The role of the vascular endothelial growth factor and the angiopoietin molecule family in hipoxic diseases

Doctoral Thesis

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Introduction

Angiogenesis plays a crucial role in several physiological and pathological processes. During the development of the human organism angiogenesis occurs continuously, contrary to adulthood, when it happens only in the female reproductive cycle and during pregnancy. Under pathological circumstances, angiogenesis can be observed during inflammation and wound-healing, and it also plays a key role in tumorigenesis.

The first steps of angiogenesis are vasodilatation and increased capillary–permeability, which make the transport of plasma-proteins (fibrinogen, plasminogen) from the lumen to the perivascular area possible. Endothelial cells begin to proliferate and migrate by the influence of these angiogenetic factors. Finally, the new endothelial cells form tubules and become fixed to the extracellular matrix surrounding them.

A number of molecules play a role in the regulation of angiogenesis. Some of them (angiogenetic factors) contribute to, others (angiostatic factors) prevent the process of angiogenesis. From among the angiogenetic factors, we examined the vascular endothelial growth factor (VEGF) and the angiopoietin molecule family.

VEGF induces endothelial cell proliferation, migration and differentation. Due to these features, VEGF is a key regulator of angiogenesis. By increasing the synthesis and the release of nitrogen monoxid and prostacyclin, VEGF enhances vascular permeability. Due to its inhibiting effect on smooth muscle cell proliferation and thrombocyta aggregation, VEGF has a vasculoprotective effect.

Following the discovery of VEGF, another growth factor family was identified, which was also essential in angiogenesis. So far, four members of the angiopoietin molecule family are known: angiopoietin-1 (Ang1), angiopoietin-2 (Ang2), angiopoietin-3 (Ang3) and angiopoietin-4 (Ang4). During our investigation, we tested Ang1 and Ang2 and also their receptor, the Tie2 receptor in detail.

The Ang1 induced Tie2 signalization regulates endothelial migration, survival and takes part in the formation of capillary tubulae. Through these effects, Ang1 plays a crucial role in vascular remodelling, cell-matrix interactions and vascular stabilization as well. The influence of Ang2 is not so definite: depending on the presence of VEGF, Ang2 is capable of enhancing both vascular development and vascular regression.

The aims of our study

1. Growing evidence supports that beside renal tubular epithelial injury, vascular endothelial injury also plays an important role in the development and progression of ischemic acute renal failure. The pathogenetic importance of VEGF, which is the main regulating factor of angiogenesis, is also known in the development and progression of renal diseases. In the first part of our research we tested the protein and mRNA expression on an in vivo ischemia/reperfusion rat model. The aim of our study was the following:

1. a. Hypoxia is the most powerful stimulus for VEGF transcription. The hypoxia-dependent regulation of VEGF occurs at transcriptional level and is mainly mediated by the hypoxia-inducible transcription factor (HIF-1). The aim of our study was to investigate the expression of HIF-1α and VEGF in postischemic renal damage.

1. b. Much evidence supports that the occurrence and progression of renal diseases are different in the male and the female body. Investigations involving human subjects and animal models indicate the protective role of the female sex in various kidney diseases. Considering the possible protective role of VEGF in renal damage, we tested the gender difference in the alteration of HIF-1α and VEGF expression in renal ischemia/reperfusion animal model.

There is only little information available in the literature about the importance of angiopoietins in kidney diseases. Evidence supports that Tie2-receptors can be found on the abluminal surface of the endothel cells, and that the podocytes produce Ang1, the mesangium cells produce Ang2 in the kidney. In vitro studies show that the synthesis of Ang2 increases in hipoxic condition.

1. c. The aim of our study was to investigate Ang/Tie2 mRNA expression in renal ischemia/reperfusion animal model.

