Contents

Souhrn 2
Summary 3
1. Introduction 4
2. Objectives 5
3. Patients and Methods 6
4. Results 9
5. Discussion 14
6. Conclusions 15
7. Selected References 16
List of Publications 20

Souhrn:

Úvod: Cirkulující endotelové progenitorové buňky (EPC) mohou představovat endogenní reparační mechanismus proti probíhajícímu poškození endotelu. ANCA-asociovaná vaskulitida (AAV) je zánětlivé onemocnění postihující cévy malého a středního kalibru, které probíhá ve formě relapsů a remisí, a jehož podstatným rysem je poškození endotelu. EPC tak mohou hrát významnou roli v patogenezi AAV a sloužit jako užitečný marker pro monitoraci aktivity AAV a stratifikaci rizika u pacientů s AAV.
Hypotézy: Počet EPC u pacientů s AAV může být alterován. Snížená schopnost regenerace endotelu spojená s deficitem EPC může zvyšovat riziko relapsu u pacientů s AAV.
Pacienti a metody: Měřili jsme počty EPC kolonií (EPC-CFU) u zdravých dobrovolníků, pacientů s AAV, pacientů s chronickým selháním ledvin a u pacientů s aterosklerozou. Zjišťovali jsme vztah mezi počtem EPC-CFU a klinickými a laboratorními charakteristikami pacientů a dlouhodobým vývojem pacientů s AAV.
Výsledky: Pacienti s AAV měli významně nižší počet EPC kolonií než zdraví dobrovolníci, ale rozdíl již nebyl statisticky významný při srovnání pacientů s AAV, chronickým selháním ledvin a aterosklerozou. Kumulativní čas přežití bez relapsu se u pacientů s AAV zvyšoval spolu s vzrůstajícím vstupním počtem EPC (seřazených ve 3 ordinálně uspořádaných skupinách).
Závěr: Pacienti s AAV mají významný a perzistentní deficit EPC. Nízký počet EPC může odrážet narušený mechanismus vaskulární reparace a přispívat k opakovaným relapsům u těchto pacientů.

Summary:

Introduction: Circulating endothelial progenitor cells (EPC) may provide an endogenous repair mechanism to counteract ongoing endothelial damage. Antineutrophil cytoplasmic antibody - associated vasculitis (AAV) is an inflammatory disorder of small- to medium-sized vessels with relapsing/remitting progression and endothelial injury is a major feature of AAV. EPC thus may play and an important role in the pathogenesis of AAV, or serve as a useful marker for monitoring and/or prediction of outcomes in patients with AAV.
Hypotheses: EPC number in patients with AAV could be altered. The decreased capacity for endothelial regeneration paralleled by low EPC numbers could increase the risk of relapse in patients with AAV.
Patients and methods: We have measured EPC in healthy volunteers, patients with AAV, chronic kidney failure (CKD) and atherosclerosis by a colony-forming assay. We have investigated the relation between the numbers of EPC, clinical and laboratory characteristics of the patients, and long-term outcomes of patients with AAV.
Results: Patients with AAV had a significantly lower number of EPC than healthy subjects, but not than patients with CKD or atherosclerosis**.** The cumulative relapse-free survival increased stepwise across three increasing baseline levels of endothelial progenitor cells.
Conclusion: Patients with AAV have a significant and persistent deficiency of circulating EPCs. A low number of EPCs could reflect an impaired mechanism of vascular repair and may contribute to repeated relapses in these patients.

Introduction:

Since resident endothelial cells infrequently proliferate [1], it has been postulated that there are other sources of vascular replenishment in response to continuous damage [2]. Recent studies have identified a population of presumably bone marrow-derived cells called endothelial progenitor cells (EPCs) that circulate in peripheral blood, express a variety of endothelial surface markers, incorporate into sites of neovascularization and home to sites of endothelial denudation [3-11]. EPC counts proved to be a useful biomarker of vascular function and injury [12, 13], and to be predictive of clinical outcome in different types of disease [14ï 19]. ANCA-associated vasculitis (AAV) is a group of inflammatory disorders, characterized by inflammation and necrosis of blood vessels and relapsing remitting progression. Patients with AAV suffer from ongoing microvascular damage, which can be halted by immunosuppressive treatment. However, this treatment is frequently toxic to bone marrow and thus may decrease the number of EPCs in patients in AAV. A decreased number of EPCs could result in inadequate microvascular repair and contribute to repeated flares of the disease observed in these patients. The role of EPCs in AAV is unclear at present. We sought to investigate the numbers of EPC in patients with AAV and 1) to compare EPC counts measured in patients with AAV, other vasculopathies and helathy volunteers, 2) to analyze the relation of EPC level to the clinical and laboratory paremeters of AAV, and 3) to evaluate the possible role of EPC as a predictive marker for long term outcome of AAV.

