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Regulation of growth region cartilage proliferation and differentiation by perichondrium

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SUMMARY

Endochondral bone formation in vertebrates requires precise coordination between proliferation and differentiation of the participating chondrocytes. We examined the role of perichondrium in this process using an organ culture system of chicken embryonic tibiotarsi. A monoclonal antibody against chicken collagen type X, specifically expressed by hypertrophic chondrocytes, was utilized to monitor the terminal differentiation of chondrocytes. Proliferation of chondrocytes was examined by a BrdU-labeling procedure. The absence of perichondrium is correlated with an extended zone of cartilage expressing collagen type X, suggesting that the perichondrium regulates chondrocyte hypertrophy in a negative manner. Removal of perichondrium, in addition, resulted in an extended zone of chondrocytes incorporating BrdU, indicating that the perichondrium also negatively regulates the proliferation of chondrocytes. Partial removal of perichondrium from one side of the tibiotarsus led to expansion of both the collagen type X-positive domain and the BrdU-positive zone at the site of removal but not where the perichondrium remained intact. This suggests that both types of regulation by the perichondrium are local effects. Furthermore, addition of bovine parathyroid hormone (PTH) to perichondrium-free cultures reversed the expansion of the collagen type X-positive domain but not that of the proliferative zone. This suggests that the regulation of differentiation is dependent upon the PTH/PTHrP receptor and that the regulation of proliferation is likely independent of it. Taken together, these results are consistent with a model where perichondrium regulates both the exit of chondrocytes from the cell cycle, and their subsequent differentiation.

Key words: Growth, Cartilage, Perichondrium, Chick, Collagen X, Parathyroid hormone

INTRODUCTION

Endochondral bone formation during vertebrate embryogenesis is a highly regulated process resulting, in the case of long bones, in increased length. During this process, young chondrocytes initially undergo rapid proliferation; then cease proliferation to become mature chondrocytes, producing a large amount of extracellular matrix, and subsequently become hypertrophic. At the stage of hypertrophy, the cells exhibit a number of changes (Nurminskaya and Linsenmayer, 1996), including de novo synthesis of collagen type X (Schmid and Conrad, 1982; Schmid and Linsenmayer, 1983; Capasso et al., 1984; Gibson et al., 1984). These events change the composition and, conceivably, the properties of the cartilage matrix in the hypertrophic zone (Chen et al., 1992), allowing the invasion of blood vessels and the ultimate replacement of the cartilage matrix by bone and a marrow cavity. Thus, the proper control of chondrocyte development, i.e., appropriate regulation of cell proliferation and the subsequent differentiation to hypertrophy, is of critical importance to the formation of a normal bone.

Recent studies have begun to reveal the molecular mechanisms involved in controlling cartilage growth and differentiation. Mice null for either parathyroid hormone related peptide (PTHrP) (Karaplis et al., 1994) or its receptor (Lanske et al., 1996), and those expressing a dominant-negative form of TGF-β receptor (Serra et al., 1997), displayed accelerated differentiation of chondrocytes and therefore abnormal endochondral bone formation. Conversely, overexpression of the signaling molecule Indian hedgehog (Ihh) suppressed chondrocyte differentiation (Vortkamp et al., 1996). Based on these observations, Vortkamp et al. (1996) proposed that PTHrP and Ihh constitute a signaling pathway negatively regulating the rate of chondrocyte differentiation.

Vortkamp and coworkers (1996) also reported that the receptor of Ihh, Patched (Ptc) (Marigo et al., 1996; Stone et al., 1996), is expressed in the perichondrium flanking the Ihh-expressing domain of the cartilage, but not in the cartilage itself. This finding suggests that Ihh acts via the perichondrium. In other words, the perichondrium may regulate the differentiation of chondrocytes. In addition, Koyama et al. (1996) reported that changes in the expression pattern of tenascin-C and syndecan-3 in the perichondrium are correlated with cartilage development.