2. Preeclampsia is one of the most frequent disorders that may often result in premature birth. Several mechanisms have been suggested to play a role in the pathogenesis of preeclampsia. One of the theories emphasizes the possible role of improper placentation and trophoblast invasion. Placentation requires the invasion of cytotrophoblast into the decidual and

intramyometrial portions of the spiral arteries of the uterus. These processes are regulated by VEGF. A great deal of data support the key role of VEGF in pathomechanism of preeclampsia. Low-birth-weight infants (≤1500 g) have an increased risk of perinatal morbidity and mortality. Because of their prematurity, several complications may occur in this population. Considerable amount of data support that there is a relation between perinatal disorders (retinopathy of prematurity, bronchopulmonary dysplasia, respiratory distress syndrome, ductus arteriosus persistent) and abnormal VEGF synthesis.

In the second part of our study we investigated the human genetic polymorphisms of angiogenetic factors in preeclampsia and perinatal complications.

2. **a.** We tested whether polymorphisms determining VEGF synthesis (VEGF $G^{+405}C$, and $C^{-2578}A$) had an influence on the development of preeclampsia?
2. **b.** Whether these polymorphisms play a role in the progression of preeclampsia?
2. **c.** We analysed the relationship between the carrier states of Ang2 $G^{-35}C$ and VEGF $C^{-2578}A$ genetic polymorphisms and retinopathy of prematurity in low-birth-weight infants.
2. **d.** We investigated the relationship between carrier states of VEGF $G^{+405}C$, VEGF $C^{-2578}A$, and VEGF $T^{-460}C$ genetic polymorphisms and premature birth with low birth-weight?
2. **e**. Moreover, we investigated the genetic background of perinatal complications focusing on the carrier state of VEGF genetic polymorphisms.

Methods

1. In vivo renal ischemia/reperfusion animal model

1.1. Animals tested in the experiments – surgical intervention

The experiments were performed on sexually mature, male and female Wistar rats. Animals were anaesthetized by intraperitoneal injection of pentobarbital sodium. After performing a midline abdominal incision, the left renal pedicle was isolated and occluded with an atraumatic microvascular clamp for 55 minutes. Before the end of the ischemic period, the right kidneys were removed. After the removal of the microvascular clamp, the abdomen was closed.

Groups of animals were bled to death from the abdominal aorta at 10 min (*n*= 6), 20 min (*n*= 6), 40 (*n*= 6), 60 (*n*= 6), 120 min (*n*= 5) and 240 min (*n*= 6) after reperfusion. Control animals (T0; n=6) underwent an identical surgical procedure without the occlusion of the left renal pedicle. There were 6 male and 6 female rats in each group. The measurement of Ang/Tie2 mRNA expression was carried out only in the case of the male rat groups at T0, T10, T40, T120, and T240.

1.2. Western Blotting

Kidney samples were solubilized in lysis buffer, then they were homogenisated. The lysed samples were centrifuged (13000 RPM, 5 min). Protein concentration of the supernatants was determined by the Bradford-assay. We used rat monoclonal antibody of VEGF and HIF-1α as primary- , and peroxidase-conjugated IgG antibody as secondary -antibody. Protein samples were separated by 12,5% SDS-polyacrylamide gel. Separated proteins were transferred to nitrocellulose membrane in transfer buffer. Then the blot membrane was incubated with the specific antibodies (VEGF, HIF-1α). After washing the membrane, it was incubated with secondary antibodies. The unnecessary secondary antibodies were removed by further washing. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) Western blotting detection protocol (Amersham Pharmacia).

1.3. RNA isolation and RT-PCR

All the RNA was isolated from kidney samples by kieselguhr, then RNS was reverse-transcipted using oligo-$(dT)_{12-18}$ primer to generate first-strand cDNA. cDNA was amplified

with real-time PCR. We used fluorescence resonance energy transfer (FRET) hybridisation probes for the determination of VEGF, Ang1, Ang2, Tie2, and SYBR Green I for the determination of HIF-1α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on a Light Cycler system (Roche Diagnostics, Mannheim, Germany). Specific primer pairs and probes were selected by the use of LightCycler ® Probe Design Software 2.0 (Roche Diagnostics, Mannheim, Germany). For eveluating the results we used LightCycler Software 3.5.3. (Roche Diagnostics, Mannheim, Germany).