Objectives:

Hypothesis 1: AAV patients are exposed to a number of factors that may decrease the pool of EPCs (acute systemic inflammatory response, widespread endothelial damage, treatment inducing bone marrow suppression, kidney dysfunction with retention of uremic toxins). We hypothesized that the EPC number in these patients could be severely altered.

Aim 1: To perform a cross-sectional study to assess the number of circulating EPCs in patients with AAV, in patients with other types of vascular injury (end-stage renal failure on maintenance hemodialysis, peripheral occlusive artery disease), and healthy volunteers. To analyze EPC counts in AAV patients with respect to their activity/remission status, level and type of ANCA antibodies, renal function and institution of immunosuppressive treatment.

Hypothesis 2: Decreased pool or impaired mobilization of EPCs could hamper the mechanism of endothelial repair and may contribute to repeated relapses in AAV. We hypothesized that low EPC numbers could predict the propensity to early relapse in patients with AAV.

Aim 2: To analyze the long-term outcome of AAV patients with respect to the number of EPC at baseline.

Patients and Controls:

A cohort of 41 patients with a diagnosis of AAV according to the international consensus definitions [20] were recruited from the Department of Nephrology, Charles University Hospital, Prague in 2004ï 05. At the same time, 25 healthy volunteers, 15 non-vasculitis patients on chronic hemodialysis and 13 patients with angiographically documented peripheral arterial occlusive disease (PAOD) were enrolled as control population samples. The EPCs were measured in all these patients by a colony-forming unit assay cross-sectionally at that time. 10 AAV patients were sampled twice throughout their treatment period or after reaching remission ï these second samples were included just in the subgroup analysis of AAV patients, but not in the comparison between different patient groups. The AAV cohort was then prospectively followed until September 2008, when the analysis of long-term outcomes was performed. At least once in their history, all patients were ANCA positive and 95% of them had histologically proven pauci-immune glomerulonephritis. WG was diagnosed according to the criteria of the ACR [21] and the Chapel Hill Consensus Conference definition [20], microscopic polyangiitis (MPA) was diagnosed according to the Chapel Hill Consensus Conference definition.

Methods:

Circulating EPCs were quantified by the colony-forming unit assay as described by Hill et al [13]. Briefly, blood was taken by venepuncture, and after discarding the first portion, 20 ml of blood anticoagulated with heparin was

centrifuged on Ficoll-Hypaque (Ammersham, Upsalla, Sweden) to obtain the mononuclear fraction. Peripheral blood mononuclear cells were washed in phosphate-buffered saline and counted on hemocytometer. 10^7 of recovered cells were resuspended in 4 ml of the Endocult™ medium (StemCell Technologies, Vancouver, Canada) and divided into two equal portions. Each of the portions, containing $5x10^6$ cells in 2 ml of Endocult™ was seeded in one well of the BD BioCoat™ Gelatin 6-well plate (BD Biosciences, Franklin Lakes, NJ, USA) and cultivated for 48 hours at 37°C in atmosphere with 5% CO_2. Nonadherent cells were then removed together with the rest of the medium, counted, and $2x10^6$ cells from each portion were seeded in two wells of the BD BioCoat™ Fibronectin 24-well plate (i.e. from each sample 4 wells containing 10^6 nonadherent mononuclear cells were seeded). After another 72 hours, endothelial colonies (EPC-CFU) were scored under inverse microscope. Only colonies with at least 20 cells, containing rounded cells in the middle and elongated cells at the periphery were considered as endothelial colonies.

The phenotype of EPC colonies was determined in five subjects. EPC colonies were grown on fibronectin coated glass chamber slides. CD31 (clone HC1/6, Chemicon, Temecula, CA, USA), VEGFR-1 (clone FLT-19, Sigma Saint Louise, Missouri, USA), VEGFR-2 (clone 89106, R&D Systems, Minneapolis, USA) and von Wildebrandt factor (clone 2F2-A9 BD Biosciences) positivity were determined by immunofluorescent (FITC resp. PE) labeled antibodies. Similarly, UEA-1 lectin binding was evaluated with FITC-labeled UEA-1 lectin. Cell nuclei were visualized with DAPI (Sigma-Aldrich, Germany) staining, which enabled to discriminate endothelial colonies from scattered adherent cells. As this procedure is a quite

laborious one, after confirming that EPC colonies are positive for the above mentioned markers, we did not repeat these studies in other subjects.

