Influence of Platelet-Rich Plasma on Chondrogenic Differentiation and Proliferation of Chondrocytes and Mesenchymal Stem Cells

Anja Drengk^a Antonia Zapf^b Ewa Klara Stürmer^a Klaus Michael Stürmer^a
Karl-Heinz Frosch^a

Departments of ^aTrauma Surgery, Plastic and Reconstructive Surgery and ^bMedical Statistics, Georg August University Göttingen, Göttingen, Germany

Key Words

Cartilage defects · Chondrocytes · Mesenchymal stem cells · Platelet-rich plasma · Reimplantation

Abstract

Background/Aims: Autologous chondrocyte (CC) transplantation has the disadvantages of requiring two surgical interventions and in vitro expansion of cells, implying the risk of cellular dedifferentiation. Our clinical aim is to develop a one-step procedure for autologous CC transplantation, i.e. harvesting, isolation and reimplantation of CC performed in one single surgical procedure. Platelet-rich plasma (PRP) is a source of autologous growth factors reported to have mitogenic effects. The objective of this study was to test the influence of PRP as an autologous scaffold on freshly isolated CC and mesenchymal stem cells (MSC). **Methods:** CC and MSC were subjected to two- or three-dimensional (3D) growth systems, either with or without PRP. Chondrogenic differentiation was determined via quantification of collagen type II mRNA and immunohistochemical staining. **Results:** We observed a proliferative effect for MSCs exposed to PRP in monolayer culture and an increase in the expression of chondrogenic markers when cells are exposed to a 3D environment. CCs exposed to PRP show a decrease in the chondrogenic phenotype with increasing proliferative ac-

tivity. **Conclusion:** PRP has a proliferative effect on CCs and MSCs. In a one-step procedure for autologous CC transplantation, this might be an advantage over other scaffold materials, but confirmation in in vivo studies is required.

Copyright © 2008 S. Karger AG, Basel

Introduction

Treatment of articular cartilage degeneration is of major concern in modern health care. Progressive loss of cartilage on joint surfaces can result in osteoarthritis [Fritz et al., 2006], which often means a painful reduction in quality of life for the patient.

Articular cartilage is known to be a tissue with limited capacity for healing spontaneously, due to lack of vascularization [Gardner, 1994]. Consequently, nutrition of the tissue depends on diffusion only. Another factor contrib-

Abbreviations used in this paper

CC	chondrocyte(s)
MSC	mesenchymal stem cell(s)
PRP	platelet-rich plasma

uting to the poor regenerative capacity of articular cartilage is the fact that it is a tissue with a restricted number of cells. The percentage of highly specialized chondrocytes (CC), which are embedded within the matrix rich in collagen fibrils and proteoglycans, is only 1–3% [Fritz et al., 2006]. CC embedded in extracellular matrix are unable to migrate to a site of injury [Buckwalter and Mankin, 1998] and, therefore, only defects <3 mm in diameter can be self-repaired [Kim et al., 1991].

Some well-established treatment procedures with good initial outcome with respect to pain relief and mobility do exist. However, these treatments are far from satisfactory as they mostly result in the formation of biomechanically inferior fibrocartilage, which in the long term does not prevent the development of osteoarthritis. Procedures such as debridement [Magnuson, 1974], abrasion arthroplasty [Johnson, 2001], drilling [Pridie, 1959], microfracturing [Steadman et al., 2001] and autologous CC transplantation are available, with the procedure of choice dependent on the depth and width of the defect. For autologous CC transplantation, a piece of cartilage must be harvested from the non-weight-bearing site of the joint. CC are then isolated and expanded under good medical practice conditions and, finally, reimplanted at the site of cartilage injury, usually embedded in a collagen fleece. The defect is then closed with a periosteal flap. This procedure suggests promising results [Niethard and Schneider, 2004] but has some disadvantages. First, two surgical interventions are required and secondly, CC expansion is a slow process and therefore bears several risks, e.g. CC dedifferentiation and contamination.

Our clinical goal was to develop a one-step procedure, allowing CC harvesting, isolation and reimplantation to be performed in a single surgical intervention. However, applying this method, there would obviously be no increase in the number of CC. The additional use of platelet-rich plasma (PRP), which has been shown to have a mitogenic effect on different cell types in vitro [Gruber et al., 2002; Weibrich et al., 2002; Arpornmaeklong et al., 2004; Gruber et al., 2004; Choi et al., 2005], could be utilized to increase the cell number. PRP is a source of autologous cytokines, chemokines and growth factors which, in the case of injury, manage and control the process of wound healing and tissue repair. The most prominent growth factors in PRP are platelet-derived growth factor (PDGF), transforming growth factor (TGF)- β_1 and TGF- β_2 , epidermal growth factor, insulin-like growth factor, basic fibroblast growth factor and vascular endothelial growth factor, which influence cell proliferation and differentiation [Anitua et al., 2004]. The aim of

this study was, therefore, to test the influence of PRP on proliferation and differentiation of freshly isolated CC and mesenchymal stem cells (MSC) of sheep in an in vitro study.

