

# Lack of effect of indomethacin on mesenchymal limb-bud cells *in vitro*

Stimulated by reports of an inhibitory effect of non-steroid anti-inflammatory drugs on fracture healing and heterotopic bone formation in clinical and experimental studies, this investigation was performed to study the effect of indomethacin on chondrogenesis and mineralization *in vitro* by chicken mesenchymal limb-bud cells. Limb-bud cell cultures were treated with indomethacin and, in order to study a possible effect of metabolites, with serum from indomethacin-treated rats.

No effects of the drug on chondrogenesis, as estimated by staining with alcian blue, could be detected. This was also the case when mineralization in the cultures induced by high phosphate concentrations was studied. It is concluded that indomethacin probably exerts its effect on bone metabolism through mechanisms other than modulation of cell differentiation or cell proliferation.

In both *in vivo* and *in vitro* systems, prostaglandins are potent stimulators of bone resorption. Bone destruction occurring in pathological conditions such as osteomyelitis and malignant tumours is associated with high local concentrations of prostaglandins. Such bone destruction may be inhibited by sodium salicylate or indomethacin (Powles et al. 1973, Dekel & Francis 1981). Experimental studies have revealed delayed fracture healing under the influence of indomethacin, although ordered bone growth has not been disturbed (Allen et al. 1980, Rö et al. 1976, Sudmann & Bang 1979, Sudmann et al. 1982, Törnkvist & Lindholm 1980).

It may thus be concluded that non-steroid anti-inflammatory drugs affect both bone-forming and bone-resorbing elements, although the mechanisms are not known. We therefore found it interesting to analyse whether indomethacin and/or metabolites of this drug affect cell differentiation, proliferation and finally formation and mineralization of cartilage in limb-bud cell cultures (Wilk et al. 1980).

## Material and methods

The methods used to culture limb-bud mesenchymal cells were adapted from those described by Umansky

**Hans Törnkvist,  
Bengt R. G.  
Danielsson,  
Lennart Dencker &  
Olle S. Nilsson**

Department of Orthopaedic Surgery, K:54 Huddinge Hospital, S-141 86, Huddinge, Sweden; Department of Toxicology, Uppsala University, Uppsala, and Department of Orthopaedic Surgery, Karolinska Hospital, Stockholm, Sweden

(1966) and Wilk et al. (1980). Fertilized white Leghorn eggs (Swedish University of Agricultural Sciences) were incubated for about 92 h in a humidified atmosphere at 37.5°C. The embryos were removed and the prechondrogenic limb buds were pooled, dissociated for 11 min at 37°C in a saline solution (Ca- and Mg-free) containing 0.1% EDTA and immediately transferred to cold growth medium/MEM, containing 10 per cent fetal calf serum, 2.5 per cent serum, 20 mM Hepes buffer and gentamycin (5 µg/ml). The cells were dispersed and filtered through a double-layer 20 µm Nitex microfilter screen to remove tissue debris. The cell concentration was adjusted to  $2 \times 10^7$  cells per ml and single 20 µl drops ( $4 \times 10^5$  cells) were plated on 35 mm plastic tissue culture dishes (Flow Laboratories). These were then incubated at 30°C for 30 min, which time sufficed for the cells to attach to the bottom of the dishes. The cultures were thereafter flooded with 2 ml of the medium as above. They were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Twenty-four hours later (day 1), the medium was exchanged and on day 2 the indomethacin (A/S Dumex, Copenhagen, Denmark) was added to yield final concentrations of 0, 25, 50, 75 or 100 µg/ml medium. To other cultures was added serum from indomethacin or vehicle-treated rats (see below) at a concentration of 10, 15 or 20 per cent of the final medium. All treatment of the cultures was performed in duplicate.

The sulphated proteoglycans in the cartilage matrix of the cultures were stained on day 7 by alcian blue, as modified by Yamada (1970) and Barka &

Anderson (1965). After the medium had been removed, the cultures were fixed (10 per cent formalin plus 0.5 per cent cetylpyridinium chloride), rinsed twice in 3 per cent acetic acid (pH 1.0) and stained for 45 min with 0.5 per cent alcian blue in 3 per cent acetic acid (pH 1.0). They were then rinsed with water.

