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Original Paper

Expression of BMP-2 in Vascular Endothelial Cells of Recipient May Predict Delayed Graft Function After Renal Transplantation

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Key Words
Delayed graft function • Renal transplantation • BMP-2 • epigastric artery • endothelial cells • recipient • outcome

Abstract
Background/Aims: Delayed graft function (DGF) is associated with adverse outcomes after renal transplantation. Bone morphogenetic protein-2 (BMP-2) is involved in both endothelial function and immunological events. We compared expression of BMP-2 in epigastric artery of renal transplant recipients with immediate graft function (IGF) and DGF. ***Methods:*** 79 patients were included in this prospective study. Patients were divided in IGF group (64 patients) and DGF group (15 patients). BMP-2 expression in intima media (BMP2m) and endothelium (BMP2e) of epigastric artery was assessed by immunohistochemistry. ***Results:*** Lower intensity of BMP2e staining was recorded in DGF compared to IGF. In DGF patients, 93% had no expression of BMP2e and 7% had 1st grade expression, compared to 45% and 41% in IGF group, respectively ($P=0.001$) ($P<0.001$ for no expression and $P = 0.015$ for 1st grade expression). Patients who had BMP2e staining positive had lower odds for DGF (OR 0.059 [0.007, 0.477]) and this remained significant even after adjustment for donor and recipient variables, cold ischemia time, and immunological matching (OR 0.038 [0.003, 0.492]). ***Conclusions:*** Our results demonstrate that BMP-2 expression in endothelial cells of epigastric arteries may predict development of DGF.

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Introduction

Reported incidence of delayed graft function varies across studies depending on definition. When defined by need for dialysis within the first 7 days after transplantation, the incidence is up to 70 % [1], especially if donors after cardiac death are used [2]. Delayed graft function was found to be an independent risk factor for decreased short and long-term graft survival. Additionally, it is associated with increased incidence of acute rejections and prolonged hospitalizations [3-5]. For these reasons, the prediction and prevention of DGF may improve outcomes of renal transplantation.

Interplay between donor, recipient and procurement factors determines early posttransplant course and DGF. Donor age, size, gender, history of hypertension or diabetes, all affect posttransplant outcome. Brain death consequences, hemodynamic instability, as well as acute injury induced during kidney recovering and preservation may have significant consequences. Recipient factors known to affect posttransplant outcome include age, cardiac status, dialysis vintage and hypotension [6]. Faced with aging dialysis population, we are also faced with aging population of potential renal transplant recipients with numerous comorbidities. However, major focus of investigations in transplantation medicine is on potentially modifiable donor risk factors. Recent study suggested the importance of recipient factors in addition to donor characteristics [7]. Except for clinical characteristics, little is known about recipient factors which may influence DGF. Biomarkers are attractive tool for prediction of posttransplant outcome. Pretransplant biopsy is used by some centers for estimation of graft quality, but also for prediction of transplant outcome. Recipients' biological materials obtained before transplantation are rarely used for investigations of the posttransplant outcome.

Endothelial cells play a leading role in the response to vascular inflammation. Inflammatory stimuli induce endothelial dysfunction and induce a proadhesive endothelial phenotype [8], with leukocyte adhesion in a central position, what is also one of the crucial steps in development of immunological response after renal transplantation. Thus, research into the molecular mechanisms of endothelial cell injury may improve our understanding of DGF.

Bone morphogenetic protein-2 (BMP-2), is a member of the transforming growth factor superfamily which was originally detected in cartilage and bone [9]. Recent studies demonstrated that vascular endothelial and smooth muscle cells are also a significant source of BMP-2 [10, 11]. Bone morphogenetic proteins (BMPs) are important regulators in blood vessel formation and vascular disease [12, 13]. Additionally, BMP2 signaling plays a role in thymus morphogenesis and T-cell differentiation [14-17]. Although BMP-2 is a well-known mediator of vascular calcification [18], its role in the development of atherosclerosis remains uncertain. Based on these characteristics we hypothesize a significant role for BMP-2 in renal transplantation.

In the present study, we compared an expression of BMP-2 in epigastric artery of renal transplant recipients with immediate and delayed graft function, and investigated relation of BMP-2 expression with posttransplant outcomes.

Materials and Methods

Patients and clinical data collection

A prospective cohort study included all patients who received renal transplant at our institution from January 2012 to March 2013. The study was approved by the Ethics committee of the University hospital center Zagreb and conducted following the Declaration of Helsinki and Istanbul. Patient demographics (age, gender), cardiovascular risk factors (smoking, diabetes mellitus, hypertension, body mass index category (normal weight, underweight, overweight or obese), dyslipidemia, renal factors (primary disease, dialysis vintage, type of dialysis) were collected. Degree of HLA mismatch, PRA and cold ischemia time were recorded. 24-hour urine collection was used to determine the creatinine clearance. Clinical data obtained for each donor included age, gender, history of hypertension, diabetes mellitus, stroke as a cause of death, cardiac resuscitation during intensive care, and serum creatinine level. Control group included 16 non-

diabetic patients, nephrectomized due to localized renal cancer (T1, N0, M0), with serum creatinine within the normal range.