Less is known about the mechanisms controlling the proliferation of chondrocytes. Although growth factors such as

IGF (Baker et al., 1993) and FGF (Rousseau et al., 1994; Shiang et al., 1994; Deng et al., 1996) have been suggested to have an effect on this process, it is unknown how these factors are coordinated *in vivo*.

In this study, we have experimentally examined the roles of perichondrium during cartilage growth and differentiation in an organ culture system. Tibiotarsi in which the perichondrium was removed exhibited increased overall growth, when compared to their counterparts in which the perichondrium remained intact. Consistent with this observation, an extended zone of proliferation was observed in the perichondrium-free samples. Also in these samples, expression of collagen type X appeared to be accelerated. These results indicate that the perichondrium may regulate both proliferation and differentiation of the growth-plate cartilage and that, in each case, the regulation is negative. Furthermore, the two types of regulation may be mediated by different pathways.

MATERIALS AND METHODS

Organ culture

Tibiotarsi were isolated from day 12 chicken embryos. Some were used intact and others had portions of the perichondrium surgically removed, as depicted in the diagrams in Results. All samples were cultured on organ culture grids (Wiremesh Co.) in serum-free medium DMEM (Life Technologies, Inc.) in the presence of penicillin and streptomycin (Life Technologies, Inc.). Cultures were maintained for up to 8 days in a 37°C incubator with 7% CO_2. For the parathyroid hormone experiments, bovine parathyroid hormone was added to the serum-free medium at a concentration of 10^{-7} M (Sigma). The choice of concentration was based on Iwamoto et al. (1994). In all experiments, the culture medium was changed daily throughout the course of culture. For analyses of growth, the contour length of either the whole organ, or its cartilaginous and bony portions after the staining (see below), was measured with a thread and a ruler under a dissecting microscope.

Whole-mount staining of bone and cartilage

Whole mounts of cultured tibiotarsi were stained with Alizarin Red (for bone) and Alcian Blue (for cartilage) according to McLeod (1980) with slight modifications. Briefly, cultured organs were rinsed in 1× PBS and fixed in 95% ethanol for 2 hours at room temperature. They were then stained at 37°C for 2-3 hours.

BrdU labeling

BrdU labeling was carried out using the 'BrdU labeling and detection kit I' from Boehringer Mannheim. Intact cartilage was dissected out from the ends of tibiotarsi that had been cultured for certain time (e.g. 2 days, see Results). To ensure penetration of the BrdU, any remaining perichondrium and bony tissue were removed. The cartilage was then reincubated (at 37°C with 7% CO_2) in serum-free medium containing 10 μM BrdU. Preliminary experiments indicated that 2.5 hours of incubation with BrdU produces an adequate signal for the proliferating cells. This incubation time was therefore used for all labeling experiments. Labeled samples were subsequently incubated in 1× PBS at 37ºC for 10-15 minutes, fixed with 1× HistoChoice (Amresco) at room temperature for 1 hour, rinsed in 1× PBS for 5 minutes with 2-3 washes and then incubated in 8% sucrose in 1× PBS for 10 minutes at room temperature. The fixed samples were then embedded and sectioned (see below).

Immunohistochemistry

Tibiotarsi for collagen type X staining were fixed with either 1× HistoChoice (see above) or 4% paraformaldehyde (Baker). Paraformaldehyde fixation was performed as previously described (Schmid and Linsenmayer, 1985). Fixed samples were subsequently embedded in OCT (Triangle Biomedical Sciences) and quick frozen over liquid N_2. Cryostat sections of 6-8 μm were cut longitudinally.

The staining procedure for type X collagen was as previously described and used a well-characterized monoclonal antibody against chicken type X collagen (Schmid and Linsenmayer, 1985). Sections from the paraformaldehyde-fixed samples were treated with 5 mg/ml type I hyaluronidase (Sigma Immunochemicals) prior to immunostaining to achieve better extracellular staining for type X collagen (Schmid and Linsenmayer, 1985). The monoclonal antibody against BrdU was included in the 'BrdU labeling and detection kit I' and was used according to the manufacturer's protocol, except that a rhodamine-conjugated secondary antibody (Pierce) was substituted for the fluorescein-conjugated antibody. Slides were examined and photographed under a fluorescence microscope (Nikon). All immunostaining results reported here are representative of three or more separate experiments.

