, Germany) in the recommended buffer. Amplification involved 45 cycles in an Eppendorf Thermocycler. The total volume of the PCR reaction was 50 µl. The primers were designed according to the known sequences of GAPDH in order to amplify a DNA segment of 657 bp (forward primer: 5'-CTG GCC GTG AGT ACG TCG-3'; reverse primer: 5'-TTG ACAAG AGTG TTG GTG A3'-3'); AT₁R in order to amplify a DNA fragment of 330 bp (forward primer: 5'-GGT CTG TTGGT GTG AT-3'; reverse primer: 5'-CCA GCC AATAG TGATG AG-3'), AT₂R in order to amplify a DNA fragment of 263 bp (forward primer: 5'-CTG CTG TTGGT GTG CCT TCAT T-3'; reverse primer: 5'-GCT CTC TTCA GCC CTG CAG CC-3'), AGT in order to amplify a DNA fragment of 499 bp (forward primer: 5'-CTG GCC CTG TTA ACACT AC-3'; reverse primer: 5'-CTG TGG CTC TCT CAT CC-3'), ACE in order to amplify a DNA fragment of 428 bp (forward primer: 5'-GGT GTG GTG GAA CGAGTA TG-3'; reverse primer: 5'-CGG GGT AAA ACTGGA GA TG-3') and VEGF in order to amplify a DNA fragment of 367 base pairs (bp) (forward primer: 5'-CGG GCC TCC GAA ACCATG AAC TTT-3'; reverse primer: 5'-CTA TGT GCC GTG CCT GCC CGC CCT GGT GAG GT-3') and Bands were visualised after electrophoresis on a 2% agarose gel (Invitrogen GmbH, Karlsruhe, Germany).

Quantitative Real-Time PCR

For quantification of VEGF, AGT and β₂-Microglobulin, corresponding kits from Applied Biosystems were used according to the instructions of the manufacturer (VEGF: Hs00173626_m1; AGT: Hs01586213_m1; β₂-Microglobulin: 4326319E). The quantity of cDNA for the genes of interest was normalised to the quantity of 18S RNA in each sample (delta-CT-method). Gene expression in the figures is presented as 1/delta CT.

Verification of the successful AGT-knockdown

In order to prove the successful knockdown of AGT, we analysed the concentration of AGT-mRNA using quantitative Real-Time PCR. Here, indeed we did not observe a complete switch-off of AGT, however it was possible to show a significant reduction of the expression of AGT (p = 0.034).

Analysis of Quantitative Real-Time PCR-rawdata

The quantity of cDNA for the genes of interest was normalised to the quantity of 18S RNA in each sample by dividing the fluorescence values for the gene amplification with the fluorescence values for the 18S RNA amplification. Since these delta ct values are negatively correlated with the amount of gene expression, they were converted to 1/delta ct in order to avoid confusion.
Statistics

Statistical analysis was performed using IBM SPSS Statistics Version 21. After verification of a normal distribution of the data received after stimulation with angiotensin II, analysis of variance was used. In case of significant data, paired comparison after Bonferroni was performed. Due to the fact, that the knockdown data showed no normal distribution, statistics was calculated according the Mann-Whitney-test. Presentation of the data is carried out using Box-Whisper-plots. Differences were considered to be significant at $p < 0.05$ and significant differences between treatment arms are marked with asterisks (*).

Results

VEGF-expression in human breast cancer cells

Experiments with human breast cancer cell lines confirmed the presence of the different components of the RAS. In both, in the HR-positive cell lines MCF-7, MDA-MB 361, and ZR-75-1 as well in the HR-negative cell lines MDA-MB 231, MDA-MB 453 and MDA-MB 468 gene expression of AT1R, AT2R, AGT, ACE, and VEGF was observed (Fig. 2).

Effect of extrinsic and intrinsic angiotensin II

Since we proofed the presence of those genes in the above mentioned breast cancer cell lines, stimulation of the cells with extrinsic angiotensin II was performed in order to investigate the effect on VEGF expression. We revealed a significant extrinsic angiotensin II-dependent upregulation of VEGF in all cell lines together. However, separated analysis of HR-positive and HR-negative cells after incubation with angiotensin II only reached borderline significance (Fig. 3). In the next step, we focused on the meaning and the functionality of intrinsic angiotensin II with regard to the expression of VEGF. In absence of any extrinsic angiotensin II, the intrinsic conversion to angiotensin II was inhibited by captopril and expression of VEGF was quantified, revealing a significant de-
crease of VEGF in HR-positive and HR-negative cell lines (▶ Fig. 4). Obviously, the angiotensin II effect is mainly mediated via AT\textsubscript{1}R, since inhibition of AT\textsubscript{1}R using candesartan also caused a significant decrease of VEGF in all cell lines (▶ Fig. 4). In contrast, inhibition of AT\textsubscript{2}R by PD 123,319 did not show any significant effects on VEGF in HR-positive and HR-negative cell lines (ct = cycle threshold).

