Bioactive Factors in Platelet-Rich Plasma Obtained by Apheresis

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Keywords

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Summary

Introduction: The use of platelet-rich plasma (PRP) in regenerative approaches in cartilage repair is becoming more common. Information about PRP composition and its content of putative bioactive chondrogenic growth factors (GF) that may support cartilage regeneration is scarce. **Methods:** GF composition of a pool of 6 PRP preparations was determined using Protein Antibody Membrane Arrays covering 507 GF, signaling molecules, and receptors. To verify the chondrogenic GF variability in PRP, Growth Factor Antibody Membrane Arrays covering 26 GF were applied to 6 individual PRP preparations. Selected GF involved in chondrogenic differentiation were quantified by Enzyme-Linked Immunosorbent Assay (ELISA). **Results:** 417 out of 507 possible detectable proteins were present in the PRP pool, including 76 GF. Quantification of selected chondrogenic GF by ELISA showed an average of 0.31 ng/ml bone morphogenetic protein-2, 0.50 ng/ml connective tissue growth factor, 0.76 ng/ml fibroblast growth factor-2, and 0.59 ng/ml transforming growth factor- β 3. **Conclusion:** PRP as a therapeutic option in regenerative cartilage repair strategies is a powerful tool for the local application of chondrogenic GF to the site of injury. Chondrogenic GF are present in PRP and may support cartilage repair by inducing cell differentiation and cartilage matrix formation.

Introduction

Advanced therapy medicinal products (ATMP) are medicinal products used for the treatment of diseases and injuries, and are based on the methods of somatic cell therapy, gene therapy, or tissue engineering [1]. Tissue engineering-based ATMP involve a range of approaches, whose key element is the use of biologically based mechanisms to achieve the repair and healing of damaged and diseased tissues. These approaches range from small blood vessel replacement over the repair of bone, tendon, and ligament to cellular-based therapies for degenerative cartilage defects [2]. One approach for cartilage repair in orthopedic surgery is autologous chondrocyte implantation (ACI) [3]. Further developments of the ACI technique led to the combination of autologous chondrocytes with scaffold materials to form three-dimensional bioactive resorbable cell grafts [4]. This procedure involves the excision of a small cartilage biopsy by arthroscopy from a less load-bearing region of the articular cartilage. The chondrocytes are then released by enzymatic digestion and expanded in vitro. After expansion the cells are placed into a resorbable biomaterial. A second surgery is then performed either by arthroscopy or arthrotomy. The defect is debrided, and the cell transplant is fixated into the cartilage defect. A new strategy for articular cartilage repair is a cell-free technique that combines a bone marrow stimulation technique with the implantation of a three-dimensional bioactive resorbable scaffold in a one-step procedure. During bone marrow stimulation, by e.g. microfracturing or drilling, the subchondral bone is perforated to induce bleeding into the defect [5]. This technique allows mesenchymal stem and progenitor cells to migrate into the scaffold which can be immersed with autologous serum [6, 7] or platelet-rich plasma (PRP) [8, 9] to support cell migration and chondrogenic differentiation.

Platelets are known to contain various growth factors (GF) [10] which can be concentrated in PRP either by apheresis or centrifugation [11]. In clinical application PRP is used to enhance regenerative processes in mesenchymal tissues such as the repair of patellar tendon [12], anterior crucial ligaments [13], bone [14], and cartilage [8]. In cartilage repair, PRP has been shown to induce the migration and chondrogenic differentiation of subchondral mesenchymal progenitor cells [15]. In a recent case series, 52 patients with focal chondral defects received resorbable polyglycolic acid-hyaluronan (PGA-HA) implants (chondrotissue®) augmented with autologous PRP after drilling. This procedure resulted in significant improvement of the patients' situation and in the formation of hyaline-like cartilage repair tissue [8, 9]. In contrast, the use of bone marrow stimulation alone often led to qualitatively inferior fibrocartilage repair tissue [16, 17] containing a high amount of collagen type I [18]. This may be due to an insufficient local stimulation necessary to induce the formation of superior hyaline-like cartilage repair tissue, which is characterized by a high amount of collagen type II [19].

PRP is defined as a plasma fraction of autologous blood with a certain platelet concentration [20]. Activation of alpha-granules which are part of the platelets leads to the release of numerous proteins [21]. In addition to cytokines, chemokines and other proteins, the alpha-granules release GF such as platelet-derived growth factor (PDGF), platelet derived angiogenesis factor (PDAF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), basic fibroblast growth factor (FGF-2), and transforming growth factor-beta (TGF-β) [22]. TGF-β as well as FGF-2 and IGF are known to enhance proteoglycan synthesis and extra cellular matrix (ECM) formation by mesenchymal stem and progenitor cells during chondrogenic differentiation [23–25]. Additionally, PDGF is involved in the maintenance of the chondrogenic phenotype of chondrocytes and induces proliferation and proteoglycan synthesis [26]. One disadvantage of using autologous PRP is patient variability. This variability can lead to differences in GF concentration and composition and therefore to an unpredictable outcome of a PRP-based cartilage defect treatment. Therefore, our aim was to investigate the content of released bioactive factors in individual PRP and to identify the specific GF or combinations of GF in PRP that are known to be involved not only in chondrogenic differentiation and but potentially also in PRP-mediated cartilage repair.

