No effect of autologous growth factors (AGF) around ungrafted loaded implants in dogs

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Abstract Autologous growth factors (AGF) is a growth-factor-rich concentrate of platelets, white blood cells and fibrinogen. Application of AGF was presumed to improve implant fixation and gap healing of non-grafted, loaded implants. We inserted one loaded titanium implant intra-articularly in each medial femoral condyle of eight dogs. Each implant was surrounded by a 0.75 mm gap. One implant in each dog was coated with AGF prior to implantation whereas the contralateral implant served as a control. AGF was prepared by isolating the buffy-coat from blood and further concentrated using an Interpore Cross UltraConcentrator. Platelet counts were increased from a median baseline of $168 \times 10^3/\mu\text{l}$ to $1003 \times 10^3/\mu\text{l}$ in AGF. However, AGF had no significant effect on implant fixation

or bone formation. Even though AGF increased ultimate shear strength and energy absorption by approximately 50%, these differences had a *p*-value less than 0.05. The sample size in this study was small and any negative conclusions should be taken with caution due to low statistical power. We have previously demonstrated that AGF significantly improves fixation and incorporation of grafted implants. AGF might require mixing with an osteoconductive grafting material in order to provide a scaffold on which to foster bone growth and to keep the growth factors on location for a prolonged period.

Introduction

Mechanical fixation and bone ongrowth on cementless implants can be enhanced by local application of single recombinant growth factors [5, 13, 21]. Risk of overdosing, enhanced catabolic activity as well as high price has limited the clinical use of purified growth factors [5, 8]. Autologous growth factors (AGF) is an autologous buffy coat (platelet and white cells) concentrate processed by a commercially available concentrator (Interpore Cross International, Irvine, California, USA) [14]. Since platelets are an autologous source of growth factors such as platelet-derived growth factor (PDGF), transforming growth factor- β (TGF- β), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) [3, 11, 17], in physiologically occurring relative concentrations, which are all known stimulators of bone growth [20], we postulated that AGF would improve osseointegration and mechanical fixation of cementless implants. The object of this study was to investigate the influence of AGF on implant fixation, bone

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Fig. 1 The implant device was inserted in the medial condyle of both knees. **a** Anchorage screw with a threaded piston. **b** Test implant mounted on the piston. **c** Titanium ring. **d** Polyethylene (PE) plug (diameter 4.5 mm) which protrudes so that load from the proximal tibia can be transmitted to the implant device. A gap of 0.75 mm between surface of the implant and border of the drill hole was either left empty or treated with autologous growth factors (AGF)

ongrowth and gap healing in a well-established loaded non-grafted canine model.

Materials and methods

Design

Eight hound dogs with an average age of 18 (range 11–32) months and a weight of 21 (range 20–23) kg were used. The same dogs were used for implantation in the proximal tibias for another study. All animal handling and AGF processing was approved by our institution's Animal Care and Use Committee.

Preparation of AGF

Under anaesthesia, immediately prior to surgery, approximately 450 ml of blood was withdrawn, using citrate as anticoagulant. With a cell separator (Medtronic Sequestra 1000, Minneapolis, Minnesota, USA), blood was divided into three fractions: platelet poor plasma, platelet and white cell rich buffy coat, and erythrocytes. Erythrocytes and platelet poor plasma were immediately re-infused. The buffy coat (60 ml) was isolated and further concentrated to 20 ml using an UltraConcentrator (Interpore Cross International). Thrombin (100 U/ml) was added to initiate the formation of a gel and to degranulate platelets. After aliquots of blood and AGF were removed to determine platelet count, the implants on one side were coated with AGF gel prior to insertion and the cavity was filled with gel. Blood and AGF samples were taken from all dogs. However, samples from three dogs were damaged during processing and platelet counts were finally only done on five dogs.

