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Influence of Osteoprotegerin (OPG) on Experimentally Induced Ectopic Bone

Krzysztof H. WŁODARSKI, Iwona KOCHANOWSKA, Marek PIEŃKOWSKI and Kazimierz OSTROWSKI

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In this paper the effect of osteoprotegerin (OPG) on slowing down the resorption process of heterotopically induced bone tissue is described. The induced ossicle is resorbed *ex inactivitate*. This system is analogous to osteoporosis in immobilised skeletal bones. Bone induction was achieved in BALB/c mice after injection of a suspension of $3x10^6$ HeLa cells into thigh muscle of animals immuno-suppressed by a single dose of hydrocortisone. To slow down the process of induced bone resorption, OPG was administered and the effect was measured quantitatively by weighing the mass of the induced ossicle after hydrolysis of soft tissues surrounding the induced ossicles. As an effect of the application of OPG, dry bone mass of the induced ossicles exceeded 340-540% of the values of the control specimens following 9 applications of 0.05 mg OPG per mouse every second day or 14 doses every day.

Key words: Osteoprotegerin, ectopic bone, bone protection.

Krzysztof H. WŁODARSKI, Kazimierz OSTROWSKI, Chair and Department of Histology, Medical Academy in Warsaw, Chałubińskiego 5, 02-004 Warszawa, Poland.
E-mail: kwlodar@ib.amwaw.edu.pl
Iwona KOCHANOWSKA, Institute for Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, 53-114 Wrocław, Poland.
E-mail: kochanie@immuno.iitd.pan.wroc.pl
Marek PIEŃKOWSKI, PienGen Corp. Knoxville, TN 37919, U.S.A.

In this paper, a new model of experimental osteoporosis is described as well as the effect of osteoprotegerin (OPG) on impeding the resorption process of bone tissue.

The two existing main populations of patients suffering from osteoporosis are menopausal women and patients undergoing long-term steroid therapy or immobilisation. In these cases one can interfere with the loss of bone mineral and resorption of skeletal tissues only by slowing the process. As no efficient therapy for treatment of osteoporosis exists, the general feeling is that the best way of preventing osteoporosis is the building of a strong bone system at a young age.

Two populations of cells – osteoblasts and osteoclasts – are responsible for the balance and regulation of the bone rebuilding process. The precursors of both kinds of these cells are found in bone marrow (BURGER 1984). Both populations are under the influence of various known factors such as some hormones, vitamins, cytokines (TAKAI *et al*. 1998) or immunological factors (TAKAI *et al.* 1998; MARTIN & NG 1994). The activity of osteoclasts is regulated mainly by the paracrinal activity of osteoblasts. The critical immunological factor is the protein on the surface of T-cells which activates bone-resorbing cells (TAKAYANAGI *et al.* 2000, 2002 a, b; SUDA *et al*. 2001; TEITELBAUM 2000).

The activity of osteoclasts is balanced in several ways. Osteoblasts are the cells forming the new bone. They produce the RANKL protein, which activates RANK on osteoclast precursor cells, stimulating these cells to differentiate into mature bone resorbing osteoclasts. Activated RANK does this by inducing the expression of c-Fos (GRIGORIADIS *et al.* 1994), which binds to DNA and activates the expression of several genes needed for osteoclast function – among others the gene for interferon-β which is also produced by the population of T-cells. The interferon-β protein binds its receptor, present on neighbouring osteoclast precursors, inhibiting osteoclast differentiation by preventing the RANK-induced expression of c-Fos. Two proteins, ISGF3 and PKR, are among the proteins that are intermediaries between the interferon-β receptor and c-Fos. The transcription factor NFAT (nuclear factor of activated T-cells) (SUN *et al*. 1997) is activated

by RANKL in bone marrow macrophages, via TRAF6 and c-Fos dependent pathways. RANKL also induces oscillatory calcium signals that activate calcineurin, leading to phosphorylation and nuclear transport of NFAT. NFAT acts as a transcriptional master switch for terminal differentiation of osteoclasts.

Osteoprotegerin (OPG) (HOFBAUER & HEUFELDER 1998) is a soluble protein, released from osteoblasts, that binds to RANKL and prevents it from binding to RANK (YASUDA *et al*. 1999). Nowadays synthetic OPG is produced and tested in clinical research.

A higher resorption rate of bone tissue, not compensated by bone formation, is the essence of osteoporosis. This leads to the loss of bone mass. Osteoprotegerin is tested in several clinics for treatment of osteoporosis. The evaluation of the effects of this treatment is not always easy in clinical conditions.