Material and Methods

Preparation of Human Platelet-Rich Plasma

PRP (n = 6) from normal, healthy blood donors (18–68 years) was extracted by apheresis at the Department of Transfusion Medicine, Charité-Universitätsmedizin Berlin, using an automated blood collection system (Trima Accel®, Terumo BCT, Lakewood, CO, USA) with ACD-A (anti-

coagulant citrate dextrose-A). Anonymized PRP was used for further analysis. The concentration of platelets was $0.6\text{--}1.3 \times 10^{10}/\text{ml}$. Leukocytes were less than $0.3 \times 10^4/\text{ml}$. PRP (approximately 250 ml per preparation) was frozen overnight at -80°C . Platelet activation was performed by repeated freeze and thawing cycles as described by Weibrich et al. [27]. The PRP was centrifuged, and the supernatant was taken and stored at -20°C . Prior to use, PRP was thawed slowly at 4°C , followed by centrifugation at 4°C at $1,600 \times g$ for 10 min to remove residual fibrinogen. The supernatant was used immediately. Total protein content of each individual PRP was determined using the bicinchoninic acid (BCA) assay (Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer's recommendations.

Human Protein Antibody Membrane Array Analysis

The presence of 507 different proteins in the PRP (pool of n = 6 preparations) was determined through the use of a human Protein Antibody Membrane Array 1 (RayBiotech, Norcross GA, USA) according to the manufacturer's instructions. First, the total protein content of the PRP pool was adjusted to approximately 3.9 mg. Thereafter 650 µg total protein of each PRP preparation was used, and the primary amines of the proteins were biotinylated for 30 min. The biotin-labeled PRP pool was added onto the array membrane and incubated at room temperature (RT) for 2 h. Detection of proteins was performed by incubation with horseradish peroxidase(HRP)-conjugated streptavidin for 2 h. Signal intensities were detected by chemiluminescence, and the membranes (n = 2) were briefly exposed to X-ray films (GE Healthcare, Munich, Germany) for 30 s. The presence or absence of each protein (indicated by black spots) was determined macroscopically by two independent observers. Only spots that were detected by both observers were considered to be present.

Human Growth Factor Antibody Membrane Array Analysis

The content of 41 GF and GF receptors was determined in individual PRP preparations (n = 6) using human Growth Factor Antibody Membrane Array 1 (RayBiotech) according to the manufacturer's recommendations. After blocking, membranes were incubated for 2 h at RT with individual PRP preparations (650 µg of total protein each) diluted in blocking buffer. Afterwards, the membranes were incubated with biotin-conjugated antibodies raised against the particular GF for 1.5 h. Detection of GF was performed by incubation with HRP-conjugated streptavidin for 2 h. GF were detected by chemiluminescence, and the membranes (n = 6) were briefly exposed to an X-ray film (GE Healthcare) for 1 min.

Images were digitized as negative slides at a resolution of 3,200 dpi, and spot intensities were determined densitometrically using Photoshop CS6 software (Adobe Systems, San Jose, CA, USA) as described previously [28]. In brief, a representative background color of the array was determined in a standard area (240×240 pixel). The number of stained pixels was determined within this given area for each spot. The mean value of the negative controls (n = 8) from each array was subtracted from values of GF (n = 2 per GF) or positive controls (n = 6).

The sample covariance of all pixels from the negative controls (n = 48) was added to the negative control with the highest pixel value. This value (450 pixels) serves as a threshold; pixel values less than 450 pixels were considered as no signal/absent. For normalization of arrays, signal values of GF were divided by the mean value of the positive controls and multiplied with 100. A value of 100 represents the spot intensity of the positive control.

Enzyme-Linked Immunosorbent Assay (ELISA)

To quantify the concentrations of bone morphogenetic protein-2 (BMP-2), fibroblast growth factor-2 (basic-FGF) and connective tissue growth factor (CTGF) in individual PRP preparations (n = 6), a sandwich ELISA (PeproTech, Rocky Hill, NJ, USA) according to the manufacturer's recommendations was performed. Samples of 100 µl were applied in triplicate to a 96-well plate (R&D Systems, Abingdon, UK) precoated

Table 1. Summary of growth factors detected in PRP pool (n = 6 donors)

A	B	C	D	E	F	G	H	I	L	M	N	P	S	T	V
Activin A	BDNF	CNTF	Dkk-1	EGF	FGF-2	GCSF	HB-EGF	IGF-II	Lefty A	M-CSF	Neurturin	PDGF-AA	SCF	TGF- β 2	VEGF
Activin B	b-NGF	CTGF		EG-VEGF	FGF-4	GDF-1	HGF	Inhibin A	LIF	MFG-E8	NOV	PDGF-AB		TGF- β 3	VEGF-B
Activin C	BMP-2			VEGF	FGF-6	GDF-3					NT-3	PDGF-C			VEGF-C
Artemin	BMP-3			Endocan	FGF-7	GDF-5					NT-4	Persephin			VEGF-D
	BMP-3b			Epiregulin	FGF-8	GDF-8						Progranulin			
	BMP-4				FGF-9	GDF-9									
	BMP-5				FGF-10	GDF-11									
	BMP-6				FGF-11	GDF-15									
	BMP-7				FGF-12	GDNF									
	BMP-8				FGF-13	GH-1									
	BMP-15				FGF-16	GM-CSF									
	BTC				FGF-17										
					FGF-18										
					FGF-19										
					FGF-20										
					FGF-21										
					FGF-23										