Materials and Methods

Isolation and Cultivation of CC and MSC

Pieces of cartilage from the femoral condyle of an adult female sheep were collected. CC were isolated via overnight digestion with collagenase type II from PAN (Biotech, Germany), with a final enzyme concentration of 2 mg/ml. Subsequently, cells were passed through a 70- μ m cell strainer (Falcon; BD Bioscience, Erembodegem, Belgium) to remove any undigested cartilage, washed twice with DMEM (Gibco, Germany) and counted. Cell viability was checked with the trypan blue exclusion test.

MSC were obtained using a standardized modified protocol according to Pittenger [1999]. With an 18-gauge syringe, bone marrow was aspirated under anesthesia from the iliac crest of the sheep mentioned above. Cells isolated by density gradient centrifugation were plated in DMEM supplemented with 10% FCS, 2 mmol/l L-glutamine and 50 μ g/ml penicillin-streptomycin. Culture medium was changed twice a week. Cells formed symmetric colonies within 10–14 days and reached confluence after 3–4 weeks. Cells were used for assays after the second passage. Under specific culture conditions [Pittenger et al., 1999], cells obtained as described were able to differentiate into a chondrogenic and osteoblastic lineage.

In all experimental approaches, the culture medium was the same for all cell types, DMEM supplemented with 10% FCS, 2 mmol/l L-glutamine, 50 μ g/ml ascorbic acid and 50 μ g/ml penicillin-streptomycin. Cells were cultured at 37°C and 5% CO₂.

Preparation of PRP

To produce PRP extracts, blood was collected from the ear vein of the anesthetized sheep into 7.5-ml monovettes supplemented with sodium citrate (3.8%) at a ratio of 9 volumes blood and 1 volume sodium citrate according to Anitua [2004]. The citrated blood was centrifuged in a standard laboratory centrifuge (Labofuge 400; Heraeus) for 10 min at 2,400 rpm. Subsequently, the yellow plasma from 6 monovettes was transferred to a 50-ml plastic tube and platelets were pelleted in a second centrifugation step for 15 min at 3,600 rpm (protocol according to Weibrich et al. [2001], slightly modified). The pellet was resuspended in 1,200 μ l of plasma and the platelets counted in a Neubauer counting chamber. The PRP was stored at 4°C until the next day when the isolated CC were ready for use. Thus, in the experimental setup, CC received autologous PRP preparations, whereas MSC were treated with homologous PRP preparations. PRP (150 μ l) was activated with 7.5 μ l CaCl₂ (10%) in a 1.5-ml reaction tube, mixed and either left until clotted, mixed with cells and carefully placed into a culture well in the form of a drop, or pipetted onto the bottom of a cell culture flask as a thin fibrin gel smear.

Treatment Groups

We had six different treatment groups, each starting with 4 $\times$ 10⁵ cells: (1) cultivation of cells in a high-density, three-dimensional (3D) pellet culture system; (2) same as (1) but with the ad-

dition of activated PRP; (3) cells suspended in a fibrin sealant clot; (4) cells suspended in a PRP clot; (5) cells growing in monolayer, and (6) same as (5) but with the addition of activated PRP (fig. 1).

To produce the 3D pellet (treatment group 1), cells were suspended in 2.5 ml of medium in a 15-ml plastic tube and centrifuged for 5 min at 500 g, according to Tallheden et al. [2004]. The pelleted cells aggregated and formed a stable micromass. For treatment group 2, 150 μ l of PRP was activated as described above and the resulting clot added to the culture medium, where it was kept buoyed up on the surface of the medium. In group 3, we employed a fibrin sealant set (Tissucol; Baxter, Heidelberg, Germany), preparing the solutions according to the manufacturer's instructions but using a final dilution of the stock solutions of 1.24×10^5 cells were resuspended in 75 μ l of the fibrinogen component and mixed with 75 μ l of the thrombin solution. The resulting cell fibrin-sealant construct was cultured in a 6-well culture dish. For treatment group 4, the same amount of cells was resuspended in 150 μ l PRP and activation was carried out as described above. The resulting cell-PRP clot was cultured analogous to group 3. Treatment groups 5 and 6 comprised cells grown as a monolayer in 75-ml culture flasks either in the absence (5) or in the presence of PRP (6).

Each individual cell isolate was submitted to all of the six treatment groups and each group (except for 5 and 6) was prepared twice, once for immunohistochemical analysis and once for RNA preparation. Culture time was 14 days. At the end of culture, cells grown in monolayer were harvested as described previously [Frosch et al., 2003] and counted in a Coulter counter.

Histochemical and Immunohistochemical Procedures

After culture, micromasses and cell-PRP/cell fibrin-sealant clots were fixed in 4% paraformaldehyde overnight, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Three-micrometer sections were cut and placed on microscope slides, deparaffinized and stained either with toluidine blue or immunoassayed with an antibody against collagen type II (mouse anti-chicken collagen, monoclonal; Dunn Labortechnik, Germany). Sections that underwent staining for collagen type II were pretreated with proteolytic enzyme (Dako, Germany) for 10 min at 37°C to improve accessibility of the antibody and to expose epitopes that may have been masked during fixation. All sections were incubated with 3% H₂O₂ for 10 min at room temperature to eliminate endogenous peroxidase activity and subsequently received a protein block (Dako). Incubation with primary antibody was carried out overnight for anti-collagen type II (dilution 1:50). The secondary horseradish-peroxidase-conjugated antibody (goat anti-mouse; Dako) was incubated for 1 h and, after rinsing, sections were developed with 3-amino-9-ethyl carbazole (Sigma, Deisenhofen, Germany) for 30 min, counterstained with hemalum (Merck, Darmstadt, Germany) and mounted in Aquamount (BDH Laboratory Supplies, Poole, UK). The stained sections were analyzed with a Leica DM IRBE microscope.