Assessment of vessel calcification

Pelvic X-rays obtained at the day of transplantation were evaluated by an experienced renal transplant surgeon for the presence of vascular calcifications which were classified as present or absent.

Sample collections and immunohistochemistry

The Gibson incision was used to access to the iliac fossa and expose the iliac vessels. After ligation of the epigastric artery, full circumference was resected, as described previously [19-23] and further processed in the Clinical Pathology and Cytology department, fixed in formalin, and embedded in paraffin. The original specimen slides stained with hematoxylin and eosin were reviewed for the presence of arteries. Corresponding paraffin blocks were retrieved and additional slides were prepared from multiple, sequential 4-μm-thick sections. Anti – human Pro-BMP-2 monoclonal antibody (1:100) (R&D Systems) was used for immunohistochemical analysis. Paraffin sections (3-4 μm) of all specimens were deparaffinized in xylene and then rehydrated through graded alcohol. Endogenous peroxidase activity was blocked with 0,3 % hydrogen peroxide for 10 min. Sections were baked in an oven at 97°C for 20 min. PT link tanks (Dako, Glostrup, Denmark) were used to remove paraffin and heat-induced epitope retrieval (EnVision FLEX + K8000, 3 in1, Solution High pH; Dako). All slides were incubated for 20 min at 97°C and left in buffer (EnVision FLEX wash buffer; Dako). Staining was performed using an automated immunostainer (AutostainerLink48; Dako). The protocol was as follows: slides were incubated for 10 min in an endogenous block (EnVision FLEX + K8000; Dako) and then incubated with antibody for 30 min. Then all sections were incubated for 30 min in labelled polymer (EnVision FLEX+ K8000; Dako). Each individual stage was followed by buffer rinses (EnVision FLEX + K8000; Dako). Staining was visualised using the chromogen 3,3′-diaminobenzidine for 10 min, counterstained with hematoxilin (EnVision FLEX+ K8000; Dako) for 10 min and manually cover. Immunoreactivity in tumor cells was accessed by grading (0 - 3) and the results were expressed as percentage of positive cells (negative < 10 %, 10-49 % reactive cells – grade 1, 50-74% reactive cells – grade 2, and more than 75 % reactive cells – grade – 3). A 40x objective was used. Control samples of renal arteries were collected after nephrectomy due to localized renal cancer. One section of each artery segment was used for the von Kossa stain to assess presence of microcalcifications. For von Kossa staining, tissue was fixed in methacarn solution and embedded in paraffin. Sections were then stained using the von Kossa method and counterstained with hematoxylin and eosin as described previously [24]. To confirm that von Kossa stain yielded the same results (positive or negative) regardless of the resected part of artery, we obtained sections of two parts (from each end of the arterial segment).

Definitions

Expanded criteria donor was defined with following characteristics: a. donor age older than 59 years; b. donor age between 50 and 59 years with, additionally two of the following: death caused by cerebrovascular accident (CVA); terminal serum creatinine more than 137 μmol/L; history of hypertension [25]. Delayed graft function was defined as the need for dialysis after the first week posttransplant. Clinical decision to initiate dialysis was made by attending nephrologists. Grafts without need for dialysis after 6 days had immediate function. Acute rejection was defined as increase in serum creatinine more than 25% and proven by biopsy. Patients were divided in delayed graft function (DGF) group and immediate graft function group (IGF) group based on definition of delayed graft function.

Immunosuppression

The maintenance immunosuppressive regimen consisted of mycophenolate mofetil, corticosteroids and either tacrolimus (77 patients) or cyclosporine (2 patients). All patients received basiliximab as induction therapy. Rejection episodes were treated with steroid bolus therapies.

Statistical analysis

Statistical analysis was performed using Stata/SE 11.2 for Windows (StataCorp LP, USA). Continuous variables were tested for normality of distribution using D'Agostino-Pearson's test. Differences between two groups of normally distributed variables were tested using Student's t-test while Mann-Whitney U test was used for non-normally distributed variables. Differences between categorical variables were tested using Fisher's exact test. Correlations between two variables were tested with Pearson's test and

Spearman's rank correlation test, depending on normality of distribution. Logistic regression was used to test independent association between a categorical dependent variable and several independent variables (i.e. potential confounders). Associations between micro-calcifications (assessed by Von Kossa staining) and BMP-2 expression, as well as between micro-calcifications or BMP-2 expression and creatinine or creatinine clearance, were tested by Cramér's V statistic. All values were deemed statistically significant for a two-tailed $p<0.05$.

Results

General characteristics of patients and comparison according to graft function

Seventy-nine patients undergoing renal transplantation (one receiving graft from a living donor) from which epigastric artery samples were taken at the time of surgery, were included in this prospective cohort study. Thirty eight percent of donors were male, average age was 51±10 (range 23-76) years. Four percent had history of diabetes and 49% had history of hypertension at the time of transplantation. Forty percent belonged to the group of expanded criteria donors.