For histological staining, cryostat sections were washed in 1× PBS for 5 minutes and then stained with eosin and hematoxylin.

RESULTS

Gross morphology of perichondrium-free cultures

To begin to examine the role(s) of the perichondrium during cartilage development, we dissected off the perichondrium along the entire length of tibiotarsi (Fig. 1B) before culturing them along with their normal counterparts with intact perichondrium. The perichondrium over the articular surface, which is tightly adherent to the cartilage, remained intact after the manipulation.

The perichondrium-free tibiotarsi exhibited considerably more growth than their counterparts with an intact perichondrium. This can be seen in the tibiotarsi cultured for 8 days shown in Fig. 1A, in which bone is stained red and cartilage blue. Quantitative analyses determined that most, if not all, of the increased growth in length can be accounted for by that in cartilage. After 8 days in culture, the cartilaginous ends of the perichondrium-free sample had grown in length approximately 50% more than their normal counterparts (Fig. 2). The bony portion in both perichondrium-free and normal samples, however, had the same length (Fig. 1A and data not shown). In addition to their increased length, the perichondrium-free cultures also showed a considerable increase in the width of the cartilage (see Fig. 1A).

Measurements of the kinetics of growth in three pairs of tibiotarsi (each pair was from the same chicken embryo) indicated that the perichondrium-free samples exhibited continuous growth in length throughout the course of culture (Fig. 3). The intact tibiotarsi in culture, on the other hand, showed some growth (at a lower rate) during the first 3 days, but then ceased growth.

Proliferation and differentiation of chondrocytes in perichondrium-free cultures

To understand the cellular basis for the increased growth in perichondrium-free samples, we examined the proliferative zone of these versus their counterparts with an intact perichondrium. For this, intact cartilage was dissected out from either the distal (tarsus) or the proximal end of the cultured tibiotarsi, labeled with BrdU and then sectioned longitudinally

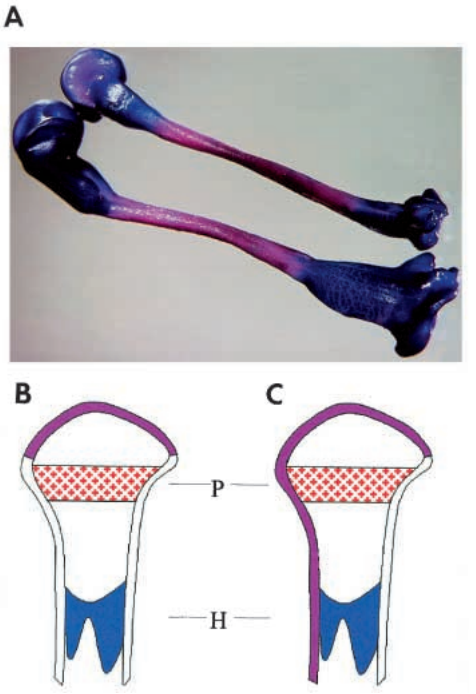

Fig. 1. (A) Morphology of tibiotarsi in culture for 8 days. Top: sample with intact perichondrium; bottom: sample without the perichondrium. Bone is stained red and cartilage blue. The distal (tarsus) ends are at the upper left corner whereas the proximal ends are at the lower right corner of the picture. Note the increased growth in both length and width at both cartilage ends of the perichondrium-free sample. This morphology is typical of approximately 2 dozen pairs of tibiotarsi. (B,C) A schematic presentation of two types of surgical procedures. The diagrams represent a longitudinal section of the proximal half of a tibiotarsus. The proliferative (P) zone and the hypertrophic (H) zone are indicated as in a normal developing tibiotarsus. The removed perichondrium is uncolored whereas the perichondrium remaining after the surgery is depicted in purple. (B) Complete removal of the perichondrium along the length of the tibiotarsus. This procedure resulted in the morphology shown in A. (C) Removal of the perichondrium along one side of the tibiotarsus. This procedure was used for studying local effects of the perichondrium (see text).