Quantitative Real-Time PCR

For total RNA preparation, we used a RNA/DNA Kit (Qiagen, Germany). Cells grown in monolayer, micromasses, cell-PRP and cell fibrin-sealant clots were harvested and transferred into lysis buffer, homogenized through an 18-gauge needle and processed following the manufacturer's instructions. After samples had

been treated with DNase to remove possible contamination of genomic DNA, first-strand complementary DNA was synthesized from 400 ng total RNA using specific reverse primers for GAPDH and collagen type II using Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). From this reaction, 2.5 μ l were used in a SYBR-Green PCR reaction (QuantiTect SYBR-Green PCR Kit; Qiagen) run in the iCycler iQ Real-Time Detection System (Bio-Rad, Germany). The mRNA expression level of the target gene in each treatment group was determined relative to the expression level of the target gene of the calibrator control group 5; each value was normalized to the reference gene GAPDH and calculated according to the Pfaffl method for different amplification efficiencies (Bio-Rad Laboratories, Real-time PCR Application Guide). Quality of the PCR products was checked by melting curve analysis and electrophoresis of the PCR product. Primer sequences were as follows: KIIE3fw: 5'-GAAACCAGG-ACCAAAGGG-3'; KIIE3rv: 5'-CTTGTTCCCCTGCAGGTC-3'; GAPDHovisfw: 5'-CCTTCATTGACCTTCACTACATGGTCT-3', and GAPDHovisrv: 5'-TGGAAGATGGTGATGGCCTTTC-CATTG-3'. The primers for GAPDH were according to Bosnakovski et al. [2004]. The identity of each PCR product was confirmed by sequence analysis.

Statistical Evaluation

Data were analyzed using SAS 9.1 software (SAS Institute, Cary, N.C., USA). To investigate the effect of the different treatments, we performed a mixed linear model analysis. Pairwise comparisons between treatment groups were performed by Tukey's procedure.

Results

Influence of PRP on Cell Proliferation

At the end of the experiment, cells grown in monolayers either in the absence (treatment group 5) or in the presence of PRP (treatment group 6) were trypsinized and counted. For MSC, there were 8 individual and independent cell isolates. In 7 of 8 cases, a higher cell count was observed for samples grown in the presence of PRP (fig. 2a). For CC, the influence on cell proliferation was not as homogeneous as for MSCs. In 6 of 9 cases, cells grown in the presence of PRP showed an increase in cell number compared to those without PRP supplement, indicating a positive influence of PRP on cell proliferation (fig. 2). Based on the starting cell number, there was an average increase of about 14-fold for MSC at the end of the experiment compared to an average increase of about 10-fold for CCs.

Influence of PRP on Immunohistochemical Evaluation

None of the MSC samples in either treatment group stained positively for collagen type II. However, quantitative real-time PCR analysis showed a weak collagen

Fig. 1. a Schematic illustration of the experimental setup showing the different treatment groups and culture conditions. **b** Data describing the quantitative analysis of chondrogenic differentiation (level of collagen type II mRNA expression rate) of each treatment group, with treatment group 5 as calibrator control group.

Fig. 2. Cell counts of MSC (a) and CC (b) grown in monolayer for 2 weeks either without or with PRP.

type II transcription activity, which was not sufficient for immunohistochemical detection of protein.

In contrast to the MSC samples, CC grown in micro-mass culture (treatment groups 1 and 2) showed distinct

staining for collagen type II protein (fig. 3). As demonstrated by immunohistochemical staining, collagen type II expression is negatively influenced by the addition of PRP (treatment group 2). Staining intensity diminished

Fig. 3. Representative pictures of the immunohistochemical analysis of collagen type II expression in groups 1–4. Scale bars = 100 μ m. Note that cells grown in micromasses (**a**, **b**) show a chondrogenic phenotype with cells sitting in lacunae being surrounded by a matrix produced entirely by the cells. This matrix stains

strong for collagen type II protein in treatment group 1 and weak in treatment group 2. Cells grown in a fibrin sealant (**c**) or PRP clot (**d**) have only small lacunae or none at all and there is no collagen type II staining in the surrounding scaffold.

and the percentage of clearly stained samples dropped to 33% in treatment group 2, compared to 75% in treatment group 1. Cells grown in fibrin sealant beads did not stain for collagen type II at all, whereas there was weak staining in 2 of 9 cases in treatment group 4.

Quantitative Analysis of Chondrogenic Differentiation

To determine the expression of collagen type II, a specific marker for articular CC [Binette et al., 1998], we performed real-time PCR, using the expression level of GAPDH as internal standard.

The amount of type II collagen mRNA determined in treatment group 5 (cells grown in monolayer without any further treatment) was used as calibrator so that we expressed the amount of collagen type II mRNA in the other treatment groups as a ratio of this control. This was calculated independently for each isolate.