Average age of recipient was 51 ± 14 years and 60% were male. The median waiting time for kidney transplantation was 2 years (range 0.083 to 7 years). Glomerulonephritis (40%) and autosomal dominant polycystic kidney disease (13%) were the most common causes of end-stage renal disease. Four recipients had type II diabetes and 8% had history of cardiovascular diseases at the time of transplantation. Vascular calcifications determined at the time of transplantation by plain X-ray were recorded in 59% of patients. Average BMI was 25.3±4.0 kg/m^2 (range 15.8-35.3 kg/m^2), with 39 (49%) patients with normal weight, 30 (38%) overweight, 8 (10%) obese and 2 (3%) underweight patients based on WHO (World Health Organization) classification. Immunological matching was favorable, with median number of 3 miss-matches (MM) (range 1-5). Two patients were sensitized, with PRA 6% and 12%. There were no differences in age or sex between 16 control subjects and renal transplant recipients with control subjects having normal serum creatinine values.

Patients were grouped according to presence of parameters for delayed graft function. There were no significant differences in general characteristics of recipients having immediate graft function (IGF) and those with delayed graft function (DGF) as shown in Tables 1 and 2. Average length of hospitalization in the whole cohort was 12 days (range 7-90 days), with interquartile range 10 to 19 days. Hospitalization was more than two times longer in patients with DGF compared to the patients with IGF (median 28 days, interquartile range 18-33 days vs. 12 days, 9-16 days, respectively, $P<0.001$). Patients with DGF had worse graft function at one year after transplantation than patients with IGF, with average creatinine clearance of 40.9±10.6 mL/min compared to 58.5±21.7 mL/min in IGF group ($P<0.001$) (Figure 1).

One year after transplantation, 15% of patients with DGF had creatinine clearance <30 mL/min, compared to 8% of patients from the IGF group ($P>0.05$), 77% DGF patients had creatinine clearance < 50 mL/min, compared to 25% in IGF group ($P<0.001$), and 51% DGF patients had creatinine clearance < 75 mL/min, compared to 8% in IGF group ($\chi^2=8.15$, $P = 0.005$). None of the DGF patients had creatinine clearance ≥75 mL/min, compared to 16% in the IGF group ($P>0.05$). Patients in the DGF group had significantly higher serum creatinine at 1 year, while no difference was observed between the groups regarding overall survival, graft loss and acute rejections (Table 3).

The 1-year recipient survivals in DGF and IGF groups were 100% vs. 96,8% respectively ($\chi^2=0.481$, $P = 1.00$), and 1-year death-censored graft survivals were 86,6% vs. 98,4% respectively ($\chi^2=1.53$, $P = 0.095$).

Association of BMP2e and BMP2m expression with delayed graft function and 1-year graft survival

In 16 control subjects, BMP2 staining was recorded in both endothelial cells and muscular cells of medial layer of renal artery. BMP-2e grade 1 staining was recorded in 50%,

Table 1. Donor characteristics according to the immediate (IGF) or delayed graft function (DGF) after transplantation

	IGF (N = 64)	DGF (N = 15)	Statistical analysis
Male gender	24 (38)	6 (40)	χ^2=0,032, p=0,857
Age	50.8 ± 10.4	54.5 ± 7.8	t=-0,129, p=0,202
BMI	26.5 ± 2.9	26.5± 2.7	t=-0,078, p=0,938
Smokers	21 (37)	4 (31)	χ^2=0,170 , p_{Fisher}=0,759
Diabetes mellitus	1(2)	2 (13)	χ^2=4,610 , p_{Fisher}=0,091
Arterial hypertension	29 (45)	10 (67)	χ^2=2,220, p=0,137
Cause of death			
Trauma	14 (22)	3 (20)	χ^2=0,035 , p_{Fisher}=1,00
Cerebrovascular	48 (76)	12 (80)	χ^2=0,099 , p_{Fisher}=1,00
Cardiovascular	11 (17)	1 (7)	χ^2=1,084 , p_{Fisher}=0,443
Hypotensive episodes	12 (19)	2 (13)	χ^2=0,269 , p_{Fisher}=1,00
Expanded criteria donor	22 (34)	9 (60)	χ^2=3,347 , p=0,067
MM (number)	3 (2-4)	3 (2-4)	z=-0,053, p=0,958
Cold ischemia time (hours)	12.9 ± 5.2	14.5 ± 5.9	t=-1,050, p=0,297

Results are presented as number (%), arithmetic mean ± standard deviation or median (interquartile range). There were no statistically significant differences between groups.