1.4. Data analysis

We used ANOVA test and Newman-Keul post-hoc test to compare the results of real-time RT-PCR and Western blotting in corresponding groups. Data were considered to be significantly different, if *P* was less than 0.05.

2. The a nalysis of VEGF and Ang2 genetic polymorphisms

2.1. Genetic polymorphisms of VEGF in preeclampsia

We consecutively enrolled 84 nulliparous pregnant women with severe preeclampsia at 1st Department of Obstetrics and Gynecology at Semmelweis University between 1998 and 2004. The diagnosis of preeclampsia was determined by the American College of Obstetricians and Gynecologists (ACOG Practice Bulletin No. 33.). 96 randomly selected nulliparous women with uncomplicated pregnancies served as controls.

2.2. Genetic polymorphisms of Ang2 and VEGF in retinopathy of prematurity

Preterm infants were delivered and treated in the Ágost Schöpf-Mérei Institute of Obstetrics, and at at Semmelweis University, 1st Department of Gynecology and Obstetrics and 2nd Department of Gynaecology and Obstetrics, between 2000 and 2003. The treated-group (n=90) consisted of preterm infants (1160±300 gramm), who had been treated with laser photocoagulation or cryotherapy because of the risk of proliferating retinopathy of prematurity (ROP). To the untreated-group (serving as a control-group) we enrolled 110 preterm infants (1200±280 gramm) without any sign of ROP, or with ROP stage 1 or 2 and without any need for therapeutic intervention for ROP. We also compared the genetic polymorphisms of preterm infants with severe ROP (ROP stage: 4–5) with those who had not got ROP or had mild ROP (ROP stage: 1–3).

2.3. Genetic polymorphisms of VEGF in perinatal complications of low-birth-weight infants

We enrolled 128 low-birth-weight (≤1500 grams) infants in our retrospective study. All of the infants were delivered and treated at Semmelweis University, the Second Department of Gynecology and Obstetrics.

We made subgroups according to the presence of perinatal complications such as respiratory distress syndrome (RDS) (n=60) enterocolitis necrotisans (NEC) (n=49), intraventricular hemorrhage (IVH) (n=42), acute renal failure (ARF) (n=41), bronchopulmonary dysplasia (BPD) (n=23), ductus arteriosus persistent (PDA) (n=47) and atrial septal defect (ASD) (n=13). Perinatal complications were defined according to internationally accepted criteria. We compared the genotype distributions of low-birth-weight infants with 200 healthy term neonates. We also compared the genotype distributions of each perinatal complication subgroup with the low-birth-weight infants who did not suffer any diseases (control groups).

2.4. Isolation of DNA

We isolated DNA from blood samples with the help of Proteinase K enzyme in order to investigate preeclampsia. For determining the genotypes of infants, we isolated the DNA with Chelex100 reagent from dried blood samples taken for metabolic screening.

2.5. Determination of VEGF $T^{-460}C$ polymorphism using real time PCR - fluorescence resonance energy transfer (FRET)

VEGF $T^{-460}C$ SNP was determined by real-time PCR quantification using fluorescence resonance energy transfer (FRET). The PCRs were performed in a final volume of 20 µl containing 2 µl DNA. The probes have been labelled with the Light Cycler Red 640 fluorophore and fluorescein.

2.6. Determination of VEGF $C^{-2578}A$, VEGF $G^{+405}C$ and Ang2 $G^{-35}C$ polymorphisms by using PCR- restriction fragment lenght polymorphism (RFLP)

VEGF $C^{-2578}A$, VEGF $G^{+405}C$ and Ang2 $G^{-35}C$ polymorphisms were detected with the PCR–RFLP method. The PCRs were performed in a final volume of 50 µl containing 2 µl DNA. For the design of primers, we used Primer3™ software. The 3' end of the reverse primer for Ang2 contained a mismatch to generate a restriction endonuclease site. The products were digested by Bgl II to determine VEGF $C^{-2578}A$ polymorphisms, by BsmF I to

determine VEGF $G^{+405}C$ polymorphisms, and by Hind III restriction endonuclease to determine Ang2 $G^{-35}C$.