The influence of indomethacin on mineralization *in vitro* was also studied. Spot cultures were prepared as above, and on day 4  $\text{KH}_2\text{PO}_4$  was added to the medium ( $9 \times 10^{-3}\text{M}$  final concentration). This procedure causes the peripheral, non-cartilaginous areas to calcify (Binderman et al. 1979). At the same time, indomethacin in different concentrations was added. The final concentrations of the drug were 0, 25, 50, 75 or 100  $\mu\text{g/ml}$  medium. After a 48-h incubation period, the medium was removed and the dishes were rinsed four times with water, and stained by the von Kossa silver nitrate method (Osdoby & Caplan 1976).

Sera from indomethacin- or placebo-treated rats were prepared as follows: 12 adult (140 g) male Sprague-Dawley rats were divided into two groups each of six animals. One group was given indomethacin orally in a dose of 2 mg/kg body weight twice daily for a week. The control group received vehicle only (1 per cent carboxymethyl cellulose).

Three hours after the last dose, the animals were sacrificed by decapitation. Sera from the two groups were collected after centrifugation. Each group was pooled, and samples were taken for determination of indomethacin by liquid-gas chromatography at A/S Dumex (Copenhagen, Denmark) (Jensen 1978). Sera were then added to the spot cultures as described above.

## Results

Treatment of the cell cultures with increasing concentrations of indomethacin (25, 50, 75 and 100  $\mu\text{g/ml}$  medium) did not induce any difference in staining of the cultures with alcian blue as compared to untreated control cultures. When the drug of the same concentrations was added to cultures with high phosphate concentrations, causing a zone of calcification in the peripheral fibroblasts outside the cartilage center, no dose-related effects could be noted on this peripheral calcification. A slight increase in unspecific calcification closer to the center of the cultures was noted in all indomethacin-treated cultures compared to untreated controls.

Increasing concentrations of rat sera induced a decreased alcian blue staining of the cultures. This inhibitory effect was equally evident with sera from indomethacin- and placebo-treated animals. Analysis of indomethacin concentration in serum from the indomethacin-treated rats revealed therapeutic levels (2.50  $\mu\text{g/ml}$ ) (Alvan et al. 1975, Rø et al. 1976).

## Discussion

Using a model of inducing new bone (Urist 1965), we have found that indomethacin has a moderate inhibitory effect on heterotopic new bone formation, an effect that could well be explained by the modification of the inflammatory response caused by inhibition of the prostaglandin synthesis (Törnkvist et al. 1984). In the developing limb bud, differentiation of cartilage is a key event and may precede development of muscle and other specialized tissues (Urist 1983). Chondrogenesis is characterized by synthesis and secretion of highly sulphated cartilage proteoglycans, which are stained by alcian blue (Zwilling 1968). These proteoglycans are regarded as specific indicators of chondrocytes (Yamada 1970, Solursh et al. 1978, Urist 1983).

No toxic or inhibitory effects by indomethacin on chondrogenesis or mineralization in this limb cell culture could be detected even after treatment with high concentrations of the drug (up to 100  $\mu\text{g/ml}$  for 4 days). The concentration in plasma required for an anti-inflammatory effect is probably less than 1  $\mu\text{g/ml}$  (Alvan et al. 1975). To a large extent, indomethacin is known to be converted into pharmacologically inactive metabolites (Duggan et al. 1972). In this study, serum from indomethacin-treated rats showed a dose-dependent inhibition of chondrogenesis, but this effect was also found in cultures treated with serum from placebo-treated rats. Thus, this effect was considered to be caused by the serum as such. A high concentration of rat serum in this cell culture medium is known to have inhibitory effects on limb bud mesenchymal cell differentiation.

Wilk et al. (1980) tested acetyl acetic acid on this culture medium and recorded a 20 per cent

inhibition of chondrogenesis by high concentrations (250 µg/ml). As a comparison, retinoic acid is effective at a concentration of 0.1 µg/ml, retinol at 1 µg/ml, and different cytostatic drugs (Wilk et al. 1980) and chromium (Cr VI) at around 0.1 µg/ml (Danielsson et al. 1982).

In conclusion, the results of Wilk et al. (1980) and the present study provide no evidence for an inhibitory effect of indomethacin nor salicylates on the differentiation or proliferation of mesenchymal limb-bud cells or their formation of cartilage and mineralized tissue under *in vitro* conditions. The inhibitory effect of indomethacin on fracture healing and experimental heterotopic bone formation must thus be explained by other mechanisms.