Table 2. Characteristics of patients with immediate (IGF) and delayed graft function (DGF). TIN, tubulointerstitial nephritis; BMI, body mass index; CRP, C-reactive protein; HD, hemodialysis; PD, peritoneal dialysis

Parameter	IGF (N = 64)	DGF (N = 15)	Statistical analysis
Male gender (N/%)	37 (58)	11 (73)	χ^2=1,23, p=0,268
Age	50.7 ± 13.4	51.3 ± 15.2	t=-0,148, p=0,883
Dialysis vintage (months)	25 (11-49)	24 (19-46)	z=-1,28, p=0,201
BMI	25.3 ± 4.0	25.2 ± 4.2	t=0,081, p=0,936
CRP (IU)	1.25 (0.4-4.0)	2.5 (1.0-3.0)	z=-1,52, p=0,128
HD/PD (N/%)	47 (75) / 16 (25)	13 (87) / 2 (13)	χ^2=0,993 , p_{Fisher}=0,498
Smoking (N/%)	19 (30)	4 (27)	χ^2=0,054 , p_{Fisher}=1,00
Dyslipidemia (N/%)	27 (42)	6 (40)	χ^2=0,024 p=0,877
Diabetes mellitus (N/%)	3 (5)	1 (7)	χ^2=0,099 , p_{Fisher}=0,577
Arterial hypertension (N/%)	55 (86)	13 (87)	χ^2=0, 005, p_{Fisher}=1,00
Cardiovascular disease (N/%)	6 (9)	0	χ^2=1,521 , p_{Fisher}=0,589
Arrhytmia	5 (8)	0	χ^2=1,250 , p_{Fisher}=0,577
Valvular disease	1 (2)	0	χ^2=0,237 , p_{Fisher}=1,00
RTG calcifications (N/%)	36 (56)	9 (60)	χ^2=0,070 , p=0,792
Primary renal disease (N/%)			
Glomerulonephritis	23 (36)	9 (60)	χ^2=2,920, p=0,088
Diabetes and vascular	10 (16)	0	χ^2=2,680, p_{Fisher}=0,194
Polycystic kidney disease	8 (13)	2 (13)	χ^2=0, 008, p_{Fisher}=1,00
Congenital anomalies	5 (8)	2 (13)	χ^2=0,459, p_{Fisher}=0,612
TIN	12 (19)	1 (7)	χ^2=1,291, p_{Fisher}=0,443
Endemic nephropathy	2 (3)	0	χ^2=0, 481, p_{Fisher}=1,00
unknown	4 (6)	1 (7)	χ^2=0, 004, p_{Fisher}=1,00

Results are presented as number (%), arithmetic mean ± standard deviation or median (interquartile range). There were no statistically significant differences between groups.

grade 2 in 19%, while 31% of samples remained negative. A similar pattern was observed for BMP-2m with grade 1 staining present in 56%, grade 2 in 13%, and no staining in 31% of renal artery samples. None of the controls had grade 3 BMP-2 staining of either endothelium or media.

Expressions of either BMP-2m or BMP-2e in renal transplant recipients did not differ regarding gender, smoking status, BMI, or the presence of hypertension, vascular disease or hyperlipoproteinemia (HLP), as well as they did not correlate with creatinine or creatinine clearance one year after transplatation. The effect of diabetes could not be analyzed due to low number of patients with this disease (4 patients).

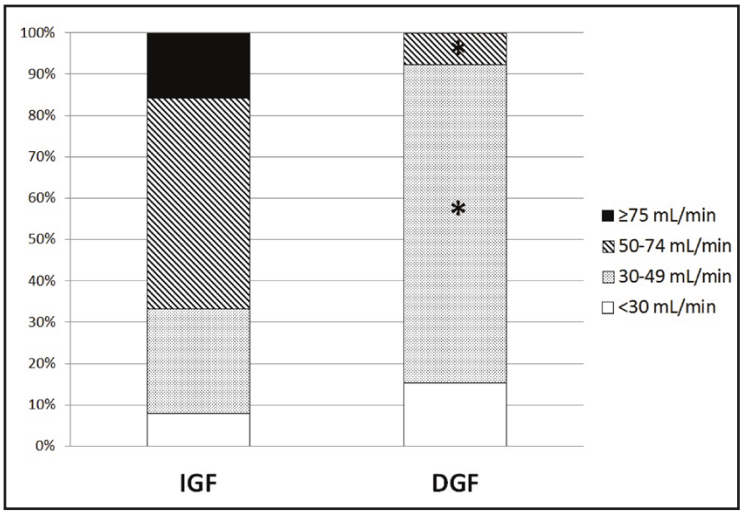

Fig. 1. Graft function at one year after transplantation in patients with IGF and DGF. Results are shown as the percent of patients in four categories of creatinine clearance (mL/min). IGF-immediate graft function, DGF-delayed graft function. *Statistically significant difference between groups for each category of creatinine clearance (Pearson's χ^2 test, $P < 0.01$).