With regard to CC, treatment group 1 showed the highest expression level of collagen type II mRNA, with a median twice above the level of the control group (fig. 1b, 4), being significantly different compared with all other treatment groups ($p < 0.05$). Adding PRP to the micromass culture dramatically reduced collagen type II mRNA expression, however the median was still above the control level and this culture condition appears superior to growing cells in a fibrin sealant clot, PRP clot, or in monolayer with or without additional PRP (fig. 1b, 4). Categories 3, 4 and 6 show a reduced median collagen type II mRNA expression level in comparison to the control group, but the difference was not significant ($p > 0.05$).

For MSC, the results are as follows: the highest expression level of collagen type II mRNA was found in treat-

Fig. 4. Box plot illustrating the collagen type II mRNA expression level of CC for each treatment group in relation to the expression level of calibrator control group 5 (zero line). Each treatment group comprised 10 independent cell isolates.

ment group 1, as it is the case in CC. However, the ratio differs, with a median of approximately 8 times more collagen type II mRNA expression in treatment group 1 compared to control group 5 (fig. 1b, 5). Again this difference was significant ($p < 0.05$). Likewise, cells submitted to treatment groups 2, 3 and 4 on average express more collagen type II mRNA than cells grown in the monolayer control group (fig. 1b, 5); however, these re-

Fig. 5. Box plot illustrating the collagen type II mRNA expression level of MSC for each treatment group in relation to the expression level of calibrator control group 5 (zero line). Each treatment group comprised 8 independent cell isolates.

sults were not significant ($p > 0.05$). Addition of PRP to the monolayer (treatment group 6) resulted in a mean collagen type II mRNA expression level below the control level.

Discussion

In recent decades, degenerative diseases of the articular knee joint have been gaining increasing clinical importance. A changed leisure behavior paralleled by an increase in the elderly population has led to a higher prevalence of articular cartilage defects, which, when untreated, often result in osteoarthritis. Treatment of such defects has evolved tremendously in recent years. PRP is currently being highly investigated, particularly in oral and maxillofacial surgery. The idea is that via application of PRP the patient's own growth factors are used to enhance healing. Marx et al. [1998] report that the addition of PRP to an autogenous cancellous bone graft results in a faster maturation rate and higher bone formation rate in alveolar defects. There are reports on the potency of PRP to improve the cohesion of an autogenous bone graft by forming a fibrin gel and to release factors leading to enhanced bone regeneration [Fuerst et al.,

2003; Fennis et al., 2004]. Anitua et al. [2004] described the stabilizing effect of PRP clots around implants in solid bone, virtually eliminating the risk of implant failure. In addition, PRP has a strong stimulant effect on capillary regeneration in wound healing [Lindeboom et al., 2007] and a neurotrophic effect in studies concerning peripheral nerve recovery [Farrag et al., 2007]. Implementation of a PRP collagen scaffold has been reported to stimulate healing of anterior cruciate ligament defects both histologically and biomechanically [Murray et al., 2006], and accelerated articular cartilage healing has been demonstrated by the use of a PRP preparation in a case of articular cartilage avulsion [Sanchez et al., 2003].

Considering all the positive effects described for PRP in various clinical applications, we aimed to establish whether the combination of MSC with PRP, or the combination of freshly isolated CC with PRP, would form a bioactive composite suited for healing of cartilage defects. This work was undertaken as a basic in vitro study to better assess the possible outcome of an in vivo experiment. For the in vitro study we added FCS to the culture medium to enhance the growth of CCs and MSCs. However, this additive would not be practical in an in vivo setting because of the risk of viral or prion-related disease transmission and foreign protein contamination. Autologous serum could be an adequate substitute for FCS, although, in the one-step procedure we propose, the cell culture step would be omitted.

PRP contains a natural cocktail of growth factors which act in concert to stop bleeding and initiate healing and restoration of damaged tissue during injury. PDGF and TGF- β are the most prominent of these growth factors and the mitogenic effect of TGF- β on osteoblasts and stem cells has been demonstrated [Marx et al., 1998]. Numerous in vitro studies demonstrate a mitogenic effect of PRP on various cell types, for example, on human trabecular-bone-derived cells [Gruber et al., 2002], human SaOS-2 osteoblasts [Celotti et al., 2006], human oral fibroblasts and osteoblasts [Graziani et al., 2006] and human foreskin fibroblasts [Lanas et al., 1994]. There are also several reports of mitogenic stimulation of human MSC by platelet releasates [Gruber et al., 2004; Vogel et al., 2006].

In this study, we also observed a mitogenic effect of PRP on marrow-derived stem cells from adult sheep. However, the effect was small, possibly due to the small amount of PRP used, suggesting that the amount of added growth factors acting on the monolayer cells may have been minimal. It might also be that a primary, stronger stimulating effect exerted by adding PRP was masked in

the course of the experiment, as the most notable effect of PRP on cell proliferation has been reported to occur after 72 h of stimulation [Graziani et al., 2006].