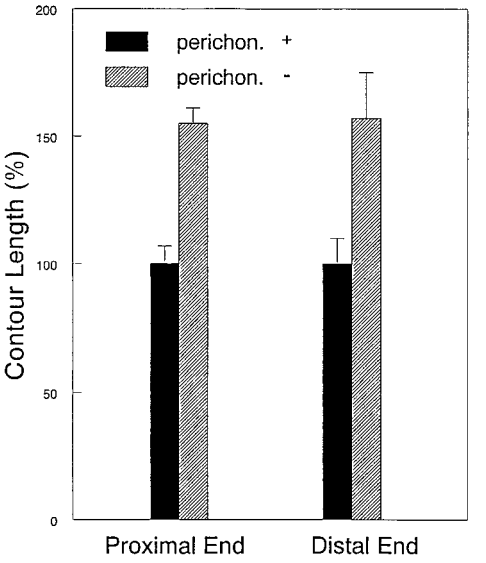

Fig. 2. Differential growth in the length of cartilage in 8-day cultures. Perichon. –, without perichondrium; perichon. +, with perichondrium. The contour length of both cartilaginous ends, i.e., the distal (tarsus) and the proximal ends, was measured separately. The measurements from tibiotarsi with the perichondrium are termed 100 %. Results are presented as mean ± s.d. *n*=6.

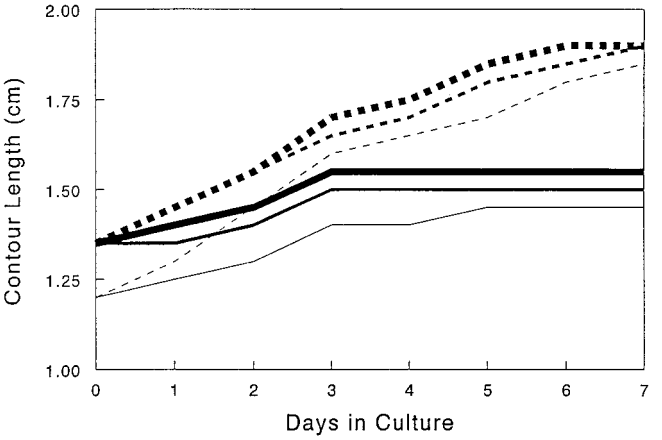

Fig. 3. Growth curves of tibiotarsi cultures. Lines of the same thickness represent samples from the same chicken. Dashed lines, samples without perichondrium; solid lines, samples with perichondrium. The contour length of each sample from the proximal to the distal end was measured daily during the course of culture.

(see Materials and Methods). A monoclonal antibody against BrdU was used to detect proliferating cells and Hoechst staining was used to visualize the nuclei throughout the whole section. As shown in Fig. 4, for the tarsus-end cartilage of 2-day cultures, the perichondrium-free sample (Fig. 4B) exhibited a widened proliferative zone ('P'), when compared to its normal counterpart (Fig. 4A). A similar result was obtained with the proximal-end cartilage (data not shown). This difference in the proliferative zone between perichondrium-free and normal tibiotarsi continued to be seen in samples cultured for 3, 4 and 5 days (data not shown). By day 8, however, the proliferative zone in both conditions (with or without perichondrium) diminished to a non-detectable level (data not shown).