Ninety three percent of DGF patients had no expression of BMP-2e, compared to 45% in IGF group (χ^2 = 11.3, P = 0.001) (Figure 2.). Grade 1 was found in only 7% of DGF patients compared to 41% IGF patients (χ^2 = 6.23, P = 0.013). Lower intensity of BMP2e staining was recorded in DGF compared to IGF and control patients. Grades 2 and 3 were absent in DGF patients, compared to 13% and 2%, in IGF patients (differences not statistically significant), respectively, and 19% and 0%, respectively, in control patients (differences not statistically significant). Statistically significant difference in BMP2m expression between DGF, IGF and control group was not found, although there was a trend towards lower expression in DGF group compared to the other groups with no DGF patients having grades 2 and 3 (Figure 3).

Table 3. Selected outcomes in patients with immediate (IGF) and delayed graft function (DGF)

	IGF (N = 64)	DGF (N = 15)
Death	2 (3)	0
Graft loss	1 (2)	2 (13)
Acute rejection	6 (9)	2 (13)
Serum creatinine at 1 y	155.7±38.6	201.1±51.0*
≥200 μmol/L	10 (16)	4 (31)
150-199 μmol/L	24 (39)	6 (46)
100-149 μmol/L	23 (38)	3 (23)
<100 μmol/L	4 (7)	0

Results are presented as number (%) or arithmetic mean ± standard deviation. *Statistically significant difference between groups (student's *t*-test,

We further wanted to examine if BMP2e and BMP2m expression is an independent predictor of graft function in our population. BMP2 expression was dichotomized as positive (grades 1, 2 and 3) or negative (no staining) and tested in both unadjusted and adjusted logistic regression models. Patients who had BMP2e staining positive (either grade) had lower odds for DGF after transplantation (OR 0.059 [0.007, 0.477]) and this remained significant even after adjustment for age, sex, BMI, smoking status and history of hypertension and DM (all for both donor and recipient), cold ischemia time, MM, and recipient's time from onset of dialysis to transplantation (OR 0.038 [0.003, 0.492]). On the other hand, BMP2m positive staining had no effect on graft function after transplantation neither prior (OR 1.41 [0.45, 4.42]) or after adjustment for multiple covariates (OR 0.86 [0.23, 3.26]). We further examined concordance between BMP2e and BMP2m expression. Simultaneous epigastric and media layer staining was found in 82.2% of patients, and it was higher in IGF than DGF group (87.7% vs. 66.7%, p=0.046). In the IGF group 40.0% of patients had positive staining for both BMP2e and BMP2m while only one patient in DGF group (7.1%) had both stains positive (p=0.02). Those with positive staining in both endothelium and muscular layer also had lower both unadjusted (OR 0.11 [0.01, 0.87]) and adjusted odds for DGF (OR 0.08 [0.01, 0.95]). Interestingly, not only that BMP2e positive staining was the strongest predictor of IGF, none of the other variables were predictors of graft function in our population.

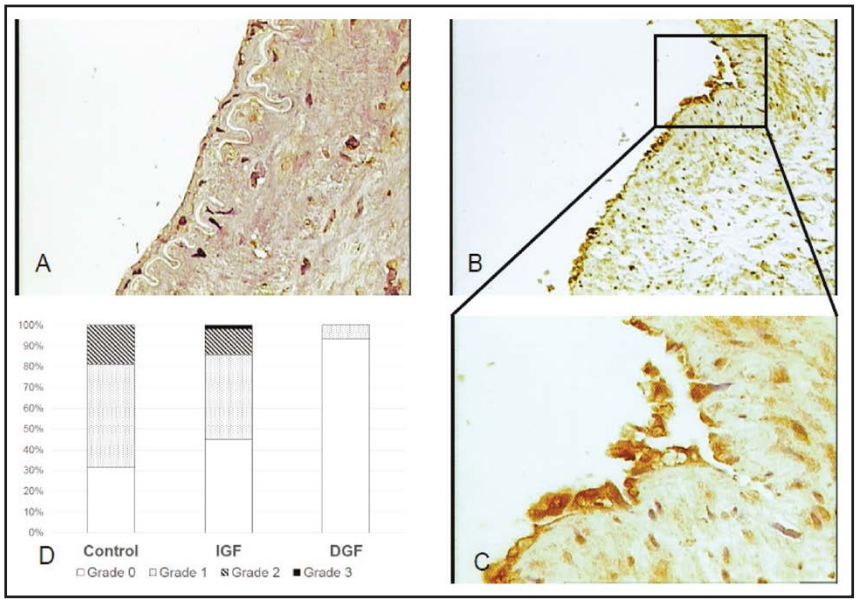

Fig. 2. BMP-2 staining in endothelial cells of epigastric artery. A. Section of a epigastric artery showing no staining for BMP. B. Immunohistochemical staining for BMP-2 demonstrates strong cytoplasmatic immunoreactivity in endothelial cells (X20). C. Enlargement x 63 D. Distribution of BMP-2 expression in endothelial cells of epigastric arteries. IGF-immediate graft function, DGF-delayed graft function. *Statistically significant difference between groups for each grade of BMP-2e expression (Pearson's χ^2 test, $P < 0.05$).

