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A comparison of Indomethacin and Diclofenac in the inhibition of experimental heterotopic new bone formation

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Summary. *The effect of the two nonsteroidal antiinflammatory drugs Diclofenac and Indomethacin on the formation of heterotropic and orthotopic bone in rats was compared. Experimental heterotopic bone formation was induced by implanting demineralized bone matrix into the abdominal wall of rats. Indomethacin (3 mg/kg), Diclofenac (3, 6 or 12 mg/kg), or saline were given as daily subcutaneous injections. 3H-proline and 45Ca were given 24 h before the animals were killed. The net amount of bone formed by induction after three weeks was decreased by 15% by Indomethacin and Diclofenac in a dose of 3 mg/kg. The higher dose of 6 mg/kg of Diclofenac caused a higher degree of inhibition (30%), while 12 mg/kg produced toxic effects. Orthotopic bone was not affected by treatment with Indomethacin or Diclofenac.*

Although both drugs inhibit prostaglandin synthesis to a different degree, they exert similar effects on induced heterotopic bone, suggesting that this action is caused by an inhibition of the inflammatory response to trauma.

Résumé. *Comparaison de l'effet de deux anti-inflammatoires non stéroïdiens, le Diclofénac et l'Indométhacine, sur la formation d'os hétérotopique et orthotopique chez le rat. La formation expérimentale d'os hétérotopique a été provoquée par implantation de matrice osseuse déminéralisée dans la paroi abdominale du rat. L'Indométhacine (3 mg/kg), le Diclofénac (3, 6, 12 mg/kg) ou du sérum salé ont été administrés quotidiennement par voie sous-cutanée. 24 heures avant que les animaux soient sacrifiés on leur injecte une quantité connue de 3H-proline et de Ca45. La quantité d'os formé par induction après 3 semaines est diminuée de 15% par l'Indométhacine ou le Diclofénac à la dose de 3 mg/kg. Une dose plus élevée, de 6 mg/kg de Diclofénac, entraîne une inhibition plus importante, de 30%, tandis qu'une dose de 12 mg/kg a des effets toxiques. L'os orthotopique n'est pas influencé par l'Indométhacine ou le Diclofénac.*

Bien que ces deux médications inhibent à différents degrés la synthèse des prostaglandines, elles ont le même effet vis-à-vis de l'induction de l'os hétérotopique, ce qui conduit à penser que cette action relève de l'inhibition de la réponse inflammatoire aux traumatismes.

Key words: *Diclofenac, Indomethacin, Osteoinduction, Heterotopic bone, Demineralized bone matrix*

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Introduction

Nonsteroidal antiinflammatory drugs exert important effects on bone metabolism. Clinical and experimental observation has shown that bone formation in fracture healing or heterotopic bone induction is retarded or completely inhibited by Indomethacin [8–11]. Bone resorption in osteolysis by malignant tumours or by osteomyelitis can be inhibited experimentally by Indomethacin and Aspirin [3, 7]. In experiments where a continual stimulus to new bone formation is present, as in the healing of fractures or in heterotopic bone formation induced by demineralized bone matrix, nonsteroidal antiinflammatory drugs cause a small decrease in the production of new bone [6, 14]. These drugs almost completely inhibit heterotopic new bone formation after total hip replacement [4, 8, 9]. They do not affect the metabolism

of orthotopic bone under physiological conditions [12, 13, 14].

Utilizing the Urist model of inducing heterotopic bone formation in rats by implanting demineralized bone matrix in the abdominal wall, we have previously shown that Indomethacin reduces bone formation in a dose responding fashion when the drug is given at the time of implantation [14, 16]. When given some time after implantation the drug does not affect the amount of bone formed [6]. The drugs appear to effect the early phase of bone induction by their ability to reduce the inflammatory response, and thus cause less favourable circumstances for bone formation. The effect may also be due to action on a specific event essential to bone induction.