Results:

Number of Circulating Endothelial Progenitors Are Lower in AAV Patients than in Healthy Controls, as well as in Hemodialysis Patients vs. Healthy Controls

In the four group comparisons (AAV, HD, PAOD patients and healthy volunteers) there was a significant difference between all groups ($p < 0.0001$, Kruskal-Wallis) and in subsequent post-test analysis (Dunnôs comparison) there were significant differences in the number of circulating endothelial precursors (expressed as CFUHill/ ml blood) between AAV patients and healthy subjects ($p < 0.0001$) and between hemodialysis patients and healthy subjects ($p < 0.05$). The difference between PAOD patients and healthy volunteers did not reach statistical significance in the multiple group comparison ($p = 0.075$), as well as there were no statistical differences between the respective patient groups.

Number of Circulating Endothelial Progenitors Do Not Correlate with Markers of Inflammation and Do Not Recover during Treatment

As expected, during the course of AAV treatment, the BVAS score decreased significantly ($p < 0.001$), as did other parameters of activity too (ANCA level, $p < 0.001$, CRP, $p = 0.003$ in Kruskal-Wallis comparison among all three treatment subgroups). The GFR improved only insignificantly ($p = 0.39$), as 4 patients in the active subgroup on treatment and 5 patients in remission required chronic dialysis treatment. However, neither did we find any significant difference between the number of circulating endothelial precursors in untreated patients with

active disease, treated patients with active disease and patients in remission, nor there was any significant correlation between the number of circulating endothelial precursors and markers of activity. There was a trend towards a higher number of CFU-Hill/ml in patients without immunosuppressive treatment (median 2.2 vs. 0.6 CFU Hill/ml), but it did not reach statistical significance ($p = 0.16$). It was also irrelevant if patients were on corticosteroids only or received cytotoxic agents (cyclophosphamide or azathioprine, data not shown). Concerning 10 patients where paired samples were available, there was no difference in the number of EPCs during the course of treatment and the pairing has not given any additional information compared to the unpaired testing.

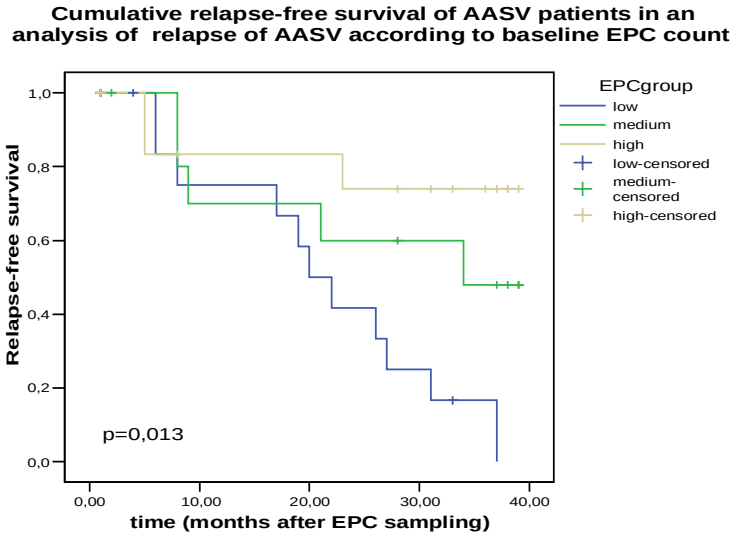
Number of Circulating EPCs Depends on the Type of ANCA and on the Severity of Renal Impairment in AAV Patients

As mentioned above, there was no significant correlation between EPC counts and levels of ANCA. However, when patients with different types of ANCA were compared, there was a trend towards higher EPC counts in patients with ANCA anti-MPO antibodies compared to patients with anti-PR3 (Mann-Whitney U test, median 3.1 vs. 0.2 CFU-Hill/ml, $p = 0.06$). When we performed the Spearman correlation between creatinine level or GFR and the number of CFUHill colonies/ml blood, we did not find any significant differences (not shown). However, when we compared two groups according to preset thresholds of GFR, we found a significantly lower number of CFU-Hill colonies in patients with a very low GFR or who were on dialysis treatment (corresponding to stage V chronic kidney disease according to K/DOQI), i.e. when patients with GFR

< 15 and > 15 ml/min were compared (median 0 vs. 1.6 CFU-Hill/ml, p = 0.015). Because of the lower number of CFU- Hill/ml in patients with AAV and significantly impaired renal function, we were interested if this lowering of circulating EPCs is not merely a surrogate marker for renal insufficiency. However, when patients with AAV and GFR < 15 ml/min or on hemodialysis were compared with patients on dialysis without vasculitis, the number of CFU-Hill/ ml were still lower for the AAV patients (median 0 vs. 1.9 CFU-Hill/ml, p = 0.03).

