The effects of ethylene-1-hydroxy-1, 1-diphosphonate on the developing mandibular condyle – a light microscopic study

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The effects of high doses of ethylene-1-hydroxy-1, 1-diphosphonate (EHDP), a potential mineralization inhibitor, were studied in the developing mandibular condyle of the rat. The animals were given one injection of EHDP/day for four consecutive days. One group of animals received 30 mg and another group 50 mg EHDP/day. Animals from each group were killed either the day after or three days after the last EHDP-injection. EHDP-administration resulted in a failure of mineralization of the cartilage and osteoid, a widening of the hypertrophic zone, an appearance of cells in lacunae at the cartilage-metaphyseal junction and in an inhibited capillary invasion. There was little evidence of a resumed mineralization in rats left to survive for three days after the last EHDP-injection. The results indicate additional effects of EHDP besides an inhibition of calcium phosphate crystallization, and also that EHDP may be a useful tool in the study of mechanisms of cellular hypertrophy and capillary invasion in mineralizing cartilage.

Key-words: Mineralization; cartilage; histology

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In the search for an agent useful in treating the adult osteoporotic skeleton or pathologic resorption in man, the properties of synthetic diphosphonates structurally related to inorganic pyrophosphate, have been rather extensively studied in the last few years. One of these compounds, ethylene-1-hydroxy-1, 1-diphosphonate (EHDP) interferes *in vivo* with bone formation, by inhibiting mineralization of the osteoid (*King et al.*, 1971; *Russell et al.*, 1973; *Schenk et al.*, 1973). As a consequence of the accumulation of unmineralized osteoid at the hard tissue surfaces resorption is also inhibited by EHDP (*Miller & Jee*, 1975).

Most of the above cited observations have been made on the epiphyseal growth plate, which is the site of both mineralization and

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resorption processes. To the present author's knowledge, there are so far no reports in the literature about the effects of EHDP or any other diphosphonate on the developing mandibular condyle. It has recently been shown in an electron microscopic study, that the mineralization and initial erosion of the condylar cartilage are essentially similar to those of the growth plate (*Larsson*, 1976). There is, however, in the condyle no columnar arrangement of the hypertrophying chondrocytes. The possibility exists that the difference in cell organization may reflect inherent differences in cellular activities in the two cartilages (cf. *Durkin et al.*, 1973).

The purpose of the present study was to examine the morphologic changes in the developing condyle of rats, using EHDP administered at high doses over a short period of time as a means of disturbing the cellular activities. It was hoped to gain further insight into the various effects of EHDP as well as into the cell activities of the condylar cartilage by comparing the results with previously reported short-term effects of EHDP upon the growth plate (*Schenk et al.*, 1973; *Miller & Jee*, 1975; *Larsson & Larsson*, 1976).