In this study we have compared the effects of Diclofenac and Indomethacin on orthotopic and induced heterotopic bone formation.

Material and methods

Animals. 50 male Sprague Dawley rats were used. They were four weeks old at the start of the experiment and had a mean body weight of 105 g (range 95–110). They were weighed weekly, and kept in cages with free access to food and water. At the end of the experiment the animals were killed by decapitation, and the serum, the implants, and the femora were collected.

Preparation of implants. The femora and tibiae were collected from the rats (200 g) and cleaned of soft tissues. The bones were then demineralized for 24 h at 4° C in 0.6 N HCl, and emptied of bone marrow. The metaphyseal parts were discarded, and the diaphyses were defatted with 1:1 chloroform-methanol at room temperature for one hour, washed in cold water, and freeze dried. The mean dry weight of the implants was 11 mg (range 9–13 mg).

Surgery. Six implants per rat were placed in muscle pouches created in the upper part of the abdominal wall. Anaesthesia was performed with Hypnorm Vet, 1.0 ml/kg body weight (Leo, Helsingborg, Sweden).

Drug treatment. Diclofenac (Voltaren, Ciba-Geigy, Västra Frolunda, Sweden) or Indomethacin (Confortid, Dumex AB, Helsingborg, Sweden) was dissolved in sterile water and the concentration adjusted so that each animal was given a daily subcutaneous injection of 1.0 ml per kg body weight. Control animals were given the same amount of NaCl (0.9 g/l) solution.

Experimental design. The rats were allocated into five groups of 10 animals. The groups were treated with Diclofenac 3, 6, or 12 mg per kg body weight, Indomethacin 3 mg per kg body weight, or NaCl. Administration was started five days before implantation of demineralized bone matrix and continued until the animals were killed 21 days after implantation.

Twenty four hours before death the animals were given a subcutaneous injection of 6 μCi carrier-free ^{45}Ca, and 40 μCi ^{3}H-L-proline (specific activity 100 Ci/mmol) per kg body weight (Amersham, England).

One femur and three implants from each animal were placed in a muffle furnace at 600° for 24 h. The ash was weighed and dissolved in 3 ml of HCl, and 10 ml Aquasol (New England Nuclear) was added to each sample. The remaining femur and implants were demineralized in 3 ml 0.6 N HCl at 4° for 24 h (the implants for 6 h), and 10 mls of Aquasol was added to the HCl supernatant. The organic residues were lyophilized, weighed, and hydrolyzed with 0.2 ml PCA and 0.4 ml H_2O_2 at 70° for 1 h [2]. Ten ml toluol/PPO and 5 ml of cellusolve was added to each sample.

All samples were counted in an Intertechnique 2000 liquid scintillation counter. Standard curves for the two isotopes were calculated. The mean values of the implants from each animal were used to determine the mean and the standard deviation of each group. All radioactive tracer activities were expressed as a percentage of the given dose (μCi/kg body weight) of the respective isotope.

Three implants from each group were prepared for histological examination. The implants were fixed in formalin, demineralized, embedded in paraffin and stained with haematoxylin, eosin, and azure.

For statistical evaluation the means of the different parameters were calculated for each animal and the groups were compared by the Wilcoxon rank sum test. The levels of significance are shown in Table 2.

Results

In all five groups a slight arrest in weight gain was noted 2–3 days after implantation of demineralized bone matrix. There was no difference in the weight gain between the different groups. The body weight at the end of the experiment ranged from 265 to 300 g. In the 12 mg/kg Diclofenac group six animals died within one week of initiation of treatment, and another two died the following week. The two remaining rats showed no sign of illness. In the other groups no sign of disease or deaths was encountered.

Neither Indomethacin nor Diclofenac affected the organic (dry weight) or inorganic (ash weight) components of the tibiae (Table 1). Similarly, the uptake of 45Ca or 3H-proline, given 24 h prior to death, was not affected.