2.7. Data analysis

Logistic regression analysis was used for the analysis of the correlation between VEGF genotypes and the risk of severe preeclampsia. Multiple linear regression analysis was used to test the correlation between the carrier state of VEGF genotypes and haplotypes and the date of the hypertension and proteinuria diagnosis. Chi-square test was used to compare allelic and genotype frequencies in groups treated and not treated with ROP, and in preterm infants with severe ROP (ROP stage:4–5) and preterm infants without or with mild ROP (ROP stage:1–3). Chi-square test was used to compare allelic and genotype frequencies in low-birth-weight infants and healthy term neonates, as well as in-low-birth-weight infants with certain perinatal complications and in corresponding control groups. For the adjustment for the known risk factors of each perinatal complication, we used logistic regression analysis. All calculations were performed with the statistical software package SPSS 10.0. Hardy-Weinberg equilibrium was calculated by the use of the Arlequin software.

Results

1.1. Determination of VEGF and HIF-1α expression in post-ischemic rat kidneys

1.1.1. Alteration of HIF-1α mRNA expression

In males, HIF-1α mRNA expression increased significantly at T120 (point of time) compared to the earlier reperfusion time and to the control group. At T240, HIF-1α mRNA expression indicated further rising. In females, HIF-1α mRNA expression increased significantly at T240 compared to the previous time and to the control group.

We found a gender difference at T120 and T240 in HIF-1α mRNA expression. In T120-males there was a significantly higher HIF-1α mRNA expression than in T120 females. At T240, HIF-1α mRNA expression was higher in females than in males.

1.1.2. Alteration of VEGF mRNA expression

In males, VEGF mRNA expression increased significantly at T120 compared to the earlier time of reperfusion and to control group. Moreover at T240, VEGF mRNA expression indicated further rising. In females, VEGF mRNA expression increased significantly at T240 compared to the earlier reperfusion time and control group.

We found a gender difference at T120 and T240 time in VEGF mRNA expression. Both at T120 ans T240, we measured significantly higher VEGF mRNA expression in males compared to females.

1.1.3. Alteration of HIF-1α protein level

Both in males and females, we measured higher HIF-1α protein level at T10 compared to the control group (T0). Protein level decreased at T60, however it remained higher than the T0 level - at each further reperfusion time. In the case of HIF-1α protein levels, we did not find any gender differences either in the control group or in post-ischemic rat kidneys.

1.1.4. Alteration of VEGF protein level

In males, VEGF protein level increased at T120, but this rise was not significant. Both in males and females we measured a significantly higher protein level at T240 compared to the earlier reperfusion time and to the control group. VEGF protein level was higher in females compared to males at T240.

1.2. Determination of Ang/Tie2 expression in post-ischemic rat kidneys

1.2.1. Alteration of Ang1 mRNA expression

Ang1 mRNA expression was significantly higher in the control group (T0) compared to post-ischemic rat kidneys.

1.2.2. Alteration of Ang2 mRNA expression

We did not find any significant alteration in Ang2 mRNA expression as a result of ischemia/reperfusion.

1.2.3. Alteration of Tie2 mRNA expression

We did not find any significant alteration in Tie2 mRNA expression after ischemia/reperfusion.