There are only a few studies reporting a proliferative effect of PRP on CC [Kaps et al., 2002; Gaissmaier et al., 2005; Akeda et al., 2006], but none of these investigations stimulated freshly isolated CC with PRP. In our study, PRP exerted a proliferative effect on freshly isolated, completely differentiated CC submitted to growth in monolayer culture. Proliferation increased in 67% of CC samples, being a less homogeneous response to PRP stimulation than that observed for MSC, in agreement with results in bovine articular CC exposed to human platelet supernatant [Kaps et al., 2002], where only 40% of platelet batches displayed growth-promoting activity, an observation explained with the cell differentiation status and donor age [Blanco et al., 1995; Guerne et al., 1995].

Another reason for the variable response of cells to stimulation with PRP may be associated with the PRP preparations utilized. There are several reported procedures for the generation of PRP concentrates [Weibrich et al., 2001; Dugrillon et al., 2002; Sanchez et al., 2003; Kitoh et al., 2004; Yazawa et al., 2004; Anitua et al., 2006] and a lack of standardization for self-concentrated PRP preparations. Platelet counts differ significantly according to the preparation method. Weibrich et al. [2001] observed no significant correlation between the platelet count of self-concentrated PRP and donor whole blood. Further, 10% of self-concentrated PRP counts were reported as lower than those of the donor whole blood and there is an additional influence of sex on platelet concentration and an interindividual variability [Weibrich et al., 2001]. A dose dependence of the PRP action has been reported previously. Choi et al. [2005] described suppression of cell viability and proliferation by high PRP concentrations for alveolar bone cells, and Graziani et al. [2006] has shown that high PRP concentrations result in a reduction in cell proliferation of human oral fibroblasts and osteoblasts. Better results for the proliferation of human FOB19 cells were obtained when PRP preparations were diluted [Ferreira et al., 2005]. Therefore, the optimal PRP concentrations remain to be determined.

We tend to see the non-uniform response of the CC samples in the current study in light of this issue. PRP preparations were not equalized in order to maintain the experimental setting as close as possible to the clinical application. PRP variability is an uncontrollable parameter in an autologous setting, similar to the donor-dependent variability of growth and differentiation competence of MSC and CC.

Our results show that cultivation of freshly isolated CC in a high-density micromass culture best preserves the chondrogenic phenotype compared to all other cultivation methods. Addition of PRP dramatically reduces collagen type II mRNA expression in categories 2, 4 and 6. This is not surprising considering the positive influence of PRP on cell proliferation discussed previously. Proliferation and differentiation cannot occur in a cell simultaneously, so an increase in proliferation obviously negatively influences the differentiation status. Addition of TGF- β decreases expression of type II mRNA in freshly isolated CC cultured in monolayer [van der Kraan et al., 1992; Fortier et al., 1998], and this observation supports our findings, as TGF- β is one of the most prominent growth factors enriched in platelet concentrations. PDGF as the other dominant growth factor in platelet concentrates also stimulates a decrease in the amount of cartilage proteoglycan, exerting a mitogenic effect [Weiser et al., 1999]. In addition, an inhibition of cartilage differentiation in vitro by platelet supernatant has been reported previously [Kaps et al., 2002; Gaissmaier et al., 2005]. However, these studies utilized CC previously expanded in monolayer culture. We show, for the first time, that PRP also leads to a reduction in collagen type II mRNA expression in freshly isolated CC. We assume that this dedifferentiating influence is even stronger on freshly isolated CC than on cells previously expanded in monolayer. This assumption is supported by the observation that a significant difference in the level of collagen type II mRNA expression is no longer obtained between treatment groups 1 and 2 in cells previously expanded in monolayer culture [unpubl. data with rabbit CC].

In our study, a negative influence of PRP on collagen type II mRNA expression was observed in freshly isolated CC in all treatment groups, but there are clear differences in the degree of impact that PRP exerts on collagen type II mRNA expression in treatment groups 2, 4 and 6. As an explanation we postulate two opposite forces acting on the cells. One is the above-mentioned dedifferentiating activity of PRP. The other is the 3D arrangement of cells, which is unique to each treatment group. In treatment group 2, cells have close cell-to-cell contact, which is a condition possibly well suited for preserving the chondrogenic phenotype. There are reports about close cell-cell contact reversing the loss of phenotype of cells grown in monolayer culture [Tallheden et al., 2004] or rather preventing such a phenotype loss [Kuettner et al., 1982; Bassler et al., 1986]. Consistently, in this treatment group, the dedifferentiating influence of PRP is the

weakest, whereas it is logically strongest when cells are grown in monolayer (treatment group 6), a condition known to promote dedifferentiation [von der Mark et al., 1977; Benya et al., 1978; Marlovits et al., 2004]. Treatment group 4 lies between these extremes, featuring 3D arrangement, but not close cell-to-cell contact. In treatment group 3, dedifferentiation was comparable to group 4. With regard to the 3D arrangement, these two groups are comparable. There are reports describing a growth-promoting activity inherent in fibrin(ogen) [Gray et al., 1993] as well as in thrombin [Zangi et al., 2006]. The decrease in collagen type II mRNA expression in treatment group 3 might therefore be due to an increase in proliferation. Concerning the collagen type II mRNA expression, culturing freshly isolated CC in a 3D arrangement using fibrin sealant or PRP seems to be inferior to a simple monolayer culture. The reason for this lies in the proliferative effect of the supplements discussed above, which result in an accelerated dedifferentiation process when compared to cells grown in monolayer. For CC previously expanded in monolayer, this seems to be different [Gaissmaier et al., 2005].