We next examined the distribution of collagen type X by immunohistochemistry. Sections from either intact or perichondrium-free tibiotarsi were reacted with a monoclonal antibody against type X collagen (Schmid and Linsenmayer, 1985). As shown for 8-day cultures in Fig. 5, the type X-positive region in the perichondrium-free sample (B) is longer than that in its normal counterpart (A). Moreover, the type X collagen in this elongated region is predominantly intracellular (see region *i* in B). This intracellular localization can be clearly seen at a higher magnification (insert, Fig. 5B). Thus, in the absence of perichondrium, additional cells acquire the ability to synthesize type X collagen, but these cells fail to secrete appreciable amounts into the extracellular matrix (see Discussion). Similar results for intracellular localization were obtained using two different fixatives, either HistoChoice or paraformaldehyde. The paraformaldehyde-fixed samples were

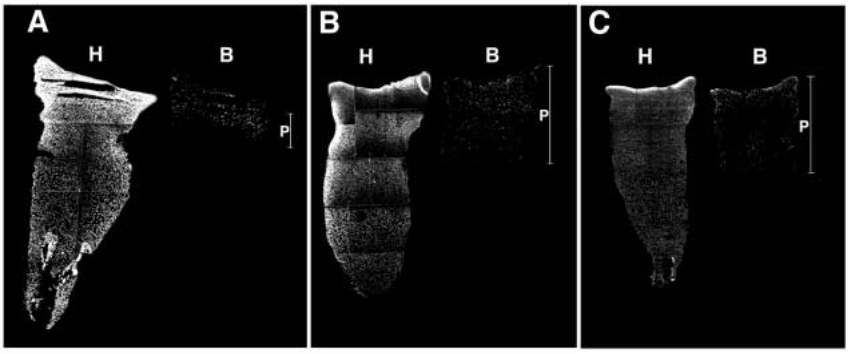

Fig. 4. BrdU labeling of the tarsus-end cartilage of tibiotarsi cultured for 2 days either with perichondrium (A) or without perichondrium (B) or without perichondrium but with PTH added in the medium (C). In each panel, B, BrdU labeling; H, Hoechst staining; P, the proliferative zone.

treated with hyaluronidase prior to immunostaining to achieve better extracellular staining for type X collagen (Schmid and Linsenmayer, 1985). Thus, removal of perichondrium expands the collagen type X-expressing domain. These results also suggest that the newly synthesized collagen type X may require a signal from the perichondrium to efficiently trigger secretion into the extracellular matrix.

The morphology of the cells expressing intracellular type X collagen was examined by histological staining. As shown in Fig. 5, these cells (panel B′, zone 1) appear not to be appreciably larger than the type X collagen-negative cells (panel A′, zone 1) in perichondrium-intact cultures.

The effect of perichondrium removal on expression of collagen type X seems temporally to occur later than its effect on proliferation. At early stages of culture (e.g., day 2 and day 3), when differences in the proliferative zone can be readily detected, the distribution of collagen type X appears to be similar for cultures with or without perichondrium (data not shown). By 6 days in culture, however, the differences are clearly detectable and these become most pronounced by day 8 (see above).

Local effects of perichondrium removal

To examine whether the effect of perichondrium was a local one, we cultured tibiotarsi in which the perichondrium had been removed from only one side (see Fig. 1C). These cultures exhibited considerable overgrowth on the perichondrium-free side, resulting in a severely deformed phenotype after 8 days in culture (data not shown). Potential effects on the proliferative zone were examined by BrdU labeling. As shown in Fig. 6, alterations in the proliferative zone were clearly detectable after 2 days in culture. The expansion of the proliferative zone occurs, but it is restricted to the side free of perichondrium. Likewise, expansion of the collagen type X-