## References

- Allen, H. L., Wase, A. & Bear, W. T. (1980) Indomethacin and aspirin: effect of non-steroid anti-inflammatory agents on the rate of fracture repair in the rat. *Acta Orthop. Scand.* **51**, 595–600.
- Alván, G., Orme, M., Bertilsson, L., Ekstrand, R. & Palmér, L. (1975) Pharmacokinetics of indomethacin. *Clin. Pharmacol. Ther.* **18**, 364–373.
- Barka, T. & Anderson, P. J. (1965) *Histochemistry: theory and practice*, pp. 77–79. Harper & Row, New York.
- Binderman, I., Greene, R. M. & Pennypacker, J. P. (1979) Calcification of differentiating skeletal mesenchyme *in vitro*. *Science* **206**, 222–225.
- Danielsson, B. R. G., Hassoun, E. & Dencker, L. (1982) Embryotoxicity of chromium: distribution in pregnant mice and effect on embryonic cells *in vitro*. *Arch. Toxicol.* **51**, 233–245.
- Dekel, S. & Francis, M. J. O. (1981) The treatment of osteomyelitis of the tibia with sodium salicylate. *J. Bone Joint Surg.* **63-B**, 178–184.
- Duggan, D. E., Hogans, A. F., Kwan, K. C. & McMahon, F. G. (1972) The metabolism of indomethacin in man. *J. Pharmacol. Exp. Ther.* **181**, 563–575.
- Jensen, K. M. (1978) Determination of indomethacin in serum by an extractive alkylation technique and gas-liquid chromatography. *J. Chromatogr.* **153**, 195–202.
- Osdoby, P. & Caplan, A. I. (1976) The possible differentiation of osteogenic elements *in vitro* from chick limb mesodermal cells. *Dev. Biol.* **52**, 283–299.
- Powles, T. J., Clark, S. A., Easty, D. M., Easty, G. C. & Neville, A. M. (1973) The inhibition by aspirin and indomethacin of osteolytic tumor deposits and hypercalcemia in rats with Walker tumor, and its possible application to human breast cancer. *Br. J. Cancer* **28**, 316–321.
- Rö, J., Sudmann, E. & Marton, P. F. (1976) Effect of indomethacin on fracture healing in rats. *Acta Orthop. Scand.* **47**, 588–599.
- Solursh, M., Ahrens, P. B. & Reiter, R. S. (1978) A tissue culture analysis of the steps in limb chondrogenesis. *In Vitro* **14**, 51–61.
- Sudmann, E. & Bang, G. (1979) Indomethacin-induced inhibition of haversian remodelling in rabbits. *Acta Orthop. Scand.* **50**, 621–627.
- Sudmann, E., Tveita, T. & Hald, J. Jr. (1982) Lack of effect of indomethacin on ordered growth of the femur in rats. *Acta Orthop. Scand.* **53**, 43–49.
- Törnkvist, H. & Lindholm, T. S. (1980) The effect of ibuprofen on mass and composition of fracture callus and bone. *Scand. J. Rheumatol.* **9**, 167–171.
- Törnkvist, H., Bauer, F. C. H. & Nilsson, O. S. (1984) Influence by indomethacin on experimental new bone formation in rats. *Clin. Orthop.* (submitted).
- Umansky, R. (1966) The effect of cell population density on the developmental fate or reaggregating mouse limb bud mesenchyme. *Dev. Biol.* **13**, 31–56.
- Urist, M. R. (1965) Bone formation by autoinduction. *Science* **150**, 893–899.
- Urist, M. R. (1983) The origin of cartilage: Investigations in quest of chondrogenic DNA. In: *Cartilage-development. Differentiation and growth*. (Ed. B. K. Hall), pp. 2–86. Academic Press, New York.
- Wilk, A. L., Greenberg, J. H., Horigan, E. A., Pratt, R. M. & Martin, G. R. (1980) Detection of teratogenic compounds using differentiating embryonic cells in culture. *In Vitro* **16**, 269–276.
- Yamada, K. (1970) Dual staining of some sulfated mucopolysaccharides with alcian blue (pH 1.0) and ruthenium red (pH 2.5). *Histochemie* **23**, 13–20.
- Zwilling, E. (1968) Morphogenetic phase in development. *Dev. Biol. Suppl.* **2**, 184–207.