For assays carried out with MSCs, culturing cells in high-density micromasses yields the highest level of collagen type II mRNA expression compared with cells grown in monolayer. It is not clear whether this is due to an increase in collagen type II expression induced through the condensed and 3D growth of the cells or whether it is the result of a better conservation of a basal collagen type II mRNA expression previously described by different authors [Majumdar et al., 2000; Worster et al., 2000]. However, several published observations seem to support the assumption that the culturing condition has led to the observed increase in collagen type II mRNA expression. Bosnakovski et al. [2004] reported that, using fetal bovine MSCs, the condensed culturing condition was a sufficient external stimulus for the cells to undergo chondrogenesis. The high cell density, which allows cell-to-cell interactions analogous to those occurring in mesenchymal condensation during limb development before cartilage formation, seems to be a specific stimulus supporting chondrogenesis of MSCs from most species [Bosnakovski et al., 2004].

To our knowledge, this is the first study on the effect of PRP on chondrogenic differentiation of MSCs, and a negative effect of PRP on the level of collagen type II mRNA expression was found. Addition of PRP to a micromass culture led to a significant reduction in collagen type II mRNA. Furthermore, the addition of PRP to the monolayer culture decreased median collagen type II

mRNA expression compared to the untreated monolayer, which can be explained by the proliferation-stimulating effect of PRP. However, in treatment groups 1–4, the median expression level of collagen type II mRNA was above the control level. Only in treatment group 6 dropped the median level of collagen type II mRNA expression below the control level. In contrast to experiments carried out with CC, it can be stressed that with regard to the chondrogenic differentiation, growing MSCs in a 3D culture system is clearly superior to monolayer culture. Growing cells in a fibrin sealant clot, a PRP gel or as a micromass with or without PRP exposure results in a higher collagen type II mRNA expression level compared with monolayer culture.

Our results from sheep MSC and PRP show a clear proliferative effect for MSC exposed to PRP in monolayer culture and an increase in the expression of the chondrogenic marker collagen type II mRNA when cells are exposed to PRP in a 3D environment. Our results indicate that MSC in combination with PRP are able to improve the healing of articular cartilage defects in vivo.

Freshly isolated sheep CC in combination with a PRP preparation lose their chondrogenic phenotype during in vitro culture. This loss of chondrogenicity seems to be connected with an increase in proliferation. Based on these observations, it remains difficult to evaluate how the cells would develop in an in vivo environment, where they would be exposed to the adequate chondrogenic stimuli. This remains to be shown in an animal study.

Acknowledgments

The authors express their thanks to R. Castro, F. Kauer and Dr. P. Krause for experimental assistance.

References

- Akeda, K., H.S. An, M. Okuma, M. Attawia, K. Miyamoto, E.J. Thonar, M.E. Lenz, R.L. Sah, K. Masuda (2006) Platelet-rich plasma stimulates porcine articular chondrocyte proliferation and matrix biosynthesis. *Osteoarthritis Cartilage* 14: 1272–1280.
- Anitua, E., I. Andia, B. Ardanza, P. Nurden, A.T. Nurden (2004) Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb Haemostasis* 91: 4–15.
- Anitua, E., M. Sanchez, A.T. Nurden, P. Nurden, G. Orive, I. Andia (2006) New insights into and novel applications for platelet-rich fibrin therapies. *Trends Biotechnol* 24: 227–234.