BDNF = Brain-derived neurotrophic factor; b-NGF = beta-nerve growth factor; BMP = bone morphogenic protein; BTC = betacellulin; CNTF = ciliary neurotrophic factor; CTGF = connective tissue growth factor; Dkk = dickkopf; EGF = epidermal growth factor; EG-VEGF = endocrine gland-derived vascular endothelial growth factor; FGF = fibroblast growth factor; GCSF = granulocyte colony-stimulating factor; GDF = growth differentiation factor; GDNF = glial derived growth factor; GH = growth hormone; GM-CSF = granulocyte-macrophage colony-stimulating factor; HB-EGF = heparin-binding epidermal growth factor; HGF = hepatocyte growth factor; IGF = insulin-like growth factor; LIF = leukemia inhibitory factor; M-CSF = macrophage colony-stimulating factor; NOV = nephroblastoma overexpressed; NT = neurotrophin; PDGF = platelet-derived growth factor; SCF = stem cell factor; TGF = transforming growth factor; VEGF = endothelial growth factor.

with a polyclonal rabbit anti-human BMP-2, FGF-2 or CTGF antibody, and incubated at RT for 2 h. Afterwards, 100 μ l biotinylated polyclonal antibodies were added and incubated for 2 h at RT, covered with 100 μ l avidin-HRP-conjugate for 30 min, and finally incubated with the substrate. Emission was measured at a wavelength of 405 nm using a microplate reader (Synergy HT; BioTek, Winooski, VT, USA) with excitation at 650 nm at 5-min- intervals for approximately 50 min. Protein concentration was determined using a standard curve (0–2,000 pg/ml for BMP-2 and 0–4,000 pg/ml for FGF-2 and CTGF).

To quantify the concentration of TGF- β 3, a sandwich ELISA (R&D Systems) was used. Samples of 100 μ l were applied in triplicate to a 96-well plate precoated with a monoclonal mouse anti-human TGF- β 3 antibody, incubated at RT for 2 h, covered with 100 μ l biotinylated polyclonal goat anti-human TGF- β 3 antibody for 2 h at RT, covered with 100 μ l HRP-conjugated streptavidin for 20 min, and finally incubated with substrate for 20 min. The reaction was terminated with 50 μ l 2 N H₂SO₄. Afterwards, emission was measured at 450 nm with an excitation wavelength of 540 nm. TGF- β 3 levels were determined using a standard curve of 0–2,000 pg/ml TGF- β 3

Results

Human Protein Antibody Membrane Array Analysis

Human Protein Antibody Membrane Array analysis was used to screen for GF present in PRP. Out of 507 possible detectable proteins, 417 were present in the PRP pool. The detected proteins included 35 intracellular proteins, 44 trans-membrane proteins, 108 receptor proteins, 69 extracellular proteins, 36 interleukins, 16 matrix metalloproteinases (MMP) and their inhibitors (TIMP), 33 chemokines, and 76 GF (data not shown). All detected GF are given in table 1. GF which are evidenced from literature to be associated with chondrogenic development of chondrocytes and/or mesenchymal stem and progenitor cells are summarized in table 2.

Human Growth Factor Antibody Membrane Array Analysis

To verify the robustness of GF presence in PRP, the GF content of 6 individual PRP preparations was determined by human Growth Factor Antibody Membrane Arrays (fig. 1A). The array configuration and the detection limits are given in figure 1B. The highest spot intensities were found for the growth factors EGF (119.2–152.7, mean 140.5), PDGF-AA (52.9–72.8, mean 60.9), PDGF-AB (92.3–140.4, mean 114.0), and PDGF-BB (97.0–142.4, mean 120.1). Differences in the presence of GF between the individual PRP preparations were found for heparin-binding epidermal growth factor (HB-EGF) (0–5.4, mean 1.3), neurotrophin-3 (NT-3) (0.0–4.2, mean 1.9), NT-4 (0.0–5.7, mean 2.9), TGF- α (0.0–13.8, mean 2.3), TGF- β (0.0–3.8, mean 1.3), and TGF- β 2 (0.0–4.3, mean 2.7). In addition to the chondrogenic GF found in the PRP pool (table 2), IGF-I was detectable in all individual PRP preparations (1.8–9.2, mean 6.0). The complete GF profile (without GF receptors) of 6 individual PRP preparations is given in table 3.

Table 2. Summary of the known effects of growth factors also found in PRP on *in vitro* differentiation of chondrocytes/cartilage and mesenchymal stem/progenitor cells

Growth factor	Chondrocytes/cartilage	Mesenchymal stem/progenitor cells	Reference
BMP-2	Stimulates synthesis of ECM Increased aggrecan degradation	Increases proliferation and ECM production Downregulates collagen type I gene expression	[38, 39]
BMP-4	Induces ECM synthesis	Inhibits chondrogenic hypertrophy Enhance production of chondrogenic components	[40, 41]
BMP-7	Stimulates ECM synthesis Decrease cartilage degradation	Inhibits MSC proliferation and stimulates ECM synthesis	[42, 43]
CTGF	Important regulator of chondrocyte proliferation and differentiation	Stimulates mesenchymal cell proliferation, migration and aggregation (condensation)	[44]
FGF-2	Decreases aggrecanase activity	Enhance proteoglycan synthesis and cell proliferation	[23, 45]
FGF-18	Increases chondrocytes proliferation and stimulates ECM synthesis	Unknown	[45]
GDF-5	Promotes cartilage formation through cartilage differentiation	Increases glycosaminoglycan and collagen type II content in hMSC	[46, 47]
TGF- β 2	Stimulates collagen type II synthesis	Induces collagen type II and proteoglycan synthesis	[24, 49]
TGF- β 3	Increases glycosaminoglycan and collagen type II content	Induces collagen type II and proteoglycan synthesis	[15, 37]

BMP = Bone morphogenic protein; CTGF = connective tissue growth factor; FGF = fibroblast growth factor; GDF = growth differentiation factor; TGF = transforming growth factor.