Loaded implant device

We used a model developed by Søballe [19] consisting of a stable loaded test implant mounted on an anchorage screw (Fig. 1). Porous coated titanium alloy (Ti-6Al-4V) test implants manufactured by Biomet Inc. (Warsaw, Indiana, USA) were used. The test implants were cylindrical in shape with a length of 10 mm and a nominal outer diameter of 6 mm. Mean pore diameter was 480 μ m and porosity was 44% as determined by the manufacturer. Implants were sterilised by gamma irradiation. On the end of the test implant, a polyethylene plug was mounted which protruded into the knee joint in order to transfer load during gait (Fig. 1). The implants were inserted in a “stable” mode, that

Fig. 2 **a** Section from an autologous growth factor (AGF) treated implant. The implant (**a**) is seen to the left. Woven bone is in contact with the implant surface and is seen throughout the gap. Lamellar bone (**c**) is found outside the original 0.75 mm gap (100X magnification, light-green, basic fuchsin staining). **b** Section from the group with no gap treatment. The implant (**a**) is covered by a thick fibrous membrane (**b**). Woven bone (**c**) is seen in the gap but it is not in contact with the implant. Lamellar bone (**d**) is found outside the original 0.75 mm gap (100X magnification, light-green, basic fuchsin staining)

Table 1 Platelet counts

Baseline (<i>n</i> =5)	Buffy coat (<i>n</i> =4)	AGF (<i>n</i> =5)	AGF baseline (<i>n</i> =5)
168 (90–247)	535 (335–945)	1003 (710–1754)	7.9 (3.8–10.4)

AGF autologous growth factor

Values given as mean (range) $\times 10^3/\mu\text{l}$

is, they did not allow micromotion between the implant and surrounding bone.

Surgery

Dogs were premedicated with Acetylpromazine (Burns Veterinary Supply, Rockville Ctr., New York, USA). Anaesthesia was induced by thiopental sodium (Pentotal, Abbott Lab, North Chicago, Illinois, USA) and was maintained on isoflurane anaesthesia. In each knee the medial condyle was approached through a parapatellar insertion. A hole of 7.5 mm diameter was drilled in the condyle, oriented with the weight-bearing axis of the femur, and the cavity was cleaned with saline. An anchoring screw with a piston was inserted on both femurs. The cavity on the left side was filled with AGF gel. Afterwards, one test implant, 6.0 mm in diameter, coated with AGF gel was inserted, leaving a gap of 0.75 mm from the implant surface to the surrounding bone (Fig. 1). On the contralateral side, the same procedures were followed but no AGF was used. After insertion of the test implant, a polyethylene plug was mounted on the top of the test implant on the threaded piston. The test implant was maintained central within the gap by the polyethylene plug bearing against a metal ring. None of the implants received bone graft.

One gram of ceftriaxone sodium (Rocephin, Roche, New Jersey, USA) was administered preoperatively and for three days following surgery. The dogs were allowed unrestricted weight bearing. Observation time was four weeks.

Preparation of tissue samples

Cultures were taken from the knee joint after euthanasia. Distal femora were stored at -20°C . Sections at a right

angle to the axis of each implant were made on a water-cooled diamond band saw (Exact, Apparatebau, Norderstedt, Germany) guided by a pin inserted in the implant's central cannula. The most superficial 5.0 mm were stored at -20°C and later used for mechanical testing, the next section of 3.5 mm in thickness was prepared for histomorphometry.

Histomorphometry

The specimens were dehydrated in 70–100% ethanol containing 0.4 % basic Fuchsin and subsequently embedded in methylmethacrylate (Technovit 7200 VLC, Exact, Apparatebau, Norderstedt, Germany). Four sections of 25- μm thickness were cut perpendicular to the long axis of the implant on a microtome (Leiden, Holland) and surface stained with 2% light green for two minutes [4].

The microscope field was transmitted to a video monitor. Histomorphometry was done using a software program (Bioquant, R&M Biometrics Inc., Tennessee, USA), which made it possible to specify grids with lines and points. Grids were superimposed on the microscopic field on the monitor. Bone ongrowth was evaluated by the linear intercept technique. Approximately 241 interceptions on the surface of the implant were counted on each implant and bone ongrowth was calculated as bone coverage of implant surface in percentage of total surface area. Volume fractions of woven bone, grafting material and non-mineralised tissue in the gap were estimated in two regions. The first was 0–300 microns from the implant surface outwards (zone 1) and the second was 350–650 microns from the implant surface (zone 2) (Fig. 2). In each zone 1,320 points were counted at 100X magnification. All specimens were blinded until data analysis.