- Arpornmaeklong, P., M. Kochel, R. Depprich, N.R. Kubler, K.K. Wurzler (2004) Influence of platelet-rich plasma (PRP) on osteogenic differentiation of rat bone marrow stromal cells. An in vitro study. *Int J Oral Maxillofac Surg* 33: 60–70.
- Bassleer, C., P. Gysen, J.M. Foidart, R. Bassleer, P. Franchimont (1986) Human chondrocytes in tridimensional culture. *In Vitro Cell Dev Biol* 22: 113–119.
- Benya, P.D., S.R. Padilla, M.E. Nimni (1978) Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell* 15: 1313–1321.
- Binette, F., D.P. McQuaid, D.R. Haudenschild, P.C. Yaeger, J.M. McPherson, R. Tubo (1998) Expression of a stable articular cartilage phenotype without evidence of hypertrophy by adult human articular chondrocytes in vitro. *J Orthop Res* 16: 207–216.
- Blanco, F.J., Y. Geng, M. Lotz (1995) Differentiation-dependent effects of IL-1 and TGF-beta on human articular chondrocyte proliferation are related to inducible nitric oxide synthase expression. *J Immunol* 154: 4018–4026.
- Bosnakovski, D., M. Mizuno, G. Kim, T. Ishiguro, M. Okumura, T. Iwanaga, T. Kadosawa, T. Fujinaga (2004) Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. *Exp Hematol* 32: 502–509.
- Buckwalter, J.A., H.J. Mankin (1998) Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instr Course Lect* 47: 477–486.
- Celotti, F., A. Colciago, P. Negri-Cesi, A. Pravettoni, R. Zaninetti, M.C. Sacchi (2006) Effect of platelet-rich plasma on migration and proliferation of SaOS-2 osteoblasts: role of platelet-derived growth factor and transforming growth factor-beta. *Wound Repair Regen* 14: 195–202.
- Choi, B.H., S.J. Zhu, B.Y. Kim, J.Y. Huh, S.H. Lee, J.H. Jung (2005) Effect of platelet-rich plasma (PRP) concentration on the viability and proliferation of alveolar bone cells: an in vitro study. *Int J Oral Maxillofac Surg* 34: 420–424.
- Dugrillon, A., H. Eichler, S. Kern, H. Kluter (2002) Autologous concentrated platelet-rich plasma (cPRP) for local application in bone regeneration. *Int J Oral Maxillofac Surg* 31: 615–619.
- Farrag, T.Y., M. Lehar, P. Verhaegen, K.A. Carson, P.J. Byrne (2007) Effect of platelet rich plasma and fibrin sealant on facial nerve regeneration in a rat model. *Laryngoscope* 117: 157–165.
- Fennis, J.P., P.J. Stoelinga, J.A. Jansen (2004) Mandibular reconstruction: a histological and histomorphometric study on the use of autogenous scaffolds, particulate cortico-cancellous bone grafts and platelet rich plasma in goats. *Int J Oral Maxillofac Surg* 33: 48–55.
- Ferreira, C.F., M.C. Carriel Gomes, J.S. Filho, J.M. Granjeiro, C.M. Oliveira Simoes, S. Magini Rde (2005) Platelet-rich plasma influence on human osteoblasts growth. *Clin Oral Implants Res* 16: 456–460.
- Fortier, L.A., A.J. Nixon, J. Williams, C.S. Cable (1998) Isolation and chondrocytic differentiation of equine bone marrow-derived mesenchymal stem cells. *Am J Vet Res* 59: 1182–1187.
- Fritz, J., C. Gaissmaier, B. Schewe, K. Weise (2006) Biologische Knorpelrekonstruktion im Kniegelenk. *Unfallchirurg* 109: 563–576.
- Frosch, K.H., I. Sondergeld, K. Dresing, T. Rudy, C.H. Lohmann, J. Rabba, D. Schild, J. Breme, K.M. Stuermer (2003) Autologous osteoblasts enhance osseointegration of porous titanium implants. *J Orthop Res* 21: 213–223.
- Fuerst, G., R. Gruber, S. Tangl, F. Sanroman, G. Watzek (2003) Enhanced bone-to-implant contact by platelet-released growth factors in mandibular cortical bone: a histomorphometric study in minipigs. *Int J Oral Maxillofac Implants* 18: 685–690.
- Gaissmaier, C., J. Fritz, T. Krackhardt, I. Flesch, W.K. Aicher, N. Ashammakhi (2005) Effect of human platelet supernatant on proliferation and matrix synthesis of human articular chondrocytes in monolayer and three-dimensional alginate cultures. *Biomaterials* 26: 1953–1960.
- Gardner, D.L. (1994) Problems and paradigms in joint pathology. *J Anat* 184: 465–476.
- Gray, A.J., J.E. Bishop, J.T. Reeves, G.J. Laurent (1993) A alpha and B beta chains of fibrinogen stimulate proliferation of human fibroblasts. *J Cell Sci* 104: 409–413.
- Graziani, F., S. Ivanovski, S. Cei, F. Ducci, M. Tonetti, M. Gabriele (2006) The in vitro effect of different PRP concentrations on osteoblasts and fibroblasts. *Clin Oral Implants Res* 17: 212–219.
- Gruber, R., F. Karreth, B. Kandler, G. Fuerst, A. Rot, M.B. Fischer, G. Watzek (2004) Platelet-released supernatants increase migration and proliferation, and decrease osteogenic differentiation of bone marrow-derived mesenchymal progenitor cells under in vitro conditions. *Platelets* 15: 29–35.
- Gruber, R., F. Varga, M.B. Fischer, G. Watzek (2002) Platelets stimulate proliferation of bone cells: involvement of platelet-derived growth factor, microparticles and membranes. *Clin Oral Implants Res* 13: 529–535.
- Guerne, P.A., F. Blanco, A. Kaelin, A. Desgeorges, M. Lotz (1995) Growth factor responsiveness of human articular chondrocytes in aging and development. *Arthritis Rheum* 38: 960–968.
- Johnson, L.L. (2001) Arthroscopic abrasion arthroplasty: a review. *Clin Orthop Relat Res* 391(suppl): S306–S317.
- Kaps, C., A. Loch, A. Haisch, H. Smolian, G.R. Burmester, T. Haupl, M. Sittlinger (2002) Human platelet supernatant promotes proliferation but not differentiation of articular chondrocytes. *Med Biol Eng Comput* 40: 485–490.
- Kim, H.K., M.E. Moran, R.B. Salter (1991) The potential for regeneration of articular cartilage in defects created by chondral shaving and subchondral abrasion. An experimental investigation in rabbits. *J Bone Joint Surg Am* 73: 1301–1315.
- Kitoh, H., T. Kitakoji, H. Tsuchiya, H. Mitsuyama, H. Nakamura, M. Katoh, N. Ishiguro (2004) Transplantation of marrow-derived mesenchymal stem cells and platelet-rich plasma during distraction osteogenesis – a preliminary result of three cases. *Bone* 35: 892–898.
- Kuettner, K.E., V.A. Memoli, B.U. Pauli, N.C. Wrobel, E.J. Thonar, J.C. Daniel (1982) Synthesis of cartilage matrix by mammalian chondrocytes in vitro. II. Maintenance of collagen and proteoglycan phenotype. *J Cell Biol* 93: 751–757.
- Lanas, A., P. Haggerty, B.I. Hirschowitz (1994) Ingestion of aspirin prevents platelet-induced human fibroblast growth. Implications for peptic ulcer healing. *Scand J Gastroenterol* 29: 17–22.
- Lindeboom, J.A., K.R. Mathura, I.H. Aartman, F.H. Kroon, D.M. Milstein, C. Ince (2007) Influence of the application of platelet-enriched plasma in oral mucosal wound healing. *Clin Oral Implants Res* 18: 133–139.
- Magnuson, P.B. (1974) The classic: joint debridement: surgical treatment of degenerative arthritis. *Clin Orthop Relat Res* 101: 4–12.
- Majumdar, M.K., V. Banks, D.P. Peluso, E.A. Morris (2000) Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. *J Cell Physiol* 185: 98–106.
- Marlovits, S., M. Hombauer, M. Truppe, V. Vecsei, W. Schlegel (2004) Changes in the ratio of type-I and type-II collagen expression during monolayer culture of human chondrocytes. *J Bone Joint Surg Br* 86: 286–295.
- Marx, R.E., E.R. Carlson, R.M. Eichstaedt, S.R. Schimmele, J.E. Strauss, K.R. Georgeff (1998) Platelet-rich plasma: growth factor enhancement for bone grafts. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 85: 638–646.
- Murray, M.M., B. Forsythe, F. Chen, S.J. Lee, J.J. Yoo, A. Atala, A. Steinert (2006) The effect of thrombin on ACL fibroblast interactions with collagen hydrogels. *J Orthop Res* 24: 508–515.
- Niethard, F.U., U. Schneider (2004) Cartilage is sturdier – progress in autologous chondrocyte transplantation (in German). *Z Orthop Ihre Grenzgeb* 142: 527–528.