Enzyme-Linked Immunosorbent Assay (ELISA)

The most promising candidates for chondrogenic bioactive factors in PRP were quantified by sandwich ELISA. Results ranged from 0.00 to 0.65 ng/ml for BMP-2 (mean 0.31 ng/ml; median 0.25 ng/ml), from 0.00 to 0.83 ng/ml for CTGF (mean 0.50 ng/ml; median 0.54 ng/ml), from 0.40 to 0.81 ng/ml for FGF-2 (mean 0.76 ng/ml; median 0.60 ng/ml), and from 0.36 to 0.76 ng/ml for TGF- β 3 (mean 0.59 ng/ml; median 0.62 ng/ml) (fig. 2).

Discussion

The effectiveness of PRP on tissue regeneration in clinical applications is controversially discussed. Especially in the field of orthopedic surgery, clinical studies addressing PRP in cartilage repair are rare [29]. The effectiveness of PRP injection in early osteoarthritis has been shown in a prospective randomized trial over 6 months compared to placebo group [30], and in a case series of 50 patients in combination with a matrix-augmented bone marrow-stimulating technique [8, 9]. In contrast, a double-blind randomized controlled trial comparing leukocyte-rich PRP and high-molecular-weight hyaluronan showed no significant differences in clinical scores after 6 and 12 months between both applications, although

both patient groups revealed significant improvement compared to baseline [29]. But in general, the encouraging clinical data lack of assured results concerning effectiveness and underlying biological mechanisms due to great variability in PRP composition, activation, application and preparation and thus presence or absence of specific factors such as GF [29]. This might be due to the fact that PRP is a relatively undefined cocktail of various GF and differentiation factors, and individual concentrations of GF depend among others on the gender of the donor and the platelet concentration [27]. Although the concentration of GF increases with an increasing number of platelets, the method used to measure platelet concentration can also affect the results [10]. Another important factor is the activation of platelets to initiate the alpha-granules to release their GF. Different activation methods lead to different GF levels [22]. Also PRP preparation methods include several variables, such as number of centrifugation steps, speed and timing, that might influence the final product in terms of concentration of different cellular types, thus making the amount of bioactive proteins unpredictable [29]. In PRP obtained by centrifugation and calcium activation, cytokines, anti- and pro-inflammatory cytokines and GF such as PDGF, IGF-1, FGF-2, TGF- β 1 and TGF- β 2 were found in different concentrations, whereas VEGF and TGF- β 3 were not detectable [31]. In contrast to *in vitro* studies on the GF

A (growth factor array)

B (array configuration)

POS	POS	NEG	NEG	AR	b-FGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
POS	POS	NEG	NEG	AR	b-FGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
				0.1 ng/ml	10 ng/ml	0.1 ng/ml	0.001 ng/ml	10 ng/ml	1 ng/ml	1 ng/ml	0.001 ng/ml
GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF1-SR
GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF1-SR
2 ng/ml	0.1 ng/ml	0.1 ng/ml	0.02 ng/ml	0.2 ng/ml	0.001 ng/ml	0.01 ng/ml	1 ng/ml	1 ng/ml	1 ng/ml	0.01 ng/ml	10 ng/ml
IGF-II	M-CSF	M-CSFR	NT-3	NT-4	PDGFR α	PDGFR β	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
IGF-II	M-CSF	M-CSFR	NT-3	NT-4	PDGFR α	PDGFR β	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
0.01 ng/ml	0.001 ng/ml	1 ng/ml	0.02 ng/ml	0.002 ng/ml	0.01 ng/ml	0.01 ng/ml	0.1 ng/ml	1 ng/ml	1 ng/ml	0.1 ng/ml	0.01 ng/ml
SCF R	TGF- α	TGF- β	TGF- β 2	TGF- β 3	VEGF	VEGF R2	VEGF R3	VEGF-D	NEG	NEG	POS
SCF R	TGF- α	TGF- β	TGF- β 2	TGF- β 3	VEGF	VEGF R2	VEGF R3	VEGF-D	NEG	NEG	POS
0.1 ng/ml	0.01 ng/ml	0.2 ng/ml	1 ng/ml	0.1 ng/ml	0.1 ng/ml	10 ng/ml	1 ng/ml	10 ng/ml			

Fig. 1. Growth factor antibody array membrane showing the growth factor profile of PRP. Representative growth factor antibody membrane array showing the presence of growth factors (black spots) in PRP obtained from one individual donor (**A**). The respective growth factors were measured in duplicates per array, antibody sensitivity is given in ng/ml (**B**). Positive controls on the array are streptavidin conjugated horseradish peroxidase and negative controls are bovine serum albumins. POS = positive; NEG = negative; AR = androgen receptor; b-NGF = beta-nerve growth factor; EGF = epidermal growth factor; FGF = fibroblast growth factor; GCSF = granulocyte colony-stimulating factor; GDNF = glial derived growth factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; HB-EGF = heparin-binding epidermal growth factor; HGF = hepatocyte growth factor; IGFBP = insulin-like growth factor binding protein; IGF = insulin-like growth factor; IGF = leukemia inhibitory factor; M-CSF = macrophage colony-stimulating factor; NT = neurotrophin; PDGF platelet derived growth factor; PIGF = placental growth factor; SCF = stem cell factor; TGF = transforming growth factor; VEGF = endothelial growth factor.

