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Targeting RANKL for reduction of bone loss around unstable implants: OPG-Fc compared to alendronate in a model for mechanically induced loosening

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Abstract

Orthopedic joint prostheses may loosen because of localized bone resorption. Despite initial optimism, there are no reports showing that bisphosphonates can stop the progression of prosthetic loosening once it has begun. This might be due to the strong resorptive stimulus, which continuously recruits new osteoclasts. Therefore, we hypothesized that a treatment targeting osteoclast recruitment would be more efficacious than a treatment reducing osteoclast activity. We used a previously described rat model for instability-induced bone resorption, and compared OPG-Fc with alendronate at a clinically relevant or an extreme dose. A titanium plate was osseointegrated at the rat tibial surface. Instability was simulated by a piston, moving perpendicularly to the bone surface. Piston movement induced bone loss via hydrostatic pressure or fluid flow. Rats were randomized to 5 groups (total n=56), of which 4 were subjected to instability and one was stable. The unstable groups were injected with either high-dose OPG-Fc (10 mg/kg, twice weekly), a high dose of alendronate (20 µg/kg/day), an extreme dose of alendronate (200 µg/kg/day) or saline. Significant protection against resorption could only be shown for OPG-Fc and the extreme alendronate dose. Both alendronate doses reduced serum levels of tartrate-resistant acid phosphatase isoform 5b to a similar extent, demonstrating that the lower dose was able to reduce resorption in the normally remodeling skeleton, although not in the osteolytic lesions caused by instability. Osteoclast numbers in the lesion were increased by the lower bisphosphonate dose and reduced by OPG-Fc. The results suggest the possibility of targeting osteoclast recruitment via the RANKL system in patients with impending prosthetic loosening.

Introduction

Loosening of orthopedic joint replacements often requires revision surgery, which may be difficult for both the surgeon and the patient, especially as many of the patients have become aged and frail during the time since the primary operation. In many cases, revision surgery has to be performed while the patient still has few symptoms, in order to avoid bone loss to an extent, which would have been too difficult to treat later on. Therefore, any treatment which could halt the progression of the loosening process might spare these patients hazardous surgery.

In a famous paper 11 years ago, Shanbhag et al demonstrated in a dog model, that bisphosphonates might be used to prevent the loosening of total hip joint replacements [1]. However, experiments in small animal models indicated that the resorptive stimulus in the loosening context might be too strong for bisphosphonates to overcome [2]. In a rat model with an unstable implant, only extremely high doses of bisphosphonates could inhibit resorption. These doses could not realistically be used in the clinic, perhaps with exception for local treatment. Attempts to block the progression of the loosening process in patients by use of systemic bisphosphonates were unsuccessful in a randomized multicenter trial [3]. So far, therefore, bisphosphonate treatment of clinically manifest loosening has been a disappointment.

Bisphosphonates can only reduce resorption after they have been internalized in osteoclasts, and this internalization requires the cells to resorb bisphosphonate-containing bone [4]. Therefore, there will always be some resorption going on with bisphosphonate treatment, although extremely high doses might reduce this to a minimum. In contrast, a treatment targeting osteoclast recruitment might reduce resorption even further, especially at sites where recruitment is strong, such as in pathological resorption.

Osteoclast recruitment is largely controlled by the RANKL/RANK system. The ligand, RANKL, can be presented to the receptor RANK, both as a solute and via cell to cell contact. This signaling is required for both osteoclast recruitment and maintenance. A blocking antibody to RANKL, denosumab, has been shown to be efficacious for osteoporosis treatment in clinical trials [5]. It is conceivable, considering the above, that denosumab could be used to inhibit the progressive bone loss occurring during the loosening of orthopedic joint replacements. Unfortunately, the human antibody denosumab is ineffective in rats. Therefore, we used an endogenous antagonist to RANKL, osteoprotegerin (OPG). The half-life of the OPG had been extended by adding an Fc-tag. OPG-Fc binds to RANKL and blocks its action, and therefore has similar effects as blocking with an antibody, such as that of denosumab in humans [6].