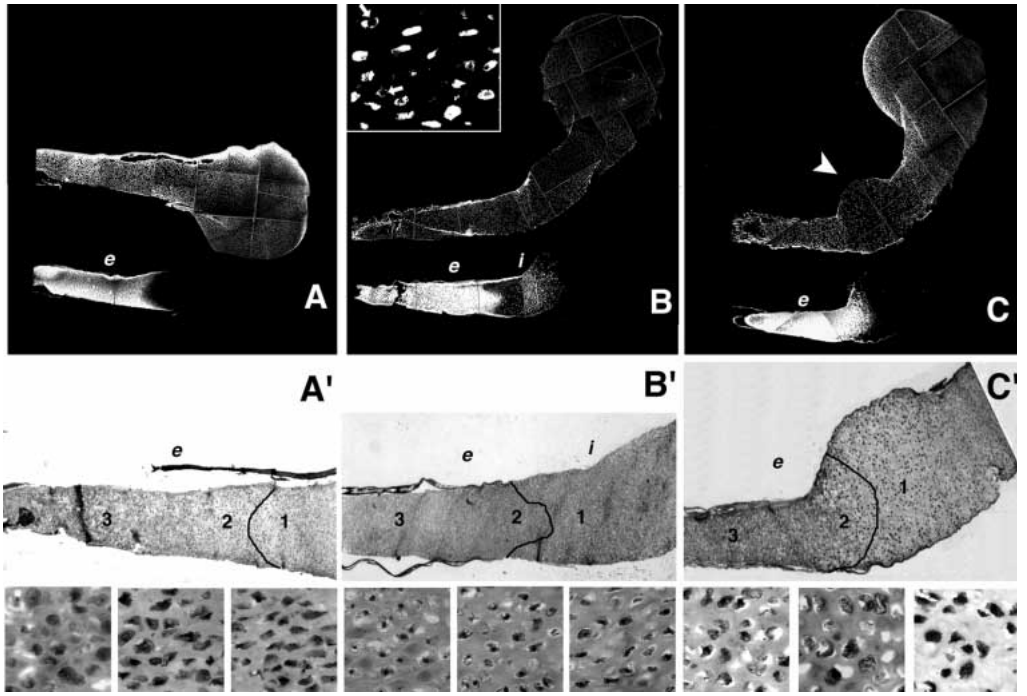

Fig. 5. Immunostaining of type X collagen (A-C) and the corresponding histological staining (A′-C′) in tibiotarsi after 8 days in culture. All sections are through the tarsus end of tibiotarsi. Immunostaining and the corresponding histological staining was performed on adjacent sections. (A,A′) With intact perichondrium; (B,B′) without perichondrium; (C,C′) without perichondrium but with PTH added in the medium. (A-C) Top: Hoechst staining to show the overall shape of the section; bottom: immunoreactivity for collagen type X. *e*, extracellular staining of collagen type X; *i*, intracellular staining of collagen type X. Inset in B: a higher magnification from the region *i*; arrows point to nuclei. The arrowhead in C denotes the 'bulge' of cartilage at the junction of bone and cartilage, often observed in cultures without the perichondrium, with or without PTH but more pronounced when with PTH. (A′-C′) The black lines demarcate the boundaries of the extracellular staining of type X collagen. Also indicated are zones 1, 2 and 3, representing either in the normal tibiotarsi (A′) nonhypertrophic, early hypertrophic and late hypertrophic regions, respectively, or the equivalent zones in the manipulated samples (B′,C′). Shown directly below are representative high-magnification pictures from each zone.

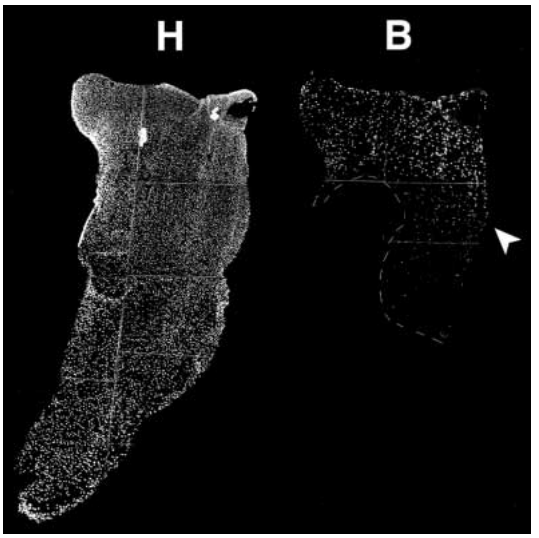

Fig. 6. BrdU labeling of the tarsus-end cartilage of tibiotarsi with partial removal of the perichondrium after 2 days in culture. H, Hoechst staining; B, BrdU staining. The arrowhead points to the side of cartilage with perichondrium removed. The dashed line marks the boundary of BrdU labeling within the section.