2. Determination of VEGF and Ang2 genetic polymorphisms

2.1. The role of VEGF genetic polymorphisms (VEGF $C^{-2578}A$ and $G^{+405}C$) in preeclampsia

The genotype distribution of the investigated VEGF polymorphisms fulfilled the Hardy-Weinberg criteria both in the preeclamptic women and the control group. The results of logistic regression analysis indicated that the carrier state of $VEGF^{+405}G$ allele was an independent protective factor against the development of severe preeclampsia. Hypertension and proteinuria were diagnosed 1.6 and 1.9 weeks earlier, respectively, in carriers of $VEGF^{-2578}A$ allele. Hypertension and proteinuria evolved 2.8, 1.8 weeks later in carriers of VEGF $^{+405}GC/^{-2578}CC$ haplotype, respectively. The results were adjusted for the risk factors of preeclampsia (maternal age, BMI before pregnancy and smoking during pregnancy).

2.2. The role of Ang2 and VEGF genetic polymorphisms (VEGF $C^{-2578}A$ and Ang2 $G^{-35}C$) in retinopathy of prematurity

The genotype distribution of the investigated VEGF and Ang2 polymorphisms fulfilled the Hardy-Weinberg criteria in all groups.

There were no differences either in the prevalence of mutant alleles or in the genotype

distributions of the polymorphisms between groups of infants treated and not treated for ROP.

The prevalence of VEGF^{-2578}A allele was lower in male preterm infants with severe ROP (ROP stage: 4–5) than in male preterm infants without or with mild ROP (ROP stage: 1–3). The result was adjusted for known risk factors of ROP (gestational age, birth weight, supplemental oxygen therapy, mechanical ventilation).

2.3. The role of VEGF genetic polymorphisms (VEGF C^{-2578}A, VEGF T^{+460}C and VEGF G^{+405}C) in perinatal complications of low-birth-weight infants

The genotype distribution of the investigated VEGF polymorphisms fulfilled the Hardy-Weinberg criteria in each group.

The prevalence of VEGF $^{+405}$ C allele was higher in low-birth-weight infants than in healthy term neonates.

The prevalence of VEGF $^{-2578}$A allele was higher in low-birth-weight infants with NEC compared with the corresponding control group. This result was adjusted for gestational age, sepsis, PDA and cardiac failure.

We also found that carriers of VEGF $^{-2578}$AA genotype were significantly less among the low-birth-weight infants with ARF than in the corresponding control group. This result was adjusted for gestational age, sepsis, NEC, RDS, PDA and cardiac failure.

Conclusion

1. Alteration of endothelial growth factors in post-ischemic rat kidneys

1.1. Investigation of VEGF and HIF-1α in post-ischemic rat kidneys

Our results suggest that both HIF-1α and VEGF mRNA and protein expression increase in acute renal failure induced by ischemia/reperfusion.

Although our earlier study emphasized the importance of post-transcriptional regulation of VEGF synthesis, in the present study we observed that an increased VEGF mRNA expression is behind the increased VEGF protein level. We postulate that the HIF-1α transcription factor plays a role in the increase of VEGF mRNS level, however further investigation would be needed to elucidate this theory on ischemic renal injury.

1.2. Gender differences in VEGF and HIF-1α expression

In females, both VEGF and HIF-1α mRNS levels increase at a later reperfusion time than in males. At T240 reperfusion time, we found higher VEGF level in females compared to males. We did not find any gender differences between males and females in the case of HIF-1α protein level.

Estrogens are believed to increase protein synthesis through inhibiting the phosphorylation of some transcription factors. It can be postulated that in ischemia/reperfusion, probably due to this effect of estrogens, there is an enhanced VEGF protein synthesis in females compared to males.

A number of data in the literature support the beneficial effects of VEGF during renal injury. It could be hypothesized that increased post-ischemic VEGF level of females play a role in gender difference observed in the susceptibility for renal ischemia.

1.3. Investigation of Ang/Tie2 in post-ischemic rat kidneys

Recent studies support that Ang1/Tie2 signalization is inhibited by hipoxia. In different tissues this inhibition is realized in different ways: partly through the decrease of Ang1 or Tie2, and partly through the increase of the antagonist Ang2 synthesis.

It was found that Ang1 mRNA expression decreased significantly after an 55- minute ischemia followed by a 10minute reperfusion in the kidneys, while Ang2 and Tie2 mRNS expression did not alter.