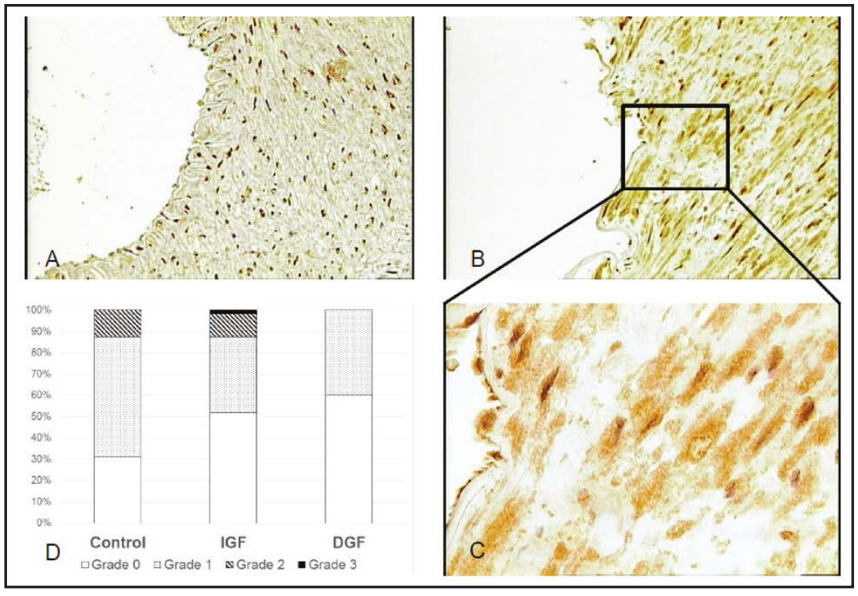

Fig. 3. BMP-2 staining in media of epigastric artery. A. Negative immunohistochemical reaction for BMP-2 in the media of the arterial wall. B. Strong cytoplasm and nuclear immunoreactivity for BMP-22 within the medial smooth muscle cells (X20). C. Enlargement x 63. D. Distribution of BMP-2 expression in the media of epigastric arteries. IGF-immediate graft function, DGF-delayed graft function. There was no statistically significant difference between two groups.

Patients who had BMP2e positive staining did not have higher odds for 1-year graft survival neither before (OR 0.54 [0.09, 3.40]) or after adjustment for multiple covariates (OR 0.85 [0.04, 20.64]). Similarly, BMP2m positive staining did not influence 1-year graft survival neither before (OR 1.35 [0.21, 8.53]) or after adjustment (OR 29.83 [0.41, 2165.96]). Those who had both stains positive also did not differ in odds of 1-year graft survival before (OR 0.31 [0.05, 2.00]) or after adjustment (0.07 [0.001, 4.35].

Von Kossa staining of epigastric arteries

Microcalcifications in the media of epigastric arteries were detected by von Kossa

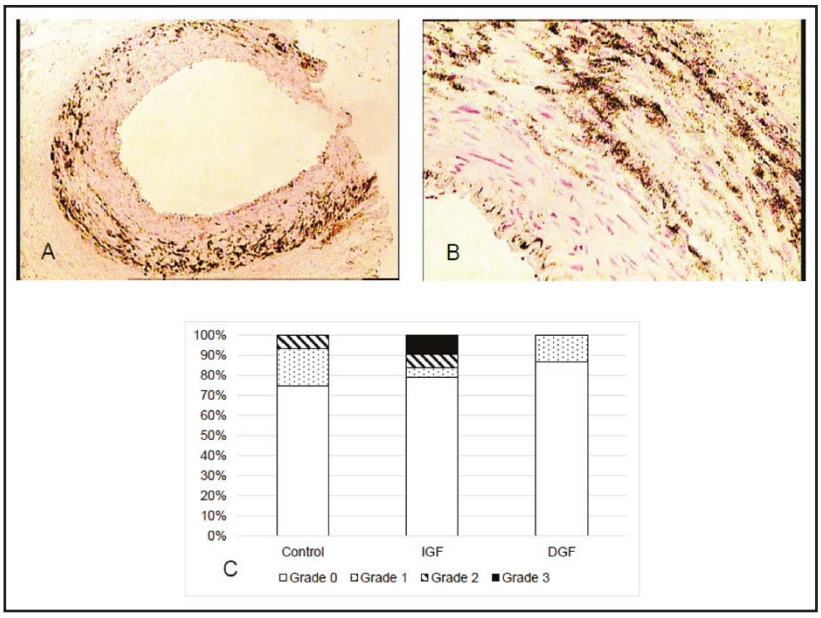

Fig. 4. von Kossa staining in vessel wall of epigastric artery. A, magnification x10, B, insert of the vessel wall showing diffuse punctuate staining. C, von-Kossa staining intensitiy in control, IGF and DGF group was without statistically significant difference.

staining in only 16,5% of the samples. They were mostly diffusely dispersed in the tissue, but in 6 patients numerous calcifications were found. There was no significant difference in von Kossa staining between groups (Figure 4).