Separation of extrinsic and intrinsic effects of angiotensin II
In order to separate the effects of extrinsic and intrinsic angiotensin II on VEGF, cells were again incubated with the ACE-inhibitor captopril to prevent intrinsic angiotensin II-expression and simultaneously incubated with extrinsic angiotensin II. In HR-negative cell lines, we detected a significant decrease of VEGF-expression after treatment of cells with the ACE-inhibitor captopril and AT\textsubscript{1}R-inhibitor candesartan shows a significant decrease in HR-positive (red) (p = 0.02) and HR-negative (blue) cell lines (p < 0.01). In contrast, inhibition of AT\textsubscript{2}R using PD 123,319 did not show any significant effects on VEGF in HR-positive and HR-negative cell lines (ct = cycle threshold).
as compared to the controls, which we could not find in HR-positive cell lines (▶ Fig. 5). In addition, simultaneous incubation with angiotensin II and candesartan decreases VEGF-expression significantly in both in HR-positive and HR-negative cell lines. Furthermore, simultaneous incubation with angiotensin II and PD 123,319 again decreases VEGF-expression significantly in both in HR-positive and HR-negative cell lines. This decrease seems even to be stronger as the decrease of VEGF after inhibition of AT1R (▶ Fig. 5).

Quantification of VEGF

In order to analyse the overall effect of the RAS on VEGF and angiogenesis in HR-positive and HR-negative breast cancer cells, the last step was quantifying VEGF-expression after having performed a knockdown of AGT in those cells. In doing so, HR-positive cells showed a highly significantly increased expression of VEGF at any time of incubation (48, 72, 96, 120, and 144 hours) as compared to controls (red lines). ct = cycle threshold.

HR-negative cells only show significantly increased expression of VEGF after 48 hours of incubation (p = 0.003). After 72, 96, and 120 hours no significant differences could be observed. However, after 144 hours a significant decrease of VEGF-expression has been detected (p = 0.04).

▶ Fig. 6 VEGF-expression after knockdown of AGT via siRNA-transfection: a HR-positive cells show a highly significantly increased expression of VEGF (red) at any time of incubation (48, 72, 96, 120, and 144 hours) (p < 0.002) as compared to controls (red lines). ct = cycle threshold. b HR-negative cells only show significantly increased expression of VEGF after 48 hours of incubation (p = 0.003). After 72, 96, and 120 hours no significant differences could be observed. However, after 144 hours a significant decrease of VEGF-expression has been detected (p = 0.04).
Discussion

Aim of this study was to investigate the RAS-dependent regulation of tumorangiogenesis as a function of the hormone-receptor-status of breast cancer cells. The important influence of the RAS on VEGF is well known and has been described for many different tumor entities [27]. However, currently little is known concerning differences in RAS-dependent VEGF-expression in HR-positive and HR-negative breast cancer. In this study, it has been shown that expression of VEGF was increased due to extrinsic as well as intrinsic angiotensin II in all investigated breast cancer cells. This stimulating effect is mediated via the AT1R whereas the AT2R has a more modulating function. In addition, it was shown that knockdown of AGT increases VEGF significantly in HR-positive breast cancer cells but decreases VEGF in HR-negative cells. This indicates that RAS-dependent tumorangiogenesis in HR-positive and HR-negative breast cancer cells is regulated differently.

For many types of tumors, an important role of the RAS has been shown including cancers of the prostate, brain, cervix, pancreas und lung [28]. In particular, this concerns presence of AT1R, which is necessary for mediation of the proangiogenic effects of angiotensin II [29, 30]. Here, we showed the expression of AGT, ACE, AT1R, AT2R, and VEGF in all analysed cell lines, fulfilling the requirement for the hypothesis that tumorangiogenesis can be regulated by the RAS basically in HR-positive and HR-negative breast cancer cells. This is in line with immunohistochemical data of Jethon et al. [31], who detected the AT1R in both HR-positive and HR-negative breast cancer tissue. Former data showed, that angiotensin II has to be considered as the main player for proliferation of tumor cells as well as endothelial cell, thus angiogenesis mediating this effect via AT1R [32, 33]. Therefore, it can be hypothesized that the RAS is able to regulate VEGF and thereby influences angiogenesis in breast cancer. Due to the worse prognosis of HR-negative as compared to HR-positive breast cancer, it might have been assumed that VEGF-expression differs between these tumor types, however this could not be confirmed. Epidemiological studies showed, that women with decreased angiotensin II levels due to defect enzymes have a reduced risk for breast cancer [34, 35]. In summary, obviously the extrinsic stimulation of angiotensin II can not explain the different regulation of angiogenesis between the different breast cancer types, but can be used in order to describe the function and interaction of the different receptors.