MATERIALS AND METHODS

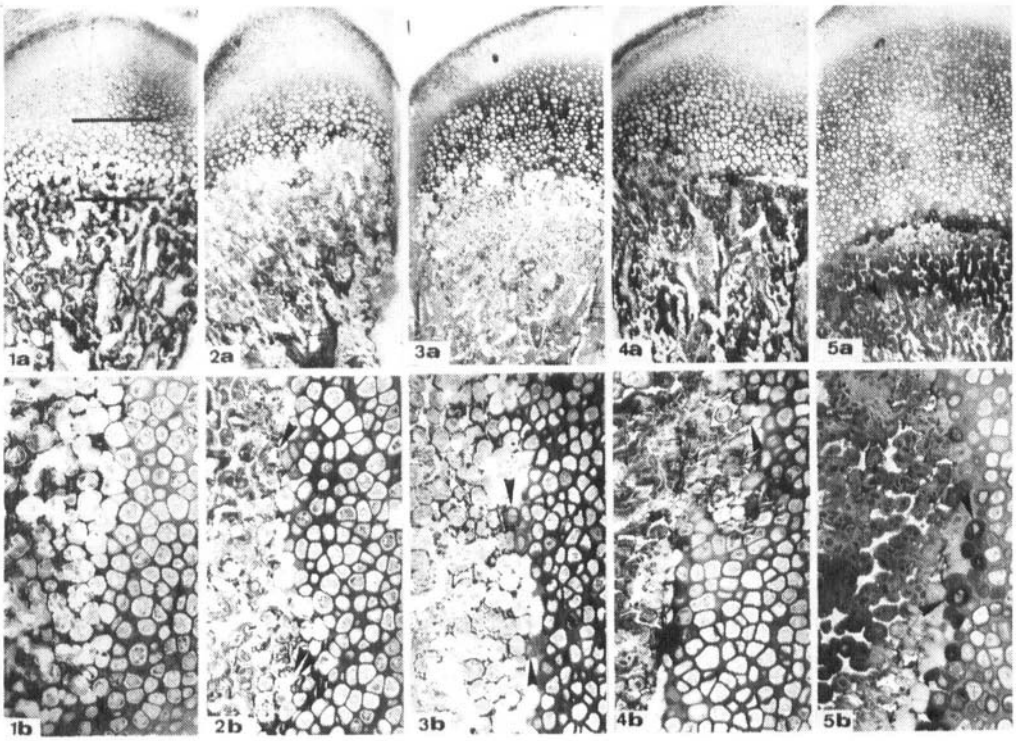
Twenty-day-old rats, 40–45 g weight, were used in the present study. Ethylene-l-hydroxy-l, l-diphosphonate (EHDP) was a gift from Henkel & Cie GmbH, Düsseldorf, West Germany. It was dissolved in 0.9% Na Cl, at a concentration of 6.75 or 12.5 mg/cc. The rats were given a total of 4 ip injections of EHDP, during 4 consecutive days. Six groups of animals, 3 per group, from two litters were used. Two groups from one litter received 30 mg EHDP/kg body weight/day given in one injection and two groups from the other litter 50 mg EHDP/kg body weight/day given in two injections/day. The third group of animals from each litter served as the controls and received no injections. The EHDP-treated rats were sacrificed at two different times. In the morning following the last injection, one of each of the groups, which received 30 and 50 mg EHDP respectively, as well as control animals were anesthetized with sodium pentobarbital (Mebumal), and fixed by perfusion followed by processing of the developing mandibular condyle for electron microscopy as described previously (*Larsson*, 1976). The remaining two EHDP-treated groups (30 and 50 mg EHDP respectively) and controls were sacrificed 3 days later and the developing condyles were processed according to the same technique.

Thick sections (1 u) were stained with toluidine blue for light microscopy. The ultrastructural findings will be described in a separate communication.

OBSERVATIONS

The histologic appearance of the developing mandibular condyle (Fig. 1a,b) has been recorded in detail in a previous communication (*Larsson*, 1976) and will not be further described here. The features to be described below in the various EHDP-treated groups all represent aberrations from the histologic appearance of the controls.

In rats receiving 30 mg EHDP/kg/day and killed the day following the last injection, there was an irregular degree of mineralization of the cartilage septa and the erosin-line was smoothed out (Fig. 2a). In isolated areas, bars of mineralized septa were clearly identified but they extended only up to a level of one lacuna of the growth cartilage (normally 2–3). Capillaries were clearly invading these areas. However, in some areas mineralized septa were absent and the capillaries had apparently halted at the cartilage front, with no clear evidence of invasion (Fig. 2b). Moreover, in areas lacking capillary invasion and mineralized septa, cell remnants or apparently intact cells were frequently seen in the hypertrophic chondrocytic lacunae adjacent



All figures are light micrographs. The magnification of figures labelled «a» is 80 x and of figures labelled «b» 145 x. The figures labelled «a» illustrate the medio-lateral plane of the developing condylar head, with the condylar cartilage at the top of the figures. The figures labelled «b» are high magnification micrographs that in each respective figure corresponds to an area equivalent to that indicated in figure 1a. Figures labelled «b» have been turned clockwise so that the condylar cartilage appears to the right side of the figure.

Fig. 1a and b. From control animal. Calcification and initial erosion of the cartilage takes place within the zone between the two horizontal lines indicated in fig. 1a. This zone is seen at higher magnification in fig. 1b, with hypertrophic chondrocytes to the right side and calcified trabeculae to the left side of the picture.