Our results suggest that in the kidney, hipoxia has an inhibition effect on Ang1/Tie2 signalization and this effect is realized through the decrease of Ang1 mRNA expression.

Data support that VEGF and Ang/Tie2 play a common role in several molecular processes. In pathogenesis of inflammatory diseases and also in regulation of vascular smooth muscle cells or endothel cells permeability, VEGF and Ang1 often have contradictory effects.

In conclusion, we observed that a decrease of Ang1 mRNA precedes the increase of VEGF, in post-ischemic kidney. We hypothesized that these molecular alterations play a-key role in capillary and tubular proliferation observed in renal injury caused by ischemia/reperfusion.

2. Polymorphisms of endothelial growth factors in preeclampsia and in low-birth-weight infants

2.1. The role of VEGF genetic polymorphisms in preeclampsia

Recently it has been revealed that some of VEGF polymorphisms determine the production of VEGF. Significant correlation was observed between the VEGF $G^{+405}C$ and VEGF $C^{-2578}A$ SNPs and the VEGF production of lipopolysaccharide-induced peripheral blood mononuclear cells (PBMC). Highest VEGF production was associated with the $VEGF^{+405}GG$ genotype, intermediate production was associated with the GC genotype, and the lowest production with the CC genotype. In the case of the VEGF $C^{-2578}A$ SNP, the CC homozygous PMBCs produced significantly more VEGF than the AA homozygous cells.

Our results suggest that genetic polymorphisms of the VEGF gene, linked to an inherited alteration of VEGF production may contribute to the pathogenesis of preeclampsia. We found that the carrier state of the $VEGF^{+405}G$ allele, which is accompanied by high VEGF producing capability, decreases the risk of severe preeclampsia.

Our evidence supports that VEGF has an impact on the regulation of systemic blood pressure and the inhibition of VEGF activity may lead to proteinuria. We found that hypertension and proteinuria appeared earlier in the presence of the $VEGF^{-2578}$ A allele. It can be postulated that the progression of preeclampsia may be accelerated in the presence of polymorphisms which predispose to low VEGF producing capacity.

2.2. Genetic polymorphisms of VEGF and Ang2 in retinopathy of prematurity

Our results suggest that there is no relation between the carrier state of VEGF $C^{-2578}A$ or Ang2 $G^{-35}C$ SNPs and the risk of severe ROP in preterm infants.

To the best of our knowledge this is the first study that investigated the relation between a promoter polymorphism of Ang2 and a disease. Previously it had been revealed that the Ang2 promoter contains a 585-bp region around the transcriptional start site (–1 to –585), which is the major determinant of the Ang2 expression. The investigated $Ang2^{-35}$ SNP is localised in this promoter region of Ang2. Moreover, we found that $Ang2^{-35}$ SNP is localised at a binding site of Pax 2 transcription factor, which is known to be involved in the retinal development. Our findings, however, do not support our original hypothesis that the carrier state of $Ang2^{-35}$ SNP has an influence on the risk or the severity of ROP.

Interestingly, in a minority of tested infants (i.e. boys with ROP stage 4–5) the presence of $VEGF^{-2578}A$ allele was less prevalent. This result suggests that the presence of the mutant allele, which predisposes to decreased VEGF producing ability, might be protective against the development of serious ROP among preterm boys.

2.3. Genetic polymorphisms of vascular endothelial growth factor in perinatal complications

The prevalence of VEGF $^{+405}$ C allele was higher in low-birth-weight infants than in healthy term neonates. Our results suggest that carriers of $VEGF^{+405}$ C allele, which are predisposed to low VEGF producing ability, have an increased susceptibility to prematurity. Analyzing the genetic background of perinatal compilactions, we found that the carrier state of $VEGF^{-2578}A$ allele is in relation with the occurrence of NEC and acute renal failure.

These findings suggest that the testing of VEGF polymorphisms would provide valuable information for the risk assessment of preterm birth and perinatal complications.

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