- Pittenger, M.F., A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284: 143–147.
- Pridie, K. (1959) A method of resurfacing osteoarthritic knee joints. *J Bone Joint Surg Br* 41: 618–619.
- Sanchez, M., J. Azofra, E. Anitua, I. Andia, S. Padilla, J. Santesteban, I. Mujika (2003) Plasma rich in growth factors to treat an articular cartilage avulsion: a case report. *Med Sci Sports Exerc* 35: 1648–1652.
- Steadman, J.R., W.G. Rodkey, J.J. Rodrigo (2001) Microfracture: surgical technique and rehabilitation to treat chondral defects. *Clin Orthop Relat Res* 391(suppl): S362–S369.
- Tallheden, T., C. Karlsson, A. Brunner, J. Van Der Lee, R. Hagg, R. Tommasini, A. Lindahl (2004) Gene expression during redifferentiation of human articular chondrocytes. *Osteoarthritis Cartilage* 12: 525–535.
- van der Kraan, P., E. Vitters, W. van den Berg (1992) Differential effect of transforming growth factor beta on freshly isolated and cultured articular chondrocytes. *J Rheumatol* 19: 140–145.
- Vogel, J.P., K. Szalay, F. Geiger, M. Kramer, W. Richter, P. Kasten (2006) Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and in vivo bone formation in calcium phosphate ceramics. *Platelets* 17: 462–469.
- von der Mark, K., V. Gauss, H. von der Mark, P. Muller (1977) Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. *Nature* 267: 531–532.
- Weibrich, G., S.H. Gnoth, M. Otto, T.E. Reichert, W. Wagner (2002) Growth stimulation of human osteoblast-like cells by thrombocyte concentrates in vitro (in German). *Mund Kiefer Gesichtschir* 6: 168–174.
- Weibrich, G., W.K. Kleis, M. Kunz-Kostomanolakis, A.H. Loos, W. Wagner (2001) Correlation of platelet concentration in platelet-rich plasma to the extraction method, age, sex, and platelet count of the donor. *Int J Oral Maxillofac Implants* 16: 693–699.
- Weiser, L., M. Bhargava, E. Attia, P.A. Torzilli (1999) Effect of serum and platelet-derived growth factor on chondrocytes grown in collagen gels. *Tissue Eng* 5: 533–544.
- Worster, A.A., A.J. Nixon, B.D. Brower-Toland, J. Williams (2000) Effect of transforming growth factor β 1 on chondrogenic differentiation of cultured equine mesenchymal stem cells. *Am J Vet Res* 61: 1003–1010.
- Yazawa, M., H. Ogata, A. Kimura, T. Nakajima, T. Mori, N. Watanabe (2004) Basic studies on the bone formation ability by platelet rich plasma in rabbits. *J Craniofac Surg* 15: 439–446.
- Zangi, L., R. Rivkin, I. Kassis, L. Leviansky, G. Marx, R. Gorodetsky (2006) High-yield isolation, expansion, and differentiation of rat bone marrow-derived mesenchymal stem cells with fibrin microbeads. *Tissue Eng* 12: 2343–2354.