Fig. 2a and b. From animal given 4x30 mg EHDP/kg and sacrificed the day after the last injection. The erosion line is smoothed out (fig. 2a) and higher magnification (fig. 2b) reveals halted capillaries at the edge of unmineralized cartilage (single arrow fig. 2b). Calcified septa may be seen in adjacent areas, with invading capillaries (double arrow fig. 2b). Note cell remnants in lacunae of unmineralized cartilage.

Fig. 3a and b. From animal given 4x30 mg EHDP/kg and killed 3 days after last injection. The hypertrophic zone is slightly wider and the hypertrophic chondrocytes smaller than in controls. There is no mineralization of cartilage septa and no invasion of cartilage by capillaries (fig. 3b). Cellular elements are seen in lacunae adjacent to the metaphysis (arrows fig. 3b). Note thin calcified trabeculae in metaphysis, covered by osteoid.

Fig. 4a and b. From animal given 4x50 mg EHDP/kg and killed the day after last injection. The hypertrophic zone is widened and the erosion line is uneven due to masses of unmineralized cartilage protruding down into the metaphysis. Intervening areas show evidence of mineralization of cartilage septa (arrow fig. 4b). No capillary invasion is seen in areas of unmineralized cartilage while capillaries are invading the calcified parts.

Fig. 5a and b. From animal given 4x50 mg EHDP/kg and killed three days after last injection. The hypertrophic zone is markedly widened and the size of the lacunae much smaller than in controls. There is a lack of mineralization of the cartilage septa and large amounts of osteoid appear in the metaphysis (fig. 5b). Some of the lacunae in the cartilage contain cellular elements. The prominance of the lacunae is due to a metachromatic stain reaction (arrows fig. 5b), which is also appearent in the metaphysis, around embedded cells. Note capillary ends far beyond the cartilage (arrow fig. 5a).

to the metaphysis (Fig. 2b). Occasionally, islands of cartilage appeared within the metaphysis, beyond and unrelated to the growth cartilage. The islands of cartilage included chondrocytic lacunae and also some degree of mineralization of the septa.

More advanced changes had developed in the growth cartilage in the 30 mg EHDP rats following a prolonged (3 days) survival time. The hypertrophic zone was wider and the hypertrophic chondrocytes were smaller (Fig. 3a). Mineralized septa were lacking except in isolated parts of the periphery of the growth cartilage, adjacent to the periosteal tissues. Thin mineralized bars were seen in the metaphysis and they were covered by a thick layer of osteoid (Fig. 3b). No capillary invasion was seen in the cartilage (Fig. 3b). Some of the lacunae at the epiphyseal-metaphyseal junction contained cell-like elements, occasionally two in the same lacuna (Fig. 3b).

In rats receiving 50 mg EHDP/kg/day and killed the day after the last injection, step-like delineations were frequently seen at the epiphyseal-metaphyseal junction (Fig. 4a). Unmineralized growth cartilage protruded into the metaphysis and no capillaries invaded this cartilage (Fig. 4b). The intervening parts of the growth cartilage exhibited various degrees of mineralization of the septa and capillaries were apparently invading such areas where mineralized septa were present (Fig. 4b). Generally, however, there was a halted invasion of capillaries opposite areas of non-mineralized septa, where quite well-preserved chondrocytes were often seen in the lacunae (Fig. 4b). Occasionally, a dilated capillary was found to penetrate deep into non-mineralized cartilage. There was an increased amount of osteoid at the surface of the bony bars in the metaphysis (Fig. 4b).

A considerable increase in the widths of growth cartilage was observed in the 50 mg EHDP rats, left to survive for 3 days after the last injection (Fig. 5a). The size of the lacunae was reduced and there was no mineralization of the septa (Fig. 5b). At the epiphyseal-metaphyseal junction, an increased metachromasia was evident in a number of lacunae. These lacunae contained cell elements, often appearing as pairs of cells (Fig. 5b). In many cases, a suggested transition was seen from not completely degenerated chondrocytes to well-preserved cells in the lacunae (Fig. 5b). Large amounts of blue-stained osteoid and metachromatically stained cartilage matrix-like material was present in the metaphysis (Fig. 5b). Numerous cells were apparently embedded in the latter material, with little or no evidence of mineralization. Capillary ends were seen far beyond the level presumed to represent the epiphyseal-metaphyseal junction (Fig. 5a).