Fig. 7. Immunostaining of collagen type X in tibiotarsi with partial removal of the perichondrium after 8 days in culture. The section was through the tarsus end of the tibiotarsus. The arrowhead points to the side of cartilage with perichondrium removed. The arrow points to the side where the perichondrium remained intact. The dots mark the boundary of the section.

expressing zone is also confined to this side, as can been seen for the 8-day culture shown in Fig. 7. Thus, these observations not only confirm that the perichondrium negatively regulates both proliferation and differentiation of chondrocytes; they also indicate that both types of regulation appear to be local effects.

Effects of PTH

According to Vortkamp et al. (1996) and Lanske et al. (1996), activation of the PTH/PTHrP receptor in prehypertrophic chondrocytes negatively regulates the rate of chondrocyte hypertrophy. To assess whether the regulation by the perichondrium is mediated by this mechanism, we examined the effect of PTH on the perichondrium-free cultures.

The results showed that PTH added in the culture medium reversed the accelerated expression of collagen type X elicited by the removal of perichondrium (Fig. 5C). This is indicated by both the overall shortening of the collagen X-positive region and by the lack of an extended domain with intracellular collagen type X. Thus, the regulation of chondrocyte differentiation by the perichondrium appeared to be dependent upon the PTH/PRHrP receptor.

The morphology of cells in the PTH-treated samples was also examined by histological staining (Fig. 5). The chondrocytes in zone 1 of these samples (panel C′), though negative for type X collagen, appear to be considerably larger than the type X-negative cells in the perichondrium-intact culture (panel A′, zone 1). In addition, the zone 1 cells in the PTH-treated samples appear to be farther apart than cells in an equivalent zone from the perichondrium-intact cultures (panel A′). This result suggests that more extracellular matrix was deposited in this region of the PTH-treated cultures.

PTH, however, failed to repress the expansion of the proliferative zone in perichondrium-free tibiotarsi. This is shown by the equivalent width of the BrdU-labeled zone in PTH-treated (Fig. 4C) versus nontreated (Fig. 4B) cultures. Thus, the regulation of chondrocyte proliferation by the perichondrium appears to be independent of PTH or the PTH/PTHrP receptor.

DISCUSSION

We report that removal of the perichondrium resulted in both acceleration of the expression of type X collagen and expansion of the proliferative zone of the developing cartilage. These results provide evidence that perichondrium participates in negative regulatory pathways that control both proliferation and differentiation of chondrocytes.

The molecular mechanisms underlying these observations, however, remain to be determined. Work by Vortkamp et al. (1996) provides one possible explanation for the accelerated differentiation in the absence of perichondrium. According to this model, removal of the perichondrium along the length of tibiotarsi would eliminate the source of Ptc and therefore would abrogate Ihh signaling. This would then relieve the negative regulation of chondrocyte differentiation and result in more chondrocytes progressing to hypertrophy. To examine whether Ihh expression was accelerated in a coordinated manner upon removal of the perichondrium, we performed in situ hybridization for this molecule. Our preliminary results, however, suggest that in these tibiotarsi cultures, the expression of Ihh is no longer restricted to the prehypertrophic/early hypertrophic region (Vortkamp et al., 1996, and our unpublished observation), but instead appears to be expressed throughout the cartilage. This ‘diffusive’ expression occurred in both perichondrium-free and -intact cultures. These observations indicate that the expression pattern of this molecule in the cultured samples, therefore, is considerably different from that in uncultured tibiotarsi in

vivo. Thus, it is unclear at present whether the regulation of chondrocyte differentiation in this organ culture system, is mediated by Ihh.