Therefore, a model of experimental osteoporosis was developed in the form of resorption of heterotopically induced ossicles, which are formed in the muscles and are resorbed *ex inactivitate*. This system mimics osteoporosis in immobilised skeletal bones.

Material and Methods

For induction of heterotopic ossicle the experimental system introduced by ANDERSON *et al.* (1961) was applied. A suspension of $3x10^6$ HeLa cells was injected into thigh muscles of BALB/c mice. The heterotopically induced ossicles are formed when HeLa cells are, at the time of injection, protected by a single dose of 3, 75 mg hydrocortisone per mouse. Enchondral osteogenesis starts on the 6th day after transplantation. The fully developed ossicle, usually containing bone marrow, is observed after 14 days. After 4-8 days the xenogenic (human) HeLa cells disappear when the immunosuppression ceases to work. After the 14th day of bone induction the ossicle starts to be resorbed by the process described in pathology as resorption *ex inactivitate* (WŁODARSKI 1969).

Measurements of the quantity of induced bone tissue were done after excision of the fragments of thigh muscles where suspension of HeLa cells was injected – on the 21-24th day after grafting. A solution of 0.1 N NaOH at 63° C overnight was used to digest the excised fragments. The deposited undigested bone mineral was weighed on an analytical scale with an accuracy of 0.1 mg.

Recipient animals were immunosuppressed by a single dose of 3,75 mg hydrocortisone (Hydrocortisonum Jelfa). Animals were divided into two groups: one receiving Osteoprotegerin (OPG), while the second, control group received PBS only. OPG was a gift from the AmGen Company.

Quantification of the yield of induced bone

Animals were killed three-four weeks after initiation of bone induction.Thigh muscles, containing the foci of heterotopically formed bone, were excised and hydrolysed in 0.1 N NaOH in 64°C overnight. The bone ossicles, released from the adjoining soft tissues, were washed in destilled water, dried overnight until constant weight and weighed on an analytical scale with an accuracy of 0.1 mg. The mean bone mass (mg) and standard deviation (SD) were calculated for OPG and PBS-treated groups. The significance of differences was analysed by using the Student's *t*-test.

Two control and two OPG-protected mice were used for histological analysis. Specimens were fixed in Bouin solution demineralised in a 10% EDTA solution, paraffin sections were stained with hematoxyline-eosine (HE) and inspected under a light microscope. No histometry was used in the course of investigation. The qualitative result is shown in Figures 1 and 2.

The quantitative results based on the amount of deposited bone mineral in the induced ossicle are shown in Table 1.

Table 1

Osteoprotective effect of systemic osteoprotegerin (OPG) administration[1] on heterotopically induced[2] bone by grafting of HeLa cells

No of animals	Treatment	The yield of bone induction[3] mean in mg ± SD	Difference OPG vs PBS in %
53	OPG	3,33 4,64	350 %
48	PBS (CONTROL)	0,95 1,35	(340-540%)

[1]OPG dissolved in PBS was injected in 9 doses of 0.05 mg/mice on alternate days or in 14 doses every day, from +10 until +20-25 th day post HeLa cell grafting. Control animals received PBS instead of OPG.

[2]$3x10^6$ HeLa cells suspended in PBS were injected into right shank muscles of CFW and Balb/c mice of both sexes. At the time of cell injection the animals were immuno-suppressed by 3.75 mg of hydrocortisone, given subcutaneously.

[3]The sites of HeLa cells grafting together with the induced bone ossicles were removed, hydrolysed overnight in 0.1 N NaOH; the dissolved soft tissues were washed out, the liberated bone ossicles were dried overnight and weighed on the analytical scale.