DISCUSSION

The toluidine-blue dye used in the present study does not specifically reveal mineralized areas of the tissue. In a previous communication (*Larsson*, 1976), the localized dark-blue staining observed at the mineralization zone of the developing mandibular condyle was found to correspond well with sites of mineral deposits simultaneously demonstrated at the ultrastructural level. Electron microscopic examination of the sections is necessary, however, in order to reveal the initial, minute deposits of apatite crystals. Nevertheless light microscopy can readily by used to demonstrate gross changes.

The present results indicate, that short-term treatment with high doses of EHDP (30 and 50 mg/kg/day) leads to an irregularly prevented mineralization of the cartilage in the developing mandibular condyle. A complete failure of mineralization of the cartilage is evident in animals left to survive for three days after four injections of 30 or 50 mg EHDP/kg. This «rachitogenic» effect of EHDP is in good agreement with the concept that EHDP acts by preventing apatite crystals

from forming at sites of mineralization (*Russel & Fleisch*, 1975). The fact that mineralization was not resumed in the cartilage three days after cessation of treatment, is in agreement with previous suggestions that high doses of EHDP given for a few days will inhibit cartilage mineralization for weeks (*Schenk et al.* unpublished, cited by *Russell & Fleisch*, 1975). However, at the ultrastructural level, signs of resumed mineralization were observed by *Larsson & Larsson* (1976) in the metaphyseal region of the growth plate 32 hours after the last injection of EHDP. It is thus possible that mineral deposition may occur shortly after EHDP-administration but only at sites of newly formed matrix, close to the vascular tree.

Another point of interest in the present study is the lack of erosion of cartilage in the EHDP-treated animals, with an absence of capillary penetration. Halted capillaries at the unmineralized cartilage septa have also been observed in the growth plate of rats receiving 2 x 15 mg EHDP/kg for 24 hours (*Schenk et al.*, 1973). From their studies on rachitic and EHDP-treated chicks, *Bisaz et al.* (1975) concluded that vascular invasion of the epiphyseal growth plate is independent of prior mineralization. From the present results as well as from those of *Schenk et al.* (1973), it may be concluded, that in spite of no mineralization of the cartilage septa, capillaries do not penetrate into the cartilage from the metaphysis in EHDP-treated animals. Normally, the capillary sprouts penetrate the unmineralized horizontal septa of the growth plate (*Schenk et al.*, 1968) and gaps in partly mineralized septa in the corresponding zone of the condyle (*Larsson*, 1976). The lack of capillary invasion in the EHDP-treated rats may thus be the result of a changed composition of the cartilage matrix, due to the EHDP-treatment, somehow also associated with a lack of primary disintegration of the matrix in front of the capillary sprouts. It may also be related to some unknown effect by EHDP upon the capillary endings. These findings deserve further investigation. The fact that vascular invasion has been found to extend up through the greatly widened hypertrophic zone of the growth plate of EHDP-treated chicks (*Bisaz et al.*, 1975), would in the first place seem to eliminate the idea of an effect by EHDP upon the matrix and/or the capillaries. However, the vascular penetration of the growth plate in chicks is quite different from that in rat growth plate and condylar cartilage (*Bisaz et al.*, 1975). Furthermore the vascular channels, protruding into the hypertrophic zone of the chick growth cartilage, have not been studied in detail at the ultrastructural level. Thus, the activities at the tips of the channels, 4–5 cells above the level of the calcification zone, are not known. It may well be that there are inherent differences between the erosive pattern of the chick growth plate and that of the rat growth plate and condyle, which may be of significance in terms of the effects of administered EHDP.