content using PRP preparations from centrifugation studies, investigations using human PRP preparations obtained by apheresis are rare. PRP obtained by an apheresis system has

the advantage to be technically standardized, whereas the PRP preparation by laboratory centrifugation is more technician-dependent, and therefore its reproducibility is biased

Table 3. Growth factor profile of PRP preparations obtained from 6 individual PRP donors

Growth factor	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
b-NGF	0.0	0.0	0.0	0.0	0.0	0.0
EGF	145.1	129.8	152.7	119.2	146.7	149.7
FGF-2	19.9	12.4	12.8	10.1	1.8	22.0
FGF-4	10	13.4	11.7	11.9	11.8	11.0
FGF-6	0.0	0.0	0.0	0.0	0.0	0.0
FGF-7	0.0	0.0	0.0	0.0	0.0	0.0
GCSF	0.0	0.0	0.0	0.0	0.0	0.0
GDNF	3.6	3.8	7.4	0.0	2.4	4.5
GM-CSF	0.0	0.0	0.0	0.0	0.0	0.0
HB-EGF	0.0	2.5	5.4	0.0	0.0	0.0
HGF	1.8	5.1	8.7	4.6	9.0	9.1
IGF-I	1.8	7.7	5.8	9.2	7.6	3.8
IGF-II	15.5	16.4	18.6	17.0	19.8	8.6
M-CSF	6.7	1.8	8.8	3.3	3.7	1.8
NT-3	0.0	3.2	4.2	0.0	1.6	2.2
NT-4	0.0	4.8	5.7	2.8	0.0	4.2
PDGF-AA	72.8	58.8	59.4	52.9	59.1	62.9
PDGF-AB	140.4	112.5	112.5	92.3	111.9	114.5
PDGF-BB	142.4	119.3	125.1	97.0	126.2	110.5
SCF	0.5	9.6	6.0	6.8	3.5	3.4
TGF- α	0.0	0.0	13.8	0.0	0.0	0.0
TGF- β	0.0	0.0	2.1	1.8	3.8	0.0
TGF- β 2	0.0	2.8	3.1	4.3	1.9	4.2
TGF- β 3	2.0	5.6	4.9	6.5	1.9	5.6
VEGF	0.0	0.0	0.0	0.0	0.0	0.0
VEGF-D	11.0	13.1	9.9	11.7	7.6	7.0

b-NGF = Beta-nerve growth factor; EGF = epidermal growth factor; FGF = fibroblast growth factor; GCSF = granulocyte colony-stimulating factor; GDNF = glial derived growth factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; HB-EGF = heparin-binding epidermal growth factor; HGF = hepatocyte growth factor; IGF = insulin-like growth factor; LIF = leukemia inhibitory factor; M-CSF = macrophage colony-stimulating factor; NT = neurotrophin; PDGF = platelet derived growth factor; SCF = stem cell factor; TGF = transforming growth factor; VEGF = endothelial growth factor.

[29]. The intention of this study was to measure GF that are present in human PRP obtained by apheresis, as it is effectively used in combination with bone marrow-stimulating techniques for cartilage regeneration. Knowledge about the GF content of PRP may enlighten the underlying mechanisms how PRP act on chondrocytes and/or mesenchymal stem and progenitor cells [8, 9]. Studies regarding the cellular, chondrogenic response of mesenchymal progenitor cells derived from subchondral bone on RNA and protein level upon PRP treatment are reported previously. The use of 5% activated human PRP obtained by apheresis ($0.6\text{--}1.3 \times 10^{10}$ platelets/ml) in a well-established three-dimensional chondrogenic differentiation assay showed an increase of the expression of chondrogenic marker genes collagen type II, aggrecan, and cartilage oligomeric matrix protein in subchondral progenitor cells compared to non-stimulated controls [15]. Additionally, progenitor cells in three-dimensional scaffolds formed an extracellular matrix rich in proteoglycans and collagen type II upon treatment with 5% PRP obtained by apheresis ($0.6\text{--}1.3 \times 10^{10}$

platelets/ml) as shown by immunohistochemical and histochemical staining [32].

Taking the stimulatory chondrogenic effect of PRP on subchondral progenitor cells into account, we aim at defining bioactive factors in PRP which possibly mediate chondrogenic induction of mesenchymal progenitor cells. In this study, we found that of the 76 detected GF present in a PRP pool of 6 PRP preparations obtained by apheresis, 9 are known from literature to play a major role in the chondrogenic differentiation of chondrocytes and/or mesenchymal stem and progenitor cells. Assessment of the GF content in 6 individual PRP preparations obtained from apheresis revealed 3 constantly detectable GF (IGF-I, FGF-2 and TGF- β 3) and 2 GF which are occasionally present. An increase in the number of PRP preparations might lead to a more homogeneous population of this highly individual substance and subsequently more detailed results concerning presence or absence of distinct GF. Therefore, the small sample size of 6 individual PRP preparations is a limitation of the study. Quantification of selected

Fig. 2. Quantification of selected growth factors by ELISA. The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the minimum and the maximum of all data. FGF-2 and TGF-β3 are robustly present in all 6 PRP preparations with a range of 0.40 ng/ml to 0.81 ng/ml for FGF-2 (median 0.60 ng/ml) and 0.36 ng/ml to 0.76 ng/ml for TGF-β3 (median 0.62 ng/ml), whereas BMP-2 and CTGF are found in 5 of 6 donors in variable concentrations between 0.00 ng/ml to 0.65 ng/ml for BMP-2 (median 0.25 ng/ml) and 0.00 ng/ml to 0.83 ng/ml for CTGF (median 0.54 ng/ml).