The etiology of prosthetic loosening is unclear. Although the role of a chronic, wear debris-induced inflammation is generally accepted, it is also clear that instability and micromotion are important factors [7]. Indeed, micromotion can induce dramatic bone

resorption in the absence of any debris particles, both in animal models and in the clinic [8]. In failed fracture surgery, where screws and plates loosen due to microinstability, resorption zones are seen around the screws. A classical example is the resorption around fibulo-tibial screws inserted at surgery for ankle fractures, which are weakly fixed and intended to loosen when the patient starts loading the joint. The mechanism behind instability-induced resorption most likely involves the effects of fluid pressure and flow, which also by themselves are potent inducers of local resorption [9]. Regarding total joint replacements, the relative importance of wear debris versus fluid pressure and flow is debated. For the present study, we chose to use a rat model for instability-induced loosening, because of its high reproducibility and obvious clinical relevance for the instability aspect of loosening [9].

We tested three hypotheses. First, we hypothesized that OPG-Fc would block instability-induced resorption. Second, that OPG-Fc would be more efficacious than a clinically relevant dose of alendronate. This dose was based on previous rat data in similar models, and the effects on serum tartrate-resistant acid phosphatase isoform 5b (TRACP5b) levels. The third hypothesis was that an exaggerated dose of alendronate would also block instability-induced resorption. In addition, effects on osteoclast numbers in the instability-exposed area were compared.

Materials and Methods

Animal model

A total of 56 male 10 week old SD rats were used in an animal model for pressure induced prosthesis loosening. Briefly, a titanium plate was fastened to the proximal tibia, and allowed to osseointegrate for 5 weeks. Thereafter, a plug in the plate was removed and replaced by a cylinder with a piston, which could be moved perpendicularly to the bone surface previously facing the plate. The piston had a resting position 0.5 mm above the bone surface. During the first 5 days, soft tissue was allowed to form in the space between the piston and the bone surface. Thereafter, loading of the piston started, by pushing it towards the bone surface. After being pressed down by an external force, the piston was moved up to its resting position by an internal spring. The velocity of the piston during this manoeuvre was approximately 14 mm/s. The exposed bone area was 6.2 mm². Each episode of instability comprised 20 cycles during 2 minutes at 0.17 Hz. The present model has been improved since previous studies [10]. It is described and validated in [9].

Drug treatment

OPG-Fc, supplied by Amgen, is a dimeric 90 kDa fusion protein comprised of the RANKL-binding TNF domains of rat OPG (amino acids 22-194) linked to the Fc portion of rat IgG1. Alendronate sodium trihydrate (Sigma, St Louis, MO) in saline was used for alendronate injections. The animals were randomly allocated to 5 groups. Four groups received a piston, and one group received a dummy piston (stable group). The groups with a piston (unstable groups) received different drugs by subcutaneous injection: OPG-Fc (10 mg/kg, days 1, 4 and 8), alendronate extreme (200 µg/kg daily), alendronate high (20 µg/kg daily), or control (saline). With exception for the stable group, all animals were subjected to twice daily instability episodes for 10 days. The experiments followed institutional guidelines for care and treatment of experimental animals, and were approved by the regional ethics committee for animal experiments.

Surgery

In a first operation, a titanium plate with a center plug was fastened at the medial aspect of the rat proximal tibia with two cortical screws. The rats were anaesthetised with 5% isoflurane gas (Forene, Abbot Scandinavia, Solna, Sweden). The rats received a preoperative subcutaneous injection of 25 mg/kg tetracycline (Engemycin; Intervet, Boxmeer, Holland) and 5 mg/kg carprofen (Rimadyl vet; Orion Pharma AB, Sollentuna, Sweden) at the first operation. At the second operation, the rats received 0.015 mg/kg buprenorphine (Temgesic; Schering-Plough, Brussels, Belgium). Under aseptic

conditions a 5–6 mm longitudinal incision was made along the rat tibia. The periosteum was reflected proximally to the physis. A depression in the tibial cortex was milled out correspond to the pressure area of the middle hole. The centre plug protruded into a milled depression in the bone cortex but not reaching the marrow cavity.

New bone was allowed to grow up to the titanium surface of the central plug to form a flat bone surface. After the depression was prepared, the titanium plate was screwed in the predominantly cortical bone approximately 7 mm from the physis. After 5 weeks, in which the titanium plate and central plug were osseointegrated, the plug was replaced by a piston. With the piston in place, a 1.4 mm distance is left for soft tissue to form between the piston and the flat bone surface. When loaded, the soft tissue is compressed by the piston, thereby creating a fluid pressure, propagated down onto the underlying bone. The piston does not reach the bone, but stops at a distance of 0.6 mm from the bone surface, thereby avoiding direct contact. The animals were subjected to pressure cycles for 10 consecutive days. The day after the last cycle, the animals were anesthetised using isoflurane and euthanized using a cardiac injection of 50 mg/kg sodium pentobarbital (Pentobarbitalnatrium vet; APL, Kungens Kurva, Sweden).