Indomethacin and Diclofenac, given in daily doses of 3 mg/kg body weight, decreased the ash weight of the implants by approximately 15% (Table 2). The higher dose of 6 mg/kg of Diclofenac caused a more pronounced decrease in ash content. The dry weight of the implants is the sum of the implant that has not been resorbed and the net amount of bone matrix formed by induction. There was no significant difference between the treated groups and controls.

The absolute values of uptake of 45Ca in the implants remained largely unaffected by treatment. There was a tendency towards higher values of specific 45Ca activities in the treated groups compared to controls, although the differences were not statistically significant.

Table 1. Ash and dry weight, 45Ca and 3H specific and absolute activities of the tibiae of rats treated with saline, indomethacin or diclofenac for four weeks

	Control	Indometh. 3 mg/kg	Diclofenac 3 mg/kg	Diclofenac 6 mg/kg	Diclofenac 12 mg/kg
n	10	10	10	10	2
Ash (mg)	143 (10)	141 (9)	143 (7)	145 (13)	148
Dry weight (mg)	75 (4)	73 (7)	78 (5)	76 (5)	79
45Ca (% of dose)	0,332 (0,052)	0,372 (0,053)	0,349 (0,019)	0,342 (0,052)	0,435
45Ca specific (% of dose/g ash)	2,31 (0,34)	2,60 (0,29)	2,44 (0,17)	2,35 (0,32)	2,63
3H (% of dose × 1000/tibia)	20,3 (3,7)	21,1 (5,1)	21,5 (2,2)	22,8 (2,5)	25,5
3H specific (% of dose/g dry wt)	0,27 (0,05)	0,29 (0,05)	0,28 (0,03)	0,30 (0,04)	0,31

Values are means with the standard deviation in parenthesis. 45Ca and 3H-proline were given 24 h prior to death. Isotope activities are expressed as a percentage of the given dose (μCi/kg body weight) of the respective isotope per tibia (absolute activity), or per gram ash of dry weight (specific activity). No statistically significant values were obtained when the treatment groups were compared to controls, using the Wilcoxon rank sum test

Table 2. Ash and dry weight, 45Ca and 3H specific and absolute activities of implants three weeks after implantation in rats treated with saline, indomethacin or diclofenac

	Control	Indometh. 3 mg/kg	Diclofenac 3 mg/kg	Diclofenac 6 mg/kg	Diclofenac 12 mg/kg
n	10	10	10	10	2
Ash (mg/impl)	6,30 (1,26)	5,15* (1,02)	5,36 (1,3)	4,43** (0,94)	4,7
Dry weight (mg/impl)	11,92 (0,86)	11,10 (1,04)	11,12 (0,61)	11,52 (0,37)	9,3
45Ca (% dose × 1000/impl)	29,4 (8,19)	28,2 (12,2)	26,3 (6,8)	23,3 (8,1)	24,7
45Ca spec (% of dose/g ash × 1000)	4,69 (0,33)	5,65 (1,9)	4,96 (0,75)	5,22 (1,48)	5,14
3H (% dose × 1000/impl)	1,70 (0,28)	1,19** (0,27)	1,33* (0,32)	1,40 (0,37)	1,15
3H specific (% of dose/g dry wt × 1000)	0,14 (0,02)	0,12* (0,02)	0,12 (0,03)	0,12 (0,04)	0,12

* $P < 0.05$; ** $P < 0.01$

Values are means with the standard deviation in parenthesis. 45Ca and 3H-proline was given 24 h prior to death. Isotope activities are expressed as a percentage of the given dose (μCi/kg body weight) of the respective isotope per implant (absolute activity), or per gram ash of dry weight (specific activity). The different treatment groups were compared to controls using the Wilcoxon rank sum test

The absolute activity of 3H was lower in the 3 mg/kg Indomethacin and Diclofenac groups; in the former group the specific activity was also decreased. In the other treatment groups a similar, although not significant, tendency towards lower values of absolute and specific activities of 3H in the implants was noted.