GF from pooled PRP and individual preparations by ELISA resulted in mean concentration of 0.31 ng/ml BMP-2, 0.50 ng/ml CTGF, 0.76 ng/ml FGF-2, and 0.59 ng/ml TGF-β3.

GF such as PDGF-BB, IGF-I and IGF-II, which are present in each of 6 individual PRP preparations, have been shown to recruit human mesenchymal progenitor cells derived from the bone marrow [33, 34], and chondrocytes [35] as assessed by in vitro migration assays using the respective GF. Besides their migratory effects, PDGF, IGF-I and FGF-2 enhance proliferation of human chondrocytes in vitro [36]. In addition, treating mesenchymal stem cells with FGF-2 in monolayer cultures results in enhanced cell proliferation and proteoglycan synthesis compared to untreated cultures [23]. Previous studies with single GF found in PRP preparation, such as BMP-2, BMP-4, BMP-7, CTGF, FGF-2, growth differentiation factor-5 (GDF-5), IGF-I, TGF-β2 and TGF-β3, are involved in chondrogenic development of chondrocytes and/or mesenchymal stem and progenitor cells. In order to assess their effects on cartilage repair in vitro or in vivo, all of these GF have been used as single inducers rather than in combination [15, 23–25, 37–49]. Only few GF have been tested in combination, suggesting additive effects; an enhanced chondrogenic development was observed when mesenchymal stem cells were induced with IGF-I and either TGF-β or BMP-2 or BMP-7, leading to a significant increase of cartilage matrix synthesis [50]. Also, a combination of BMP-2, BMP-4, BMP-7 or FGF-2 with TGF-β supports the chondrogenic induction of MSC in vitro [51]. Our results show that BMP-2, which stimulates the synthesis of ECM by chondrocytes [38], and CTGF, known as an important regulator of chondrocyte proliferation and differentiation [44], are not always present in all PRP preparations. However, TGF-β3, which induces collagen type II and proteoglycan synthesis by mesenchymal progenitor cells and chondrocytes [15, 37], and FGF-2, which was found to enhance proteoglycan synthesis and cell proliferation by

MSC [48], are detected in all individual PRP preparations. Additionally, other GF identified in array analyses, such as brain-derived neurotrophic factor (BDNF), FGF-17 and FGF-18, are suspected to have an influence on chondrogenic development in cartilage repair and/or homeostasis [52–54]. However, the effect of PRP on mesenchymal stem or progenitor cells is controversially discussed. On the one hand, PRP induces the proliferation of mesenchymal stem cells and activates osteogenic and chondrogenic marker transcription factors such as runt-related transcription factor-2 (RUNX2) and SOX-9 in monolayer cultures [55]. On the other hand, PRP promotes chondrogenic but not osteogenic or adipogenic differentiation of human subchondral progenitor cells in pellet cultures [15]. To overcome the problems of GF composition and concentration due to different production and activation techniques, the GF content should be determined for each batch. The prediction of specific GF concentrations based on PRP thrombocyte counts or by correlation with other GF within the PRP is not reliable [27]. For clinical use, a standardized PRP preparation by apheresis may decrease technical bias. Therefore, qualitative and quantitative analysis of GF content in PRP preparation by apheresis is the basis for further studies on cell differentiation in vitro and tissue regeneration in vivo.

Different preparation techniques might be useful for different applications. Therefore, the used PRP preparation system, such as apheresis, centrifugation or PRP-processing kits, should be tested concerning its distinct properties of the resulting PRP.

In conclusion, PRP as a therapeutic option in regenerative cartilage repair strategies might be a powerful tool for the local application of chondrogenic GF to the site of injury. Concentration and content of bioactive factors present in PRP depend on the preparation and activation method and should be individually adapted to the respective application.

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Disclosure Statement

JPK; UF; SV; KN; ME and CK are employees of TransTissue Technologies GmbH. TransTissue develops regeneration medicine therapies based on mesenchymal cells.