Von Kossa staining was not associated with BMP2 staining in endothelium or media of epigastric artery. Also, there were no differences in sex, age, smoking status, BMI, or the presence of arterial hypertension, vascular disease or HLP. Von Kossa staining did not correlate with creatinine or creatinine clearance one year after transplantation. Of note, only four patients had a history of diabetes. All of them had calcifications on plain pelvic x-rays, but none had positive von Kossa staining. Iliac artery calcifications on plain pelvic x-rays were found in 46 patients (58%) from both groups. Among 46 patients with iliac calcifications, von Kossa staining was successfully performed in 45 patients, among which 9 patients (20%) were positive for micro-calcifications. On the other hand, in patients with positive staining for micro-calcifications, 60% (9 out of 15 patients) had calcifications on the plain pelvic x-ray. Microcalcifications were also not a predictor of DGF or 1-year graft survival in our population (P>0.05).

Discussion

Analysis of recipients' tissue in combination with donor characteristics seems as an interesting possibility for estimation of the posttransplant prognosis. We have shown that renal transplant recipients with high BMP-2 protein expression in endothelial cells of epigastric artery have immediate graft function and better one year graft function compared to patients without BMP-2 protein expression who suffer from delayed graft function and consequently have worse graft function one year after transplantation.

We did not observe statistically significant influence of donors' or recipients' characteristics on DGF. Group of patients with DGF more frequently received allografts from expanded criteria donors, as well as from donors who were older; more frequently had diabetes, and/or hypertension, and also had longer cold-ischemia time. However, none of these differences reached statistical significance. Our study may not be sufficiently powered to detect statistically significant association between different donor factors and early posttransplant allograft function. Additionally, cold ischaemia time was relatively short for the whole cohort, thus decreasing the influence of this parameter on outcomes after transplantation. For example, Bronzatto et al. have shown that cold ischemia time >24 hours

significantly increase risk for DGF [26], while the longest cold ischemia time in our patients was 23 hours.

In our cohort, male patients previously treated with hemodialysis and with chronic glomerulonephritis as primary disease more frequently develop DGF after renal transplantation, but without statistical significance. In previous reports in large group of patients, distinct characteristics of the recipient were found to increase risk of DGF. Maintenance dialysis vintage prior to transplant was found to be perhaps the most important contributor [27], followed by obesity and diabetes [28, 29]. Male patients, older than 55 years, of African-American race, with prolonged waiting-time period, sensitized and those who received small-for-size organs all have increased risk for DGF [3, 4, 30-32].

As expected, and already demonstrated in previous studies [6], DGF significantly prolonged initial posttransplant hospitalization (median 28 days in DGF vs. 12 days in IGF group) in our cohort. However, hospitalizations were much shorter in our patients, both in the IGF and DGF group probably because of our large centre size and extensive experience, with as short hospitalizations as possible. Also, DGF group had significantly worse allograft function at one year as determined by serum creatinine and creatinine clearance. However, no difference was observed between the groups regarding overall survival, graft loss and acute rejections, what is in contrast to findings of a meta-analysis of 34 studies performed from 1988 through 2007 which demonstrated that patients with DGF had a 49% pooled incidence of acute rejection compared to 35% incidence in non-DGF patients [33]. However, we do not perform protocol biopsies in patients with DGF thus some cases of subclinical acute rejections might be missed.

Bone morphogenetic proteins (BMP) are pleiotropic growth factors that regulate growth, differentiation, chemotaxis and apoptosis of different cell types [34]. They regulate kidney development and maintain structure and function in mature kidneys [35]. In kidney transplant recipients, only one study of biopsy series investigated the role of BMP7 in early fibrogenesis activation after transplantation [36], and our group investigated pattern of BMPs expression in kidney graphtectomy specimens, where BMP 4, 6 and 7 were downregulated in allografts with interstitial fibrosis and tubular atrophy [37].

In blood vessels, BMP-2 is expressed in atheroprone regions and is down-regulated by statins in the endothelium [38-41]. Also, endothelium-derived BMPs are osteoinductive [42], thus they may also contribute to vascular calcification during the development of atherosclerotic plaques [11, 42]. A vascular BMP-2 was found to have an important role in vascular physiology in patients with primary pulmonary hypertension [43, 44], and to participate in smooth muscle cell chemotaxis in response to vascular injury [45]. Homocystein was found to increase BMP-2 expression by vascular smooth muscle cells [46]. Additionally, BMP2 expression is increased by exposure of endothelial cells to proinflammatory stimuli with consequent induction of a proinflammatory endothelial phenotype resulting in enhanced leukocyte adhesion to the endothelial surface in vitro [39, 47]. Increased levels of BMP-2 exert proinflammatory, proatherogenic effects by inducing oxidative stress and endothelial dysfunction and have been shown to promote plaque calcification by inducing an osteogenic phenotype in vascular smooth muscle cells [45, 48]. Based on these findings, we hypothesized that BMP-2 may be involved in vascular remodeling in patients with end-stage renal disease and investigated association between their expression in blood vessels of recipients at time of transplantation, with the early and late posttransplant outcome. Artery wall evaluation at the time of transplantation may help to elucidate pathophysiologic relationship between donor and recipent.