In many tumors, the AT1R is overexpressed [29] and angiotensin II-dependent upregulation of VEGF is mediated mainly via AT1R [33]. In contrast, there is only rare and inconsistent data concerning the meaning of the AT2R for tumorangiogenesis. Concerning VEGF there is data for agonistic as well as antagonistic effects mediated by AT2R [36 – 39]. According to the published literature, at first glance parts of our data seem also to be conflicting, since on the one hand we showed that inhibition of the AT2R with PD 123,391 and simultaneous stimulation with extrinsic angiotensin II decreased VEGF. On the other hand, the sole inhibition of AT1R did not influence the amount of VEGF. Obviously, the AT2R does not act exclusively antagonistically, but has a more modulating effect in case of simultaneous activation of AT1R. Therefore, it seems that angiotensin II can perform its increasing effect on VEGF only after co-activation of AT2R. This hypothesis is also supported by Cleré et al., who showed that AT2R-mediated effects differ depending on the type of cells and the physiological context [40].

As discussed above, an extrinsic angiotensin II-dependent pathway controlling tumorangiogenesis in breast cancer is rather unlikely. It has much more to be assumed that the intrinsic angiotensin II, which is expressed by cancer cells themselves, is more important. Here, an autocrine stimulation of cancer cells followed by upregulation of VEGF seems possible. Incubation of the breast cancer cell lines with the ACE-inhibitor captopril prevented the synthesis of intrinsic angiotensin II, which might initiate an autocrine stimulation of VEGF-expression. Accordingly, a significant decrease of VEGF was detected. This result is in line with Koh et al. [34] und Gonzalez-Zuloeta et al. [35], who showed that genetically altered activity of ACE is associated with increased or decreased risk for breast cancer. However, there was no difference between the risk for HR-positive and HR-negative breast cancer. In contrast, only in HR-positive breast cancer cells, this decrease of VEGF after having prevented the production of intrinsic angiotensin II can be avoided by treatment with extrinsic angiotensin II.

Besides ACE, there are a couple of further enzymes such as chymase, catalysing the conversion from angiotensin I to angiotensin II [41, 42]. Therefore it has to be assumed that a possible therapeutic anti-angiogenic strategy via the RAS should focus on inhibition of the AT1R instead on ACE, but up to date, concerning this consideration, there is no data available. Treatment with candesartan still allows production of intrinsic angiotensin II, but obviously the level of VEGF is decreased due to the prevented autocrine stimulation. Consistent with our data in breast cancer cells, this has been presented for other tumor entities such as ovary [43], prostate [44] and pancreas [45]. However, inhibition of the AT2R does not increase VEGF, which allows to hypothesize that in contrast to others [46] inhibition of the AT2R does not antagonize the effect of AT1R-mediated angiogenesis directly.

Despite many published studies concerning the RAS and its role for tumorangiogenesis, the exact mechanism remains still unclear. In order to evaluate the total effect of the RAS on tumorangiogenesis in breast cancer, we performed a knockdown of AGT and revealed different results for HR-positive and HR-negative breast cancer cells. Although a complete knockoff of AGT was not achieved, we detected for the first time a significant upregulation of VEGF in HR-positive cells but a significant decrease of VEGF in HR-negative cells after 144 hours of incubation. Obviously, although we achieved only a partial knockdown of AGT, this knockdown was still strong enough in order to influence VEGF as described. These results can be explained by looking at a further member of the RAS, angiotensin 1-7. In the mouse model as well as in lung cancer cells, angiotensin 1-7 reduced growth of cancer cells and/or inhibited angiogenesis [47, 48]. This anti-proliferating and anti-angiogenic effect is thereby mediated by the suppression of VEGF [46, 48].

In summary, the percentage of the multiple RAS-mediated effects differs between HR-positive and HR-negative breast cancer cells. It seems possible that in HR-positive cells, the RAS acts more anti-angiogenic by influencing the angiotensin 1-7/mas-pathway, antagonising a high intrinsic VEGF-expression. In contrast, in HR-
negative cells the focus of the RAS-effects is more angiotensin II
AT₁R-based and therefore pro-angiogenic via increased intrinsic
VEGF. However, further data is needed in order to estimate, if in-
fluencing the RAS might be a future anti-angiogenic acting com-
ponent in the multi-modal therapy of breast cancer patients.

Conclusion

RAS-dependent regulation of VEGF between HR-positive and HR-
negative human breast cancer cells seems do be different. These
findings provide evidence for a possible future therapeutic strat-
egy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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