References

- Lucas-Samuel S: Advanced therapy: From European regulatory framework to national regulatory framework (in French). *Transfus Clin Biol* 2013; 20:221–224.
- Langer R, Vacanti JP: Tissue engineering. *Science* 1993;260:920–926.
- Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L: Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* 1994;331:889–895.
- Endres M, Neumann K, Schroder SE, Vetterlein S, Morawietz L, Ringe J, Sittering M, Kaps C: Human polymer-based cartilage grafts for the regeneration of articular cartilage defects. *Tissue Cell* 2007;39: 293–301.
- Steadman JR, Rodkey WG, Briggs KK, Rodrigo JJ: The microfracture technic in the management of complete cartilage defects in the knee joint (in German). *Orthopade* 1999;28:26–32.
- Patrascu JM, Freymann U, Kaps C, Poenaru DV: Repair of a post-traumatic cartilage defect with a cell-free polymer-based cartilage implant: a follow-up at two years by MRI and histological review. *J Bone Joint Surg Br* 2010;92:1160–1163.
- Zantop T, Petersen W: Arthroscopic implantation of a matrix to cover large chondral defect during microfracture. *Arthroscopy* 2009;25:1354–1360.
- Siclari A, Mascaro G, Gentili C, Kaps C, Cancedda R, Boux E: Cartilage repair in the knee with subchondral drilling augmented with a platelet-rich plasma-immersed polymer-based implant. *Knee Surg Sports Traumatol Arthrosc* 2013; DOI: 10.1007/s00167-013-2484-1.
- Siclari A, Mascaro G, Gentili C, Cancedda R, Boux E: A cell-free scaffold-based cartilage repair provides improved function hyaline-like repair at one year. *Clin Orthop Relat Res* 2012;470:910–919.
- Alsousou J, Thompson M, Hulley P, Noble A, Willett K: The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery: a review of the literature. *J Bone Joint Surg Br* 2009; 91:987–996.
- van der Meer PF: Platelet concentrates, from whole blood or collected by apheresis? *Transfus Apher Sci* 2013;48:129–131.
- de Almeida AM, Demange MK, Sobrado MF, Rodrigues MB, Pedrinelli A, Hernandez AJ: Patellar tendon healing with platelet-rich plasma: a prospective randomized controlled trial. *Am J Sports Med* 2012;40:1282–1288.
- Silva A, Sampaio R: Anatomic ACL reconstruction: does the platelet-rich plasma accelerate tendon healing? *Knee Surg Sports Traumatol Arthrosc* 2009;17:676–682.
- Eskandar MA, Greenwell H, Hill M, Morton D, Vidal R, Shumway B, Girouard ME: Platelet-rich plasma assisted guided bone regeneration for ridge augmentation: a randomized, controlled clinical trial. *J Periodontol* 2013; doi: 10.1902/jop.2013.130260.
- Krüger JP, Hondke S, Endres M, Pruss A, Siclari A, Kaps C: Human platelet-rich plasma stimulates migration and chondrogenic differentiation of human subchondral progenitor cells. *J Orthop Res* 2012;30:845–852.
- Knutsen G, Engebretsen L, Ludvigsen TC, Drogset JO, Grøntvedt T, Solheim E, Strand T, Roberts S, Isaksen V, Johansen O: Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. *J Bone Joint Surg Am* 2004;86A:455–464.
- Gudas R, Kalesinskas RJ, Kimtys V, Stankevicius E, Toliulis V, Bernotavicius G, Smailys A: A prospective randomized clinical study of mosaic osteochondral autologous transplantation versus microfracture for the treatment of osteochondral defects in the knee joint in young athletes. *Arthroscopy* 2005;21:1066–1075.
- Frishie DD, Oxford JT, Southwood L, Trotter GW, Rodkey WG, Steadman JR, Goodnight JL, McIlwraith CW: Early events in cartilage repair after subchondral bone microfracture. *Clin Orthop Relat Res* 2003;407:215–227.
- Goldring MB, Tsuchimochi K, Ijiri K: The control of chondrogenesis. *J Cell Biochem* 2006;97:33–44.
- Marx RE: Platelet-rich plasma (PRP): what is PRP and what is not PRP? *Implant Dent* 2001;10:225–228.
- Nikolidakis D, Jansen JA: The biology of platelet-rich plasma and its application in oral surgery: literature review. *Tissue Eng Part B Rev* 2008;14: 249–258.
- Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT: Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb Haemost* 2004;91:4–15.
- Stewart AA, Byron CR, Ponden H, Stewart MC: Effect of fibroblast growth factor-2 on equine mesenchymal stem cell monolayer expansion and chondrogenesis. *Am J Vet Res* 2007;68:941–945.
- Barry F, Boynton RE, Liu B, Murphy JM: Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. *Exp Cell Res* 2001;268:189–200.
- Longobardi L, O'Rear L, Aakula S, Johnstone B, Shimer K, Chytil A, Horton WA, Moses HL, Spagnoli A: Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-beta signaling. *J Bone Miner Res* 2006;21:626–636.
- Kon E, Buda R, Filardo G, Di Martino A, Timoncini A, Cenacchi A, Fornasari PM, Giannini S, Marcacci M: Platelet-rich plasma: intra-articular knee injections produced favorable results on degenerative cartilage lesions. *Knee Surg Sports Traumatol Arthrosc* 2010;18:472–479.
- Weibrich G, Kleis WK, Hafner G, Hitzler WE: Growth factor levels in platelet-rich plasma and correlations with donor age, sex, and platelet count. *J Craniomaxillofac Surg* 2002;30:97–102.
- Kaps C, Bramlage C, Smolian H, Haisch A, Ungethüm U, Burmester GR, Sittering M, Gross G, Hauptl T: Bone morphogenetic proteins promote cartilage differentiation and protect engineered artificial cartilage from fibroblast invasion and destruction. *Arthritis Rheum* 2002;46:149–162.
- Kon E, Filardo G, Matteo BD, Marcacci M: PRP for the treatment of cartilage pathology. *Open Orthop J* 2013;7:120–128.
- Patel S, Dhillon MS, Aggarwal S, Marwaha N, Jain A: Treatment with platelet-rich plasma is more effective than placebo for knee osteoarthritis: a prospective, double-blind, randomized trial. *Am J Sports Med* 2013;41:356–364.
- Amable PR, Carias RB, Teixeira MV, da Cruz Pacheco I, Correa do Amaral RJ, Granjeiro JM, Borojovic R: Platelet-rich plasma preparation for regenerative medicine: optimization and quantification of cytokines and growth factors. *Stem Cell Res Ther* 2013;4:67.
- Krüger JP, Ketzmar AK, Endres M, Pruß A, Siclari A, Kaps C: Human platelet-rich plasma induces chondrogenic differentiation of subchondral progenitor cells in polyglycolic acid-hyaluronan scaffolds. *J Orthop Res* 2012;30:845–852.
- Fiedler J, Roderer G, Gunther KP, Brenner RE: BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. *J Cell Biochem* 2002;87:305–312.
- Fiedler J, Brill C, Blum WF, Brenner RE: IGF-I and IGF-II stimulate directed cell migration of bone-marrow-derived human mesenchymal progenitor cells. *Biochem Biophys Res Commun* 2006; 345:1177–1183.
- Mishima Y, Lotz M: Chemotaxis of human articular chondrocytes and mesenchymal stem cells. *J Orthop Res* 2008;26:1407–1412.
- Gaissmaier C, Fritz J, Krackhardt T, Flesch I, Aicher WK, Ashammakhi N: Effect of human platelet supernatant on proliferation and matrix synthesis of human articular chondrocytes in monolayer and three-dimensional alginate cultures. *Biomaterials* 2005;26:1953–1960.
- Tang QO, Shakib K, Heliotis M, Tsiridis E, Mantalas A, Ripamonti U: TGF-beta3: a potential biological therapy for enhancing chondrogenesis. *Expert Opin Biol Ther* 2009;9:689–701.