Prior to euthanasia a blood sample was taken by cardiac puncture and used for measurement of serum markers. Following euthanasia the tibia was harvested and cut at the level of the proximal and distal screw hole for further analysis.

Histology and immunohistochemistry

The tibia specimens were fixed in neutral buffered formaldehyde (10 %, pH 7.4), decalcified in formic acid, embedded in paraffin and processed for histological and immunohistochemical evaluation. Hematoxylin and eosin (H&E) staining was used for quantitative histology.

For immunohistochemistry, mouse anti-rat Cathepsin K [1:600] [11] were used to visualize bone resorbing osteoclasts. Briefly, deparaffinized sections were blocked with 3 % H₂O₂ (Fluka, Sigma-Aldrich, St Louis, MO) for 5 minutes. A second block was performed using 0.5 % bovine serum albumin (Sigma, St Louis, MO) for 20 minutes. The sections were incubated with primary monoclonal antibody for 1 hour and 20 minutes after which they were rinsed and incubated with a biotinylated, polyclonal antibody for 30 minutes. To detect the bound antibodies, sections were incubated with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) for 30 minutes followed by 5 minutes of 3,3'-diaminobenzidine (DAB, D12384, Sigma, St Louis, MO). Thereafter, sections were counterstained with Mayer's hematoxylin for 30 seconds before mounted.

Histology and immunohistochemistry to describe the osteolytic process were assessed under light microscopy by an investigator blinded to treatment. Osteoclasts were

defined as Cathepsin K positive multi-nuclear cells located within 0.05 mm from a bone surface.

The bone resorption area was measured in two representative sections (glasses) with a distance of 450 μm between the sections, using image analysis software (Cell[^]D, Olympus Europe GmbH, Hamburg, Germany). First the area of the bone plate was defined as being rectangular, spanning the width of the pressure piston and the height of the bone plate. By using a trace tool, osteolytic cavities and other lesions within the bone plate were encircled. The combined areas of these cavities were then added to form a resorbed area.

Biochemical analyses

Serum rat osteoclast-derived TRACP5b was determined by a solid-phase immunofixed enzyme activity assay (RatTRAP, Immunodiagnostic Systems Ltd., Boldon, UK). The RatTRAP assay uses a specific monoclonal antibody prepared using purified recombinant rat TRACP as antigen [12].

Statistical analysis

Results were analyzed with non-parametric methods. Data is expressed as median and interquartile range. Resorbed area was regarded as the primary variable. All groups were compared with Kruskal-Wallis ANOVA, followed by intergroup comparisons using the Mann-Whitney test. The intergroup comparisons were performed in the following order, after relevance: 1) Pressure treatment (control) versus no pressure, 2) OPG-Fc versus control, 3) OPG-Fc versus alendronate 20 $\mu\text{g}/\text{kg}/\text{day}$, 4) Alendronate 200 $\mu\text{g}/\text{kg}/\text{day}$ versus control, 5) Alendronate 200 $\mu\text{g}/\text{kg}/\text{day}$ versus alendronate 20 $\mu\text{g}/\text{kg}/\text{day}$.

Results

Instability led to resorption

Unstable implants showed dramatic resorption, with resorptive lesions in the bone underlying the pressure piston (Figure 1), but also in areas peripheral to the piston, as previously described [9] (Table 1). Numerous osteoclasts were seen, actively resorbing bone. The osteolytic lesions appeared to contain mostly granulation tissue (Table 2).

OPG-Fc inhibited resorption completely

Unstable implants with OPG-Fc treatment showed virtually no osteoclasts at all (Figure 2d), and there were no typical osteolytic lesions (Table 1). The resorbed area was similar to stable controls, and significantly different from unstable controls and rats given alendronate at 20 µg/kg/day. Osteoclasts were extremely few, and significantly fewer than in all other groups, including the stable controls (Table 2).

Preserving the bone with alendronate required an extreme dose

Alendronate at an extreme dose also preserved the bone, so that the resorbed area was different from unstable controls, and similar to that of OPG-Fc. In contrast, in the group given a high dose, there were several osteolytic lesions, and the resorbed area was similar to unstable controls. It differed significantly from the extreme alendronate dose and from OPG-Fc (Table 1, Figure 3A).