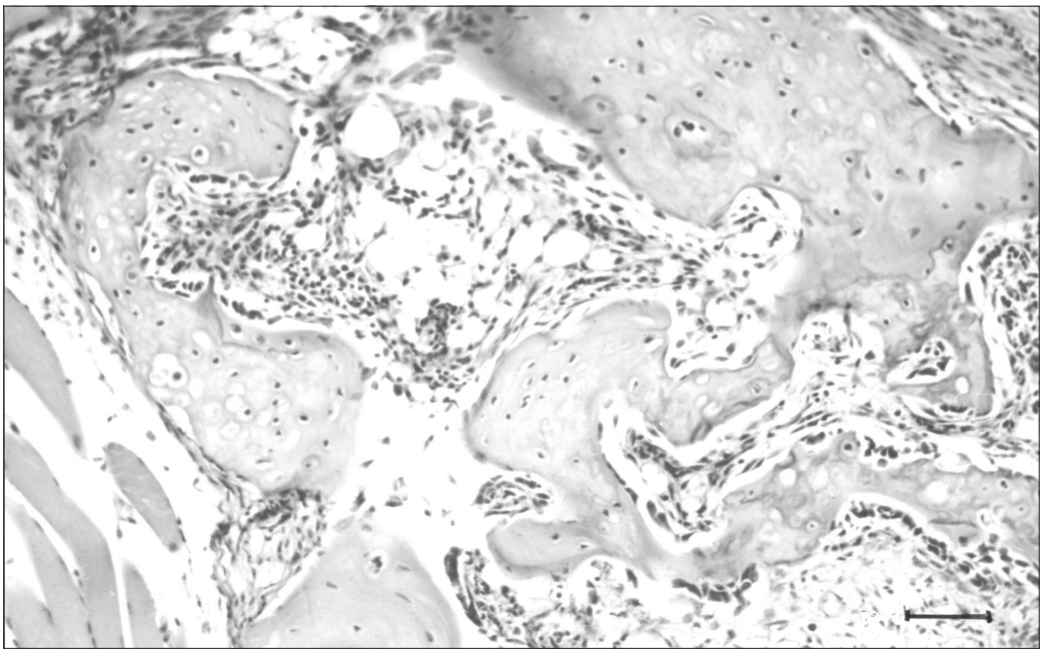

Fig. 1 The induced ossicle as seen 20 days post inoculation of heLa cells into thigh muscles of mice treated with 12 doses of 0,05 mg/mouse every day from day +10th until day 21st . The induced trabeculae are covered with osteoblasts and no osteoclastic erosion is seen. HE staining. Bar = 100 μm.

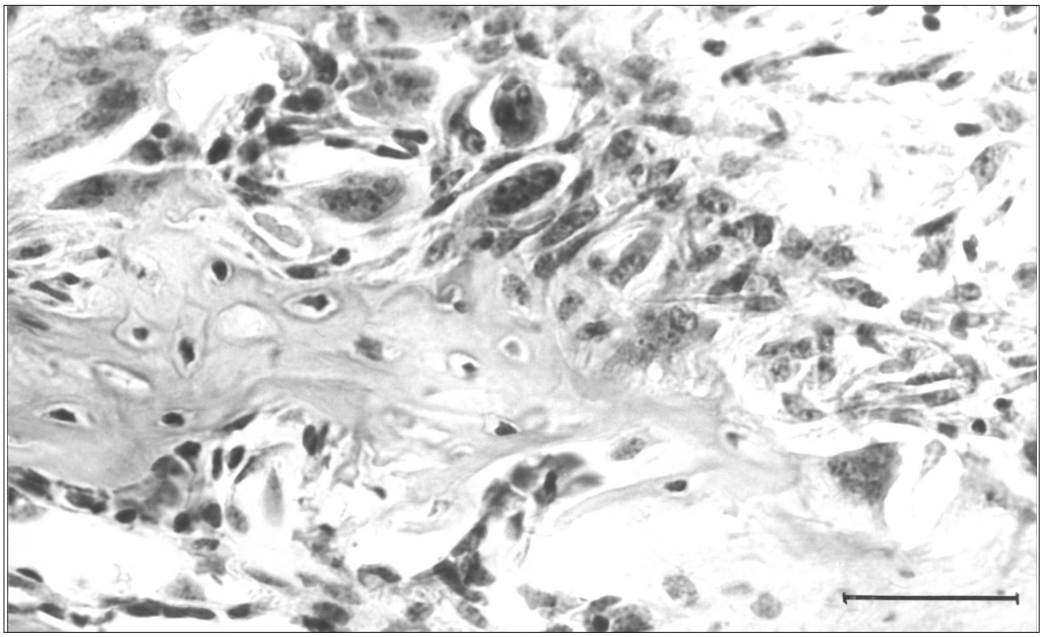

Fig. 2 Intensive osteoclastic erosion of HeLa cells induced ossicles in control, no OPG-protected mouse, four weeks post HeLa cells inoculation. HE staining. Bar = 100 μm.

Results

It was shown that the mineral content of the induced ossicle protected by OPG treatment is 350% higher than in the unprotected control ossicles.