- 38 van Beuningen HM, Glansbeek HL, van der Kraan PM, van den Berg WB: Differential effects of local application of BMP-2 or TGF-beta 1 on both articular cartilage composition and osteophyte formation. *Osteoarthritis Cartilage* 1998;6:306–317.
- 39 Schmitt B, Ringe J, Haupl T, Notter M, Manz R, Burmester GR, Sittertinger M, Kaps C: BMP2 initiates chondrogenic lineage development of adult human mesenchymal stem cells in high-density culture. *Differentiation* 2003;71:567–577.
- 40 Jiang Y, Chen LK, Zhu DC, Zhang GR, Guo C, Qi YY, Ouyang HW: The inductive effect of bone morphogenetic protein-4 on chondral-lineage differentiation and in situ cartilage repair. *Tissue Eng Part A* 2010;16:1621–1632.
- 41 Steinert A, Weber M, Dimmler A, Julius C, Schutze N, Noth U, Cramer H, Eulert J, Zimmermann U, Hendrich C: Chondrogenic differentiation of mesenchymal progenitor cells encapsulated in ultrahigh-viscosity alginate. *J Orthop Res* 2003;21:1090–1097.
- 42 Hicks DL, Sage AB, Shelton E, Schumacher BL, Sah RL, Watson D: Effect of bone morphogenetic proteins 2 and 7 on septal chondrocytes in alginate. *Otolaryngol Head Neck Surg* 2007;136:373–379.
- 43 Shen B, Wei A, Whittaker S, Williams LA, Tao H, Ma DD, Diwan AD: The role of BMP-7 in chondrogenic and osteogenic differentiation of human bone marrow multipotent mesenchymal stromal cells in vitro. *J Cell Biochem* 2010;109:406–416.
- 44 Arnott JA, Lambi AG, Mundy C, Hendsi H, Pixley RA, Owen TA, Safadi FF, Popoff SN: The role of connective tissue growth factor (CTGF/CCN2) in skeletogenesis. *Crit Rev Eukaryot Gene Expr* 2011;21:43–69.
- 45 Ellman MB, An HS, Muddasani P, Im HJ: Biological impact of the fibroblast growth factor family on articular cartilage and intervertebral disc homeostasis. *Gene* 2008;420:82–89.
- 46 Chhabra A, Zijerdi D, Zhang J, Kline A, Balian G, Hurwitz S: BMP-14 deficiency inhibits long bone fracture healing: a biochemical, histologic, and radiographic assessment. *J Orthop Trauma* 2005;19:629–634.
- 47 Zhang B, Yang S, Sun Z, Zhang Y, Xia T, Xu W, Ye S: Human mesenchymal stem cells induced by growth differentiation factor 5: an improved self-assembly tissue engineering method for cartilage repair. *Tissue Eng Part C Methods* 2011;17:1189–1199.
- 48 Boehm AK, Seth M, Mayr KG, Fortier LA: Hsp90 mediates insulin-like growth factor 1 and interleukin-1beta signaling in an age-dependent manner in equine articular chondrocytes. *Arthritis Rheum* 2007;56:2335–2343.
- 49 Van Osch GJ, Van Der Veen SW, Burger EH, Verwoerd-Verhoef HL: Chondrogenic potential of in vitro multiplied rabbit perichondrium cells cultured in alginate beads in defined medium. *Tissue Eng* 2000;6:321–330.
- 50 Danisovic L, Varga I, Polak S: Growth factors and chondrogenic differentiation of mesenchymal stem cells. *Tissue Cell* 2012;44:69–73.
- 51 Weiss S, Hennig T, Bock R, Steck E, Richter W: Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. *J Cell Physiol* 2010;223:84–93.
- 52 Hutchison MR: BDNF alters ERK/p38 MAPK activity ratios to promote differentiation in growth plate chondrocytes. *Mol Endocrinol* 2012;26:1406–1416.
- 53 Krejci P, Krakow D, Mekikian PB, Wilcox WR: Fibroblast growth factors 1, 2, 17, and 19 are the predominant FGF ligands expressed in human fetal growth plate cartilage. *Pediatr Res* 2007;61:267–272.
- 54 Moore EE, Bendele AM, Thompson DL, Littau A, Waggle KS, Reardon B, Ellsworth JL: Fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair in a rat model of injury-induced osteoarthritis. *Osteoarthritis Cartilage* 2005;13:623–631.
- 55 Mishra A, Tummala P, King A, Lee B, Kraus M, Tse V, Jacobs CR: Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. *Tissue Eng Part C Methods* 2009;15:431–435.