A very recent study (Serra et al., 1997) suggests that TGF-β signalling may be involved in the regulation of chondrocyte differentiation by the perichondrium. In this study, the authors created transgenic mice expressing a dominant-negative form of the TGF-β type II receptor. In these mice, the defective receptor was expressed by the perichondrium. Concomitantly, chondrocyte hypertrophy in the growth plate cartilage was accelerated. These results indicate that loss of TGF-β responsiveness in the perichondrium may promote chondrocyte hypertrophy. The TGF-β itself most likely originates from the hypertrophic chondrocytes themselves (Nurminskaya and Linsenmayer, 1996; D'Angelo and Pacifici, 1997).

Alternatively, the accelerated expression of collagen type X could be an event secondary to the increased proliferation. In this scenario, chondrocytes would respond to removal of the perichondrium by increasing cell proliferation. This would result in an increased number of chondrocytes. These differentiate on a normal schedule but their increased number leads to more cells expressing collagen type X. In support of this, the effect of perichondrium removal on expression of collagen type X seems to be a late response. Whereas the expansion of the proliferative zone is readily detectable at 2 days in culture, the differential expression of collagen X is not noticeable until days later (see Results).

The molecular basis for the regulation of proliferation also remains to be investigated. FGFs have been implicated in regulating bone growth. One of the FGF receptors, FGFR-3 is mainly expressed in the proliferating/resting zone of the developing long bones (Peters et al., 1993). Mice null for FGFR-3 exhibited an expanded proliferative zone in the epiphysis, indicating that, in a normal mouse, FGFR-3 acts as a negative regulator of proliferation of chondrocytes (Deng et al., 1996). Our preliminary experiments with FGF1 added to perichondrium-free cultures, however, failed to reverse the expansion of the BrdU-labeled zone (data not shown), suggesting that the perichondrium and FGFR-3 might not be acting through the same pathway to regulate chondrocyte proliferation.

The physiological importance of a negative regulation of chondrocyte proliferation may lie in the fact that young dividing chondrocytes at least in vivo cease proliferation before they embark on maturation. In a normal developing cartilage, the negative regulation of proliferation via the perichondrium may act on a subset of chondrocytes, resulting in their exit from the proliferative zone.

An additional component contributing to the increased growth in the perichondrium-free cultures could be increased matrix deposition (Dr Qian Chen, personal communication). Consistent with this, our preliminary observations indicate that, at regions where increased growth in width is most obvious (usually at the junction of bone and cartilage), cells appear to be spaced farther apart than those in the equivalent regions of the corresponding normal culture. This is most pronounced in cultures treated with PTH (see Fig. 5C′), suggesting that PTH may also affect matrix synthesis/deposition.

The predominantly intracellular staining of collagen type X in regions of the perichondrium-free cultures most likely represents newly synthesized molecules. The retention of collagen type X intracellularly for a period of time before secretion has been previously observed in vertebral chondrocytes (Linsenmayer et al., 1986). This intermediate step, however, has not been observed to any appreciable extent in developing long bones, presumably due to the rapid secretion of this molecule into the extracellular matrix. Our present manipulations of the perichondrium may have uncoupled efficient secretion of collagen X from its synthesis. Secretion of this molecule into cartilage matrix in vivo seems to require a signal from the surrounding perichondrium. The signal is currently unknown.

Collagen type X has been long considered as a molecular marker for hypertrophic chondrocytes. In our present study, however, we have noted that many cells containing intracellular collagen type X in perichondrium-free cultures did not appear to be appreciably larger than the collagen type X-negative cells (Fig. 5B′, zone 1). Conversely, in the PTH-treated perichondrium-free cultures, the cells residing in the 'bulge', though negative for type X collagen, are bigger than normal type X collagen-negative chondrocytes (Fig. 5C′, zone 1). It remains to be seen whether these cells also have acquired other characteristics (Nurminskaya and Linsenmayer, 1996) of the hypertrophic program.

Overall, the present study has demonstrated that the perichondrium regulates both proliferation and differentiation of chondrocytes, in a negative manner. Moreover, our data support the proposition that the regulation of certain parameters of differentiation and maturation may be mediated by the PTHrP/PTH receptor. The regulation of proliferation, however, is most likely independent of this receptor. The molecular basis for both those types of regulation by the perichondrium remains to be determined.

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