In animals protected by OPG treatment for 10-14 days, the ectopic bone trabeculae were absent or seen only occasionally in osteoclasts induced by HeLa cell inoculation. (Fig. 1). In contrast, bone trabeculae of the control, non OPG-protected animals were vigorously resorbed by osteoclasts (Fig. 2). Some of them exhibited an active zone and were located in the resorption lacunae, which is a manifestation of their active status.

The quantitative results based on the amount of dry bone mass of the induced ossicles are presented in Table 1. From the data obtained it is concluded that the bone mass of induced ossicles in mice protected by OPG administration is c-ca 350% higher than of the unprotected, PBS-treated animals.

Discussion

The model of ectopically induced osteogenesis by HeLa cell inoculation is easy to perform and is equal to the model of ectopic bone formation by transitional epithelium of some species such as dogs, guinea pigs or hamsters and to the model of bone formation by implantation of demineralised bone matrix. These three models of ectopic bone formation mimic endochondral osteogenesis during embryonal development (WŁODARSKI 1991).

The inhibition of bone resorption in the animal model of immobilisation osteoporosis can be evaluated in two weeks or less. In the experimental model of ectopic osteogenesis presented here, OPG was administered for two weeks, starting from the beginning of resorption of the induced ossicles. In this way the present model of bone induction is similar to skeletal unloading. Bone loss and changes in trabecular architecture in the rat model of disuse osteoporosis were observed in a time dependent manner for 7, 13 and 23 days (BAROU *et al*. 2002). Most cases of disuse osteoporosis require a long time to recover bone mineral density and strength (TAKATA & YASUNI 2001). In the present model of prevention by OPG, a beneficial effect of the procedure applied was observed earlier. As in the model of ectopic osteogenesis, an immobilisation osteopenia is chracterised by extensive trabecular bone loss associated by an increase of concentration of osteoclasts.

A dose-response effect of OPG was not evaluated in this paper, since the aim was to establish the biological (but not pharmacological) influence of OPG on the heterotopically induced bone. The OPG doses were administered according to the MORONEY *et al*. (1999) protocol. The dosage and the scheme of OPG administration were proved effective. As OPG was a gift, the range of our experiments was limited.

Ectopically induced bone does not develop a true periosteal membrane (WŁODARSKI & REDDI 1986) and the ossicles, in part formed by endochondral mode of osteogenesis, resemble primary bone trabeculae. Such bone is more prone to resorption. In the immobilisation induced osteoporosis of long bones, the spongy bone vanishes faster than in compact bones (KAWATA *et al.* 1988; TAKATA & YASUNI 2001). It was established that bisphosphonates reduce bone loss in Paget's disease and osteoporosis (ADAMI 1986; REID *et al*. 1988). However, they prevent heterotopic osteogenesis after total hip arthroplasty, but in animal models do not prevent formation of heterotopic osteogenesis by demineralised bone matrix (HU *et al.* 1991). The effect of bisphosphonates on heterotopically induced bone is not equivocally established. Some of them (EHDP) inhibit the mineralisation of osteoid (HU *et al*. 1991; NAKAHARA *et al*. 1986) while others (Cl2MDP) do not (OSTROWSKI *et al*. 1988), thus the evaluation of their effect on bone depends on the time lapse after bisphosphonates withdrawal, as the inhibition is reversible (HU *et al*. 1991; NAKAHARA *et al*. 1988) Also, *in vitro* examination of effects of various bisphosphonates has revealed quantitative and qualitative differences among them (PLUIJM *et al*. 1991).

The mechanism of heterotopic bone induction by transplantation of xenogenic (human) HeLa cells into thigh muscles of mice is not fully understood. It is only partly elucidated by previous research. It was found that it is based on the secretion of bone morphogenetic proteins BMP-4 and BMP-6 by the grafted HeLa cells (KOCHANOWSKA *et al*. 2002a). Various HeLa cell lines express the BMP genes in a different way, which was proved by RT-PCR for mRNA (KOCHANOWSKA *et al*. 2002b). The NIH Skeletal Genes Database holds information on some 200 genes involved in normal and abnormal bone and cartilage formation in mice and humans. It will take time until the full description of the mechanism of bone formation will be formulated.

Evaluation of the effect of OPG on slowing down the resorption process of heterotopically induced ossicle by weighing the amount of deposited bone mineral seems to be more accurate than any way of morphometry. The protection against resorption is proven by about 340-540 % (mean value 350 %) of higher mass of bone mineral in OPG treated animals.

It seems that the proposed system of quantitative measurements of the rate of bone resorption in the experimental model of osteoporosis observed in heterotopically induced ossicles might be useful in research on new approaches in the treatment of clinical osteoporosis.

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