Osteoclast numbers were increased by the high dose, as compared to unstable controls (Figure 2 e versus b). This seemingly paradoxical effect has been described previously in this model [10], and is consistent with the concept that osteoclasts become inactivated, but survive. With the extreme alendronate dose, osteoclast numbers were similar to stable controls (Figure 2 f versus c, Table 2, Figure 3B).

No difference was shown in serum TRACP5b between stable and unstable control groups, suggesting that serum TRACP5b levels primarily reflect systemic resorption, with minimal contributions from the small region of instability. Both alendronate doses reduced the serum level of TRACP5b to a similar extent, suggesting that the two doses cause similar effects on the normally remodeling skeleton, and that the dose dependency for alendronate is only seen at the local level under the pathological conditions, such as at the unstable implant (Table 3, Figure 3C).

Discussion

In this model for an unstable implant, both OPG-Fc and the extreme dose of alendronate protected against instability-induced resorption, whereas no protective effect was shown for a less excessive alendronate dose. This dose-dependency for alendronate has previously been demonstrated in a similar rat model, where resorption was induced by sliding implant motion [2]. In that study, it was shown that the lower alendronate dose was relevant, as it increased the density of the secondary spongiosa at the growth plate. In the present study we instead measured serum TRACP5b, and this marker indicated that both alendronate doses reduced bone resorption of the whole skeleton to a similar degree.

Translation of drug doses from rat experiments to humans is difficult. However, the findings that the high alendronate dose of 20 µg/kg/day (our lower dose) was able to increase the density of remodeling bone and reduce serum levels TRACP5b, suggest that this dose is sufficiently high to model clinical treatment in humans. By body weight, it is more than 100 times higher than the human standard dose, considering that the bioavailability of oral alendronate in humans is less than 1 percent. Still, this dose was too low to protect effectively against instability-induced bone resorption. Therefore, we believe that bisphosphonates at clinically applicable doses are unlikely to be efficacious for pharmacological treatment of impending or manifest prosthetic loosening. The likely explanation lies in the fact that some resorptive action is always necessary for the osteoclasts to ingest the bisphosphonate [4]. In a pathological condition, where large numbers of osteoclasts are continuously recruited, and each osteoclast takes a “test bite” of bone before becoming inactivated, this cumulated effect may be sufficient to cause unaffordable bone loss. Consistent with this notion, bisphosphonates impaired the function of osteoclasts in a model for disuse osteopenia in dogs, but as they were unable to overcome the strong stimulus for osteoclast recruitment, resorption was reduced only to a moderate extent [13]. In contrast, targeting RANKL by OPG-Fc or antibodies such as denosumab might be efficacious, because this treatment aims at osteoclast recruitment, and “test bites” can be avoided. The dose of OPG-Fc is used in our model is only a few times higher than the human dose of 3 mg/kg, which has been used successfully in clinical trials [14]. This is perhaps the first published study describing the effects of a fully rat version of OPG-Fc. Previous studies in rats showed that a single injection of human OPG-Fc at 5 mg/kg could reduce osteoclast numbers by 95% [15], whereas repeated injections of human OPG-Fc for 6 weeks invoked strong anti-drug immune responses in some rats [16]. Because rat OPG-Fc did not appear to elicit anti-drug immune responses over this 10-day experimental period, it might be feasible to determine in future studies whether “clinical” doses of rat OPG-Fc might also be capable of preventing this type of focal osteolysis. Rat OPG-Fc effectively inhibited systemic bone resorption in young mice at doses as low as 0.1 mg/kg/day (Amgen Inc., data on file).

We believe our model is relevant for prosthetic loosening. Although it is generally accepted that resorption around joint prosthesis is often related to a chronic inflammation elicited by wear particles, it is undisputable that instability might play an important role. As mentioned in the introduction to this article, this is common knowledge in the context of loose fracture fixation devices, where no wear debris is present [8]. The role of mechanical factors for prosthesis loosening is also supported by the observation that the surgeon's experience and prosthetic design influence the risk of loosening [17]. Moreover, prosthetic migration over the first postoperative years is a good predictor of later, clinically manifest loosening, again suggesting an important role of instability [18]. It may be that the osteolytic zones often seen around cemented prostheses are more the results of instability, whereas focal osteolytic lesions adjacent to non-cemented prostheses are more related to chronic inflammation. However, regardless if one considers the reaction to wear debris or fluid flow to be the only cause of loosening, both putative mechanisms converge on RANKL/RANK signaling.