The next point emerging from the present study is the increased width of the hypertrophic zone. Obviously, the degree of widening of this zone is dose-dependant and more osteoid is also produced in the metaphysis at the higher doses (50 mg/kg) than at the lower doses (30 mg/kg). A widened hypertrophic zone is also seen in the condyle and growth plate of rachitic rats (*Durkin et al.*, 1971) and in the growth plate of EHDP-treated rats and chicks (*Schenk et al.*, 1973, *Bisaz et al.*, 1975, *Miller & Jee*, 1975, *Larsson & Larsson*, 1976). This widening is a result of an increased number of hypertrophic cells similar to what to be seen in the present study. The question arises, is the widening of the hypertrophic zone in rachitic animals the result of the same basic disturbance as that caused by EHDP, or are different mechanisms involved?

In rats, but not in chicks, the vitamin-D deficient diet must also be low in phosphate in order to produce rickets. In the rachitic chick growth plate, there is a widened proliferative zone, contrary to the widened hypertrophic zone seen in the rachitic rat, thus suggesting some significance of the low phosphate intake in the rat (*Bisaz et al.*, 1975). Since the effects

of EHDP are similar in the chick and rat growth plates, i.e. a widened hypertrophic zone, *Bisaz et al.* (1975) consequently suggested that somehow the EHDP-effects might be related to a disturbed phosphate metabolism. They discussed the significance of an EHDP-suppressed synthesis of 1.25-DHCC, associated with a low plasma phosphate. In a recent review, *Russell & Fleisch* (1975) have forwarded a somewhat modified opinion, namely that EHDP inhibits mineralization directly and that the suppressed synthesis of 1.25-DHCC is secondary to the mineralization defect, to prevent from rises in plasma calcium. It is not clear how such a proposed mechanism would affect the cellular proliferation in the growth plate and also in the condylar cartilage. Evidently, this is an area which requires further research.

Finally, the appearance of intact cells at the presumptive calcification zone of the EHDP-treated rats is an interesting and intriguing observation. Assuming that the primary effect of EHDP is the prevention of calcium phosphate crystallization (*Russell & Fleisch*, 1975), the reason why chondrocytes apparently persist and why intact cells appear in the condylar cartilage at the cartilage-metaphyseal junction of the EHDP-treated rats can still not readily be explained solely as a lack of mineralization. It has long been agreed that the normal disintegration and final death of the chondrocytes is not the direct result of the mineralization of the cartilage septa. However, it has become a matter of some dispute, whether the hypertrophic cells of the growth cartilages actually die. Based on ultrastructural and cell cultivation studies, *Holtrop* (1972), *Silbermann & Frommer* (1974) and *Shimomura et al.* (1975) have expressed the opinion that a number of these cells may ultimately become bone cells, producing bone in the metaphysis. It is possible that the whole problem is a matter of adequate fixation (*Holtrop*, 1972). Judging from the present results, the administration of EHDP will not only result in a widened hypertrophic zone but also in the appearance of a number of cells in the lacunae at the cartilage metaphyseal junction, which are not normally seen here. Based on the cited studies, these cells may represent reactivated chondrocytes. The reason why they are so numerous in the EHDP-treated rats may be due to the fact that EHDP-administration will not only cause a widening of the hypertrophic zone but also of a «reactivating zone», which is normally insignificant. Since the cells are readily demonstrable by conventional fixation techniques, it is also possible that the EHDP-treated tissues are more accessible to the fixatives than normal tissues.

To summarize, the present study of the condylar cartilage has shown that the administration of high doses of EHDP will result in an inhibited mineralization of cartilage and osteoid, a widening of the hypertrophic zone, an appearance of cells in lacunae at the presumptive mineralization zone and also in an inhibited capillary invasion. These results can not merely be explained by a lack of crystal formation in the tissues. They also point at some additional effect by EHDP directly upon the cartilage matrix, resulting not only in a reduced disintegration of the matrix, but also in changed staining qualities at the cartilage-metaphyseal junction. Furthermore, no major differences seem to exist between the condylar cartilage and the growth plate with respect to the EHDP-effects. The present results suggest that our understanding of some of the cellular activities in mineralizing cartilages, i.e. the mechanism of cellular hypertrophy and capillary invasion, may very well be increased by further and especially by ultrastructural studies of the EHDP-effects on these tissues. Such studies are in progress.

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