Discussion

Indomethacin inhibits experimental and clinical heterotopic new bone formation, delays the healing of experimental fractures in the rat, slows the remodelling of diaphyseal bone after injury in the rabbit, and slows the remodelling of trabecular

bone [1, 4, 6, 8, 9, 11]. The mechanism through which these effects are mediated is not known, although the inhibition of prostaglandin synthesis by Indomethacin and other nonsteroidal antiinflammatory drugs indicates that the action may be mediated through an effect on the levels of prostaglandins in the target tissue. Similar effects on clinical and experimental heterotopic new bone formation have been seen with drugs such as Ibuprofen [4].

Prostaglandins act as local messengers and regulators of cellular activities, thus affecting essential events in bone induction and new bone formation. They also influence the inflammatory response. Their mode of action appears complicated, acting in a synergistic or antagonistic fashion depending upon their type and the concentration in the target tissue. Their inhibitory effect on fracture healing may be mediated through a nonspecific decrease in the inflammatory reaction, or by a more specific effect such as decreasing the proliferation of mesenchymal cells essential to new bone formation. Physiological bone turnover is not affected [12, 13, 14].

In this study the formation of matrix and the rate of mineralisation of the tibiae were not affected by the treatment when studied as the net amount of either compartment (dry and ash weights), or as a rate of synthesis of collagen (incorporation of 3H-proline), or accretion rate (45Ca incorporation). This is in agreement with previous findings of unaltered turnover of orthotopic bone during treatment with this group of drugs [6, 12, 14].

Heterotopic bone formation induced by implants of demineralized bone matrix provides a method to study bone induction, bone formation and turnover under well-defined experimental conditions [15]. The process of bone induction has been analysed in detail by Urist et al. [16]. The inducing agent, designated bone morphogenetic protein, is a non-collagenous protein of the bone matrix in many species. The demineralized bone matrix used in the present study contains no living cells. When transplanted to an allogeneic host, bone morphogenic protein is released and induces the mesenchymal cells of the transplant recipient to proliferate and differentiate, first into cartilage and then, by enchondral mineralisation, into bone [16]. The time required for this process varies, depending upon the species and the age of the implant recipient. In young rats, mineralized tissue forms within 10 days, and a complete ossicle with remodelling bone and marrow is formed within three weeks of implantation.

The net amount of heterotopic bone formed by induction was similarly decreased by treatment with Indomethacin and Diclofenac 3 mg/kg. The degree of inhibition increased with higher doses of Diclofenac, while higher doses of Indomethacin are toxic to the rat [14]. The incorporation of tritiated proline into implants was decreased in the Indomethacin and the Diclofenac (3 mg/kg) treated groups, indicating an inhibitory effect on matrix synthesis three weeks after implantation. The rate of mineralisation (45Ca uptake) was not affected by treatment with Indomethacin or Diclofenac [2].

The inhibition of induced heterotopic bone formation by nonsteroidal antiinflammatory drugs can be caused by different mechanisms. There may be a decrease in the nonspecific inflammatory response to the trauma of implantation, thus causing a less vascular and cellular reaction, which may result in a lower degree of bone induction. An altered balance between prostaglandins may influence the proliferation of mesenchymal cells, which are necessary for new bone formation [17]. However, the latter explanation is probably not correct, since Indomethacin does not affect the development of limb-bud cells in vitro [13].

Both drugs used in this study inhibit the transformation of arachidonic acid to prostaglandins by an irreversible, competitive inhibition of prostaglandin cyclo-oxygenase, and are characterized by antiinflammatory, analgesic and antipyretic effects [5]. However, they differ markedly in therapeutic index, Diclofenac not being as toxic in effects on the gastric mucosa and kidneys, and with less analgesic and antipyretic potency. We found heterotopic bone formation induced by demineralized bone matrix to be inhibited by both compounds to an equal extent, supporting the view that this effect is produced by their common effect on prostaglandin synthesis.

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