Bisphosphonate treatment increased the number of osteoclasts in this experiment. We have noted this previously in this model [2], and it has later also been described in several species, including humans [19]. In this experiment, the phenomenon probably reflects the strong recruitment of osteoclasts due to the mechanical stimulus. Apparently the cells become inactivated by the bisphosphonate, but remain in the area for a long time.

Wear debris not only may stimulate resorption, but also inhibits bone formation [20]. If the growth of osteolytic lesions were caused by reduced osteoblastic activity rather than increased resorption, pharmacological treatment would have to target the osteoblasts by applying some kind of anabolic treatment. We have tried this in the present model by giving PTH [10]. This treatment did not prevent the development of osteolytic lesions.

Although bisphosphonates may be insufficient to inhibit ongoing bone resorption around unstable prostheses, prophylaxis is another matter: bisphosphonates appear to be efficacious for improvement of the early postoperative stability, and might thereby reduce the risk of a loosening process ever getting started [21].

In conclusion, our findings suggest that it might be possible to inhibit ongoing bone resorption around loose implants by blocking RANKL signaling. Because this might be a great clinical advantage, and save patients from extensive surgery, a clinical study might be warranted.

Table 1. Area of osteolytic lesions (% of total bone area under the piston). Kruskal-Wallis test for all groups gave $p < 0.001$.

Group	N	Pair-wise comparisons			P-value
Control	9	Control	vs	Stable	0.015
High Alendronate	12	OPG-Fc	vs	Control	0.002
Extreme Alendronate	10	OPG-Fc	vs	Alendronate High	0.016
OPG-Fc	11	Alendronate extreme	vs	Control	0.008
Stable	11	Alendronate extreme	vs	Alendronate High	0.06

Table 2: Number of osteoclasts present in the area under the piston. Kruskal-Wallis test for all groups gave $p < 0.001$.

Group	N	Pair-wise comparisons			P-value
Control	9	Control	vs	Stable	0.002
High Alendronate	12	OPG-Fc	vs	Control	<0.001
Extreme Alendronate	10	OPG-Fc	vs	Alendronate High	<0.001
OPG-Fc	11	Alendronate extreme	vs	Control	0.17
Stable	11	Alendronate extreme	vs	Alendronate High	0.03

Table 3: Serum TRACP5b concentration (U/L). For OPG-Fc, all measurements were under the detection limit of 0.1 U/L. Kruskal-Wallis test for all groups gave $p < 0.001$.

Group	N	Pair-wise comparisons			P-value
Control	9	Control	vs	Stable	0.9
High Alendronate	12	OPG-Fc	vs	Control	<0.001
Extreme Alendronate	10	OPG-Fc	vs	Alendronate High	<0.001
OPG-Fc	11	Alendronate extreme	vs	Control	0.008
Stable	11	Alendronate extreme	vs	Alendronate High	0.6

Figure 1: Transverse section of proximal tibia (A) with schematic plate (B) and pressure piston (C). Area of histomorphometry is located within dashed rectangle. With the piston in place a 1.4 mm space is created (D), allowing a soft tissue interface to form. When loaded, the soft tissue is compressed by the piston. After loading, the piston returns to its original position, due to the internal spring (E). A silicon membrane (dark grey) ensures a tight seal.

Figure 2 Overview of the bone plate stained by H&E (a). Osteoclasts stained for Cathepsin K beneath the piston with stable implant (b), unstable implant (c), unstable implant treated with Fc-OPG (d), unstable implant treated with a high dose alendronate (20 µg/kg/day) (e) and unstable implant treated with extreme dose alendronate (200 µg/kg/day) (f).

Figure 3: A) Area of osteolytic lesions under the piston (%). Note the large increase in resorptive area caused by instability. OPG-Fc and extreme alendronate dosage reduce the area of the lesions to be comparable to stable controls. B) Number of osteoclasts under the piston. OPG-Fc treatment reduced the number to almost 0. C) Rat serum TRACP5b concentration. OPG-Fc treatment reduced the concentration to under the detection limit of 0.1 U/L. Line at median values. High = 20 µg/kg/day of alendronate, Extreme = 200 µg/kg/day of alendronate.

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