Mechanical testing

Push-out testing was performed using an Instron Universal test machine (Instron Ltd., High Wycombe, UK). The specimen was placed on a metal support jig and the implant, with a diameter of 6 mm in diameter, was centralised over a 7-mm circular opening. A preload of 2 N defined the contact position for the start of the test. The implant was displaced at a velocity of 5 mm/minute. Data was transferred to an Excel spreadsheet and load-deformation curves were

Table 2 Push-out values

Group	Ultimate shear strength (MPa)	Energy absorption (J/m^2)	Apparent stiffness (MPa/mm)
Empty (<i>n</i> =7)	0.13 (0.04–0.28)	24 (1–48)	0.69 (0.25–1.82)
AGF (<i>n</i> =8)	0.18 (0.06–0.91); <i>p</i> =0.16	35 (9–159); <i>p</i> =0.30	0.89 (0.26–5.25); <i>p</i> =0.22

AGF autologous growth factor

Values given as mean (range). None of the differences between the two groups were statistically significant

Table 3 Bone ongrowth in percentage of total implant surface

Group	Bone	Fibrous membrane	Other tissue
Empty (<i>n</i> =8)	1.5 (0.0–11.4)	62.7 (20.4–99.3)	35.7 (0.7–71.3)
AGF (<i>n</i> =8)	1.2 (0.8–12.1); <i>p</i> =0.46	61.9 (20.0–96.3); <i>p</i> =0.84	31.6 (2.3–73.7); <i>p</i> =0.84

AGF autologous growth factor

Values given as mean (range). None of the differences between the two groups were statistically significant

obtained. Ultimate shear strength (σ_u) was determined from the maximal force (F) and was calculated as $\sigma_u = F/\pi DL$ where D is diameter and L the length of the implant. Apparent shear stiffness was obtained from the slope of the straight-line part of the load-displacement curve and calculated as stiffness = $(\delta F/\pi DL)/\delta L$. Energy absorption was calculated from the area beneath the curve until failure.

Statistics

Due to the wide animal-to-animal variation in our data, we chose to present all data as median values and ranges in brackets and apply a non-parametric statistical test. The groups were compared all pairwise using Wilcoxon signed rank test. *P*-values less than 0.05 were considered significant.

Results

All dogs were fully mobilised one day after surgery. There were no signs of infections postoperatively or at the time of sacrifice and all cultures were negative.

Platelet count was increased from 168×10^3 (range 90– 247×10^3) platelets/ μ l to 1003×10^3 (710 – 1754×10^3) platelets/ μ l with an average 7.9-fold increase (Table 1).

During mechanical testing, one implant from the non-AGF treated group was not centralised over the support jig and the test failed. This implant was excluded from the study, but the corresponding histomorphometry section was

included. A preload of 2 N was obtained on all implants. AGF increased ultimate shear strength, energy absorption and apparent stiffness in five of seven dogs. AGF treatment increased ultimate shear strength from 0.13 MPa (range 0.04–0.28 MPa) to 0.19 MPa (range 0.06–0.91 MPa) (Table 2); however, those differences were not statistically significant ($P=0.16$). Note the limitation in statistical power for the mechanical tests due to small sample size and sample loss. Also, energy absorption and apparent stiffness were not statistically significant increased.

Woven bone was stained green and non-mineralised tissue was mainly soft and cell-rich (Fig. 2a, b). A fibrous membrane could be detected around some of the implants (Fig. 2b). The fibrous membrane could be distinguished from other non-mineralised tissue by having fibres parallel to the implant surface (Fig. 2b).

AGF showed no effect on any of the measured histological parameters (Tables 3 and 4).

Discussion

At the time of implantation, the surface of the prosthesis is in intimate contact with the host bone in some areas, whereas gaps between the implant surface and host bone will be found in other areas. Ideally, such gaps should be filled with newly-formed bone to obtain a sufficient biological anchorage of the prosthesis. We used a well-established loaded implant model to mimic the clinical situation. In this model, a well-defined gap of 0.75 mm was created and used to determine if AGF increases bone formation and implant fixation. We designed the study without bone graft as our intent was to represent implantation in a healthy bone bed, which occurs in primary implantation where bone graft is rarely necessary.