Impact of baseline EPC counts on Relapse:

A total of 19 patients (46%) relapsed during the study period: 11 in the low EPC group, 5 in the medium group, and 3 in the high EPC group (p=0.01). This trend was most striking with respect to renal relapse, when 7 patients (50%) in the low-EPC group but none in the medium and high-EPC group suffered from renal relapse (p<0.001). There was no significant difference in the relapse rate in other organs between the EPC-groups. Cumulative relapse-free survival increased in a stepwise fashion across increasing levels of EPC-CFUs (see figure).



In univariate analyses, both the EPC group (p=0.008) and type of disease (WG vs. MPA, p=0.02) were found to be associated with a propensity to earlier relapse. There also was a trend toward early relapse in patients with anti-PR3 antibodies as compared to anti-MPO antibodies (p=0.09). When these 3 suggested risk factors were combined in the risk analysis (type of ANCA, type of disease, EPC group), just the EPC group was independently associated with the time to relapse (hazard ratio for relapse of 0.42 (CI 0.21, 0.85) with an increase from low to medium or medium to high EPC group, (p=0.02). The frequency of outcomes and results of subsequent analyses are shown in Tables 3 and 4. Similar results were obtained when analyzing solely the 31 patients *sampled in active disease*: 9 (90%), 4 (40%), and 3 (27%) of these patients relapsed (p=0.01), and 7 (70%), 0

(0%), and 0 (0%) patients suffered from renal relapse ($p<0.001$) in the low, medium and high EPC groups respectively. The survival analysis for time to any relapse in this smaller subgroup was not significant ($p=0.1$), but was significant for time to renal relapse ($p<0.001$).

Impact of baseline EPC counts on death from any cause and renal outcome:

There was no significant difference in terms of incidence of death of any cause or measures of renal outcome (such as the mean time-averaged change in serum creatinine or dependence on dialysis treatment) with respect to baseline EPC levels.

Discussion:

We have conducted a pilot study to examine the numbers of EPC in patients with AAV. In the first part of our project, we have shown that contrary to circulating (detached) endothelial cells, the putative endothelial progenitor cells are significantly lower in patients with AAV than in healthy subjects. This could be caused by the widespread endothelial damage (with resulting depletion of EPC pool), systemic inflammatory response, uremic toxins, and other factors, such as the ANCA subtype and/or immunosuppressive treatment.
In the second part of our project we sought to investigate the potential of baseline EPC level to predict long-term outcomes in AAV patients. Our results suggest that assessment of endothelial progenitor cells in patients with ANCA-associated vasculitis may be helpful in the identification of patients at an increased risk for early relapse of the disease. Previous studies [22, 68] have identified a diagnosis of WG or PR3-ANCA at diagnosis as risk factors for relapse. Consistently, in our cohort both these factors proved to have significant role in univariate analyses, but in a combined analysis just EPC level remained to be predictive. EPC level may thus represent a conjoint surrogate marker of the balance between protective and pathogenic vascular mechanisms. If the inverse relation between EPC and risk of relapse is confirmed in further studies, measurement of EPC could become a useful tool in clinical practice, e.g. to tailor the strategy of induction and maintenance immunosuppressive treatment, and to adjust the frequency of follow-up visits according to risk stratification based on the EPC levels.

Conclusions:

- Patients with AAV have a significant and persistent deficiency of circulating EPCs.

- Low numbers of circulating EPCs are predictive of early relapse of the disease.

Selected references:

1. Schwartz SM, Benditt EP. Clustering of replicating cells in aortic endothelium. Proc Natl Acad Sci U S A 1976;73:651-65

2. Op den Buijs J, Musters M, Verrips T, Post JA, Braam B, van Riel N. Mathematical modeling of vascular endothelial layer maintenance: the role of endothelial cell division, progenitor cell homing, and telomere shortening. Am J Physiol Heart Circ Physiol 2004;287:H2651-8.

3. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964-967.

4. Shi Q, Rafii S, Wu M, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood. 1998; 92: 362ï 367.

5. Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest. 2000; 105: 71ï 77.

6. Peichev M, Naiyer AJ, Pereira D,et al. Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors. Blood. 2000; 95: 952ï 958.

7. Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med. 1999; 5: 434ï 438.

8. Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. Circulation. 2003; 108: 2511ï 2516

9. Walter D, Rittig K, Bahlmann F,et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrowï derived endothelial progenitor cells. Circulation. 2002; 105: 3017ï 3024.