In our cohort, BMP2 staining was recorded in the cytoplasm of endothelial cells of epigastric arteries. Statistically significant difference was recorded for BMP-2e expression between groups with lower expression intensity in DGF patients compared to IGF patients. BMP2 staining was recorded in the cytoplasm of muscular cells within the media layer of epigastric arteries. Although statistically significant difference in BMP-2m expression between DGF and IGF was not found, patients with DGF tend to have lower expression. There was no correlation of recipients' gender, smoking status, hypertension, vascular disease, BMI or HLP on expression of either BMP-2m or BMP-2e. The effect of diabetes could not be analyzed due to low number of patients with this disease (4 patients).

Postischemic events induce a continuum of complicated set of molecular, cellular, and extracellular responses that determine the function and the remodeling of the ischemic tissue [49]. Bone morphogenetic protein-7 (BMP-7) was found to decrease extent of acute renal injury in a rat model [50]. Also, exogenous BMP-7 has a neuroprotective effect after cerebral ischemia injury and promotes motor function recovery [51-53]. Less is known about the effects of BMP-2 in ischemic events. In a mouse model of acute myocardial infarction, BMP-2 reduced infarct size. Reduced rate of apoptotic cardiomyocytes both in the border zone of the infarcts and in the remote myocardium were found in mice treated with BMP-2. BMP-2 levels are increased in coronary artery disease patients with type 2 diabetes mellitus and correlate positively with the extent and complexity of coronary atherosclerotic disease as well as the degree of plaque calcification. These finding suggest that BMP-2 may be an important mediator of hyperglycemia-induced plaque progression and calcification [54]. In vitro, BMP-2 preserves cellular adenosine triphosphate stores, and decreases the rate of apoptosis. Additionally, BMP-2 induced Smad1/5/8 phosphorylation and protected adult cardiomyocytes from long-lasting hypoxia-induced cellular damage and oxidative stress avoiding activation of the transforming growth factor-β pathway, which is well known profibrotic factor [55]. If it is possible to translate these results in renal transplantation, it is possible that endogenous BMP-2 may ameliorate ischaemia-reperfusion injury in the early posttransplant period which is crucial for development of DGF. BMP family members, including BMP-2, act as proangiogenic factors, inducing endothelial cells proliferation, migration, and pseudotube formation in vitro and in vivo in a model of hindlimb ischemia. In vitro, recombinant BMP-2 activated BMP receptors, as witnessed by Id2 gene activation, increase proliferation, migration and pseudotube formation [*56]. Also,* BMP-2–muscle segment homeobox homologue (Msx2) signalling cascade can be activated by mural oxidative stress and inflammatory cytokines, suggesting that these signals participate in the arterial calcification as it is also observed in diabetic patients and animal model of chronic renal failure and the metabolic syndrome [57]. Finally, in patients with chronic kidney disease BMP-2 has recently been suggested to represent a link between oxidative stress and arterial stiffness due to vascular calcification [58]. Possible association with our results needs further investigations.

On the other hand, it was found that CD4+/CD8+ knockout mice are resistant to ischemia-reperfusion injury [59, 60]. By influencing CD4+/CD8+ lymphocytes differentiation [14, 15], BMP-2 may have additional role in development of DGF, which needs to be investigated.

Clinical relevance of vascular calcifications is not clear [61-63]. Our results additionally contribute to the opinion that endothelial dysfunction may be even more important factor than changes within the medial layer of blood vessels.

Some limitations of our study deserve future research. We did not assess the plasma levels of BMP-2. Also, histological findings of epigastric artery may not be generalizable to other arterial territories. The cross-sectional design of the study also makes determining a causal relationship between BMP-2 and DGF challenging. Finally, this study was limited by a relatively small sample size. Further prospective studies are required to determine the role that BMP-2 levels have on clinical outcomes in renal transplant recipients.

This study provides the evidence that histologic materials obtained not only from donor, but also from recipient may have prognostic value after renal transplantation. We have shown that increased BMP-2 expression in endothelial, but not muscular cells of media layer of the epigastric artery of recipient, may predict development of DGF, as well as graft function at 1 year after transplantation. By expanding the knowledge about molecular events associated with development of DGF we may discover novel preventive and/or treatment options in renal transplantation.

Conclusion

In this study, lack of BMP-2 expression in endothelial cells is strongly associated with delayed graft function. Whether BMP-2 expression has beneficial effect on primary allograft

function by influencing ischaemia-reperfusion injury, modifying immunological response or both, or by some other mechanisms, remains to be elucidated.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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