Although this study did not demonstrate a significantly favourable effect of AGF in ungrafted loaded implants (and the negative results should be interpreted with the statistical power limitations due to small sample size and sample loss), AGF was active in the same animals when mixed with morselised bone allograft with unloaded extraarticular

Table 4 Volume fractions of woven bone, fibrous membrane and non-mineralised tissue in percentage of total area

Group	Woven bone		Fibrous membrane		Other tissue	
	Zone 1	Zone 2	Zone 1	Zone 2	Zone 1	Zone 2
Empty (<i>n</i> =8)	13.2 (3.1–21.7)	11.4 (3.6–24.6)	0.0 (0.0–7.4)	9.7 (0.2–65.0)	86.6 (77.0–96.9)	71.5 (28.0–90.4)
AGF (<i>n</i> =8)	9.0 (4.6–18.2); <i>p</i> =0.74	9.9 (3.8–24.4); <i>p</i> =0.25	0.0 (0.0–11.1); <i>p</i> =0.74	6.4 (0.3–71.7); <i>p</i> =1.00	91.0 (78.5–95.4); <i>p</i> =0.64	82.7 (24.6–90.8); <i>p</i> =0.46

AGF autologous growth factor

Values given as mean (range). None of the differences between the two groups were statistically significant

implants inserted in the proximal tibia. In that setting, AGF improved implant fixation and bone ongrowth and decreased the formation of a periprosthetic fibrous membrane [7]. Therefore, we conclude that AGF was capable of stimulating bone under certain conditions.

Previously, Lind et al. succeeded in increasing implant fixation and bone incorporation by coating the implant surface with TGF- β using the same model as in our study [12]. Furthermore, HA and RGD coatings have shown dramatic results using our loaded non-grafted implant model [2, 18].

There might be several explanations for the negative results in our study. The gap surrounding the implant was in intimate contact with the joint cavity (Fig. 1). Therefore, growth factors might have been washed out by the joint fluid. Moreover the implants were inserted in a newly created cavity with poor haemostasis. The bleeding might have washed out the gel or the growth factors after implantation. These factors might however also be expected in a clinical situation inserting non-cemented arthroplasties.

A higher concentration of fibrinogen in the plasma could be one method of keeping the growth factors in place. The primary difference between AGF and other platelet concentrates is the use of an UltraConcentrator to further concentrate the buffy coat. This allows more plasma to be added in the buffycoat since the use of the UltraConcentrator filters off water leaving higher levels of blood cells and elements in the plasma such as fibrinogen [9]. Hence, it is our experience that a more solid gel can be formed compared to manually prepared platelet concentrates.

Another approach to improve the fibrin network is to add thrombin receptor activator peptide-6 (TRAP) instead of thrombin, thereby realising growth factors for a longer period [10, 22]. However, this has not yet been tested in vivo.

Positive results on PRP were first published by Marx et al. [15]. In that study, mandibular defects treated with PRP and autograft showed enhanced maturity and bone area compared to autograft alone in humans. In that study, AGF enhanced osseointegration of bone grafted implants in canines [7]. The same positive effect of AGF was found using a bone chamber where AGF increased bone and tissue ingrowth in porous coralline hydroxyapatite [16]. However, in other clinical studies [1, 7, 23] AGF did not improve results when added to autograft in patients undergoing posterolateral spine fusion.

With a different PRP preparation though with a comparable level of platelets using non-weight-bearing HA-coated implants surrounded by a large (2.5 mm) gap, we found no improvement with PRP when used alone or mixed with bone allograft [6]. The success of using PRPs such as AGF seems therefore to be highly dependent on the animal model being used or clinical application.

Most positive results on PRPs have been reported mixing PRP and bone graft or bone substitute, apparently because the growth factors such as PDGF and TGF- β are retained by the grafting material. Bone substitutes have been shown to significantly prolong release of growth factors to more than 14 days in vitro [10, 22]. So far, no studies have shown in vivo effects of platelet concentrates on bone formation when used alone.

In conclusion, although AGF previously stimulated bone formation in a grafted implant model, we found no effect of AGF on non-grafted implants in our loaded implant model. Even though a number of commercial kits are now available and a number of articles describe how to use PRPs, only a few studies showing positive results of PRP in well-designed investigations have been published. Defining conditions under which PRP is most beneficial is needed before the use of PRP can be recommended for clinical use.

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In memory of Milo Radotich (1953–2002).

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