10 Griese D, Ehsan A, Melo L, Kong, et al.. Isolation and transplantation of autologous circulating endothelial cells into denuded vessels and prosthetic grafts: implications for cell-based vascular therapy. Circulation. 2003; 108: 2710ï 2715

11. Fujiyama S, Amano K, Uehira K, et al.. Bone marrow monocyte lineage cells adhere on injured endothelium in a monocyte chemoattractant protein-1-dependent manner and accelerate reendothelialization as endothelial progenitor cells. Circ Res. 2003; 10: 980ï 989.

12. Vasa M, Fichtlscherer S, Aicher A, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res. 2001; 89: e1ï e7.

13. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med. 2003; 348: 593ï 600

14. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Böhm M, Nickenig G. Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med. 2005 Sep 8;353(10):999-1007.

15. Yip HK, Chang LT, Chang WN, Lu CH, et al. Level and value of circulating endothelial progenitor cells in patients after acute ischemic stroke. Stroke. 2008 Jan;39(1):69-74.

16. Bunham E, Moss M. Progenitor cells in acute lung injury. Minerva Anestesiol. 2006 Jun;72(6):369-74.

17. Rafat N, Hanusch C, Brinkkoetter PT, et al. Increased circulating endothelial progenitor cells in septic patients: correlation with survival. Crit Care Med. 2007 Jul;35(7):1677-84.

18. Michowitz Y, Goldstein E, Wexler D, Sheps D, Keren G, George J. Circulating endothelial progenitor cells and clinical outcome in patients with congestive heart failure. Heart. 2007 Sep;93(9):1046-50.

19. Sobrino T, Hurtado O, Moro MA, Rodríguez-Yáñez M, Castellanos M, Brea D, et al. The increase of circulating endothelial progenitor cells after acute ischemic stroke is associated with good outcome. Stroke. 2007 Oct;38(10):2759-64.

20. Jennette JC, Falk RJ, Andrassy K, Bacon P, Churg J, Gross WL, et al. Nomenclature of systemic vasculitides: the proposal of an International Consensus Conference. Arthritis Rheum 1994; 37: 187-92.

21. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, et al. The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. Arthritis Rheum 1990; 33: 1101-7.

22. Hogan SL, Falk RJ, Chin H, Cai J, Jennette CE, Jennette JC, et al. Predictors of relapse and treatment resistance in antineutrophil cytoplasmic antibody-associated small-vessel vasculitis. Ann Intern Med. 2005 Nov 1;143(9):621-31.

23. Booth AD, Almond MK, Burns A, Ellis P, Gaskin G, Neild GH, et al.; Pan-Thames Renal Research Group. Outcome of ANCA-associated renal vasculitis: a 5-year retrospective study. Am J Kidney Dis. 2003 Apr;41(4):776-84

Publications that formed the basis of the Thesis:

- **Závada J**, Kideryová L, Pytlík R, Vanková Z, Tesar V. Circulating endothelial progenitor cells in patients with ANCA-associated vasculitis. Kidney Blood Press Res. 2008;31(4):247-54. ISSN 1420-4096, **IF 1,268**

- **Závada J**, Kideryová L, Pytlík R, Tesar V. Circulating endothelial cells and circulating endothelial progenitors in kidney disease--victims, witnesses, or accomplices? Folia Biol (Praha). 2008;54(3):73-80. Review. ISSN 0015-5500, **IF 1,140**

- **Závada J**, Kideryová L, Pytlík R, Hrusková Z, Tesar V. Reduced number of endothelial progenitor cells is predictive of early relapse in anti-neutrophil cytoplasmic antibody-associated vasculitis. Rheumatology (Oxford). 2009 Oct;48(10):1197-201. ISSN 1462-0324, **IF 4,136**

Further publications related to the topic of the Thesis

- Rihova Z, Honsova E, **Zavada J**.: Two familial cases of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Rheumatology (Oxford). 2006 Mar;45(3):356-7 (Letter). **IF 4,052**

- Hruskova Z, Rihova Z, Mareckova H, Jancova E, Rysava R, **Zavada J**, Merta M, Löster T, Tesar V. Intracellular cytokine production in ANCA-associated vasculitis: low levels of interleukin-10 in remission are associated with a higher relapse rate in the long-term follow-up. Arch Med Res. 2009 May;40(4):276-84. **IF 1,703**

Unrelated publications:

- **Závada J**, Hoste E, Cartin-Ceba R, Calzavacca P, Gajic O, Clermont G, Bellomo R, Kellum JA; for the AKI6 investigators. A comparison of three methods to estimate baseline creatinine for RIFLE classification. Nephrol Dial Transplant. 2010 Jan 25. [Epub ahead of print] **IF 3.568**