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Perichondrial-mediated TGF-beta regulation of cartilage growth in avian long bone development

MARSHA L. CROCHIERE, JAMES K. KUBILUS and THOMAS F. LINSENMAYER*

Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts, USA

ABSTRACT We previously observed using cultured tibiotarsal long-bone rudiments from which the perichondrium (PC) and periosteum (PO) was removed that the PC regulates cartilage growth by the secretion of soluble negative regulatory factors. This regulation is "precise" in that it compensates exactly for removal of the endogenous PC and is mediated through at least three independent mechanisms, one of which involves a response to TGF-β. PC cell cultures treated with 2 ng/ml TGF-β1 produced a conditioned medium which when added to PC/PO-free organ cultures effected precise regulation of cartilage growth. In the present study, we have investigated the possibility that TGF-β itself might be the negative regulator which is produced by the PC cells in response to their treatment with TGF-β1. Using a TGF-β responsive reporter assay, we determined that PC cell cultures, when treated with 2 ng/ml or greater exogenous TGF-β1, produce 300 pg/ml of active TGF-β. Then we observed that this concentration (300 pg/ml) of active TGF-β1, when added to PC/PO-free tibiotarsal organ cultures, effected precise regulation of cartilage growth, whereas concentrations of TGF-β1 either greater or less than 300 pg/ml produced abnormally small cartilages. These results suggest that one mechanism by which the PC effects normal cartilage growth is through the production of a precisely regulated amount of TGF-β which the PC produces in response to treatment with exogenous TGF-β itself.

KEY WORDS: *perichondrium, periosteum, transforming growth factor-β, tibiotarsus, cartilage*

Introduction

Most bones of the vertebrate skeleton, including those in the limb, form by a process in which a cartilage model of the skeletal element is eventually replaced by bone (i.e. endochondral ossification). Throughout the period of endochondral bone development, the cartilage is surrounded by a fibrous connective tissue, the perichondrium (PC), the cells of which differentiate into chondrocytes and contribute to appositional growth of the cartilage. In addition, however, we (Long and Linsenmayer, 1998; Di Nino *et al.*, 2001) and others (Alvarez *et al.*, 2001) have obtained evidence that the PC also functions during development to regulate the growth of the cartilage, thus ensuring that the proper length and size of the bone is achieved.

In our initial study we observed that when pairs of tibiotarsi from day E 12 chicken (*Gallus gallus*) embryos were grown in organ culture, removal of the perichondrium and periosteum from one of the tibiotarsi resulted in increased growth of its cartilagenous component - as compared to that of the intact, contralateral tibiotarsus. Additional analyses showed that this regulation affected both chondrocyte proliferation and hypertrophy (Long and Linsenmayer, 1998) and suggested that during development, the PC [and/or the periosteum (PO)] negatively regulates the growth of the cartilage.

In subsequent studies (Di Nino *et al.*, 2001; Di Nino *et al.*, 2002), we obtained evidence that the PC effects this negative regulation through multiple mechanisms. One of these mechanisms involves the cooperative action of soluble factors secreted by the PC and the PO (Di Nino *et al.*, 2001). When conditioned medium from the PC and PO cell cultures were mixed together and added to organ cultures of tibiotarsi from which the PC/PO were removed (PC/PO-free cultures), this "mixed medium" negatively regulated cartilage growth. Importantly, this negative regulation of cartilage by the PC/PO mixed conditioned medium was

Abbreviations used in this paper: Ihh, Indian hedgehog; MMP, matrix metalloprotease; PAI-1, plasminogen activator inhibitor; PAIL, PAI-1 promoter construct linked to a luciferase reporter; PC, perichondrium; PO, periosteum; PTHrP, parathyroid hormone related peptide; TGF-β, transforming growth factor-β.

***Address correspondence to:** Thomas F. Linsenmayer. 136 Harrison Avenue, Boston, MA 02111, USA. Fax: +1-617-636-3676.
e-mail: thomas.linsenmayer@tufts.edu

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"precise" in that it compensated exactly for the removal of the endogenous PC and PO [i.e. the cartilage growth of the PC/PO-free tibiotarsi were the same as those of the tibiotarsi in which the PC and PO were left intact (intact tibiotarsi)].

We (Di Nino *et al.*, 2002) also observed that another way that the PC can negatively regulate cartilage growth is by producing a secondary factor(s) in response to treatment with TGF-β1. Cultures of PC cells when treated with a high concentration of TGF-β1 (2 ng/ml) produced a conditioned medium that, when added to the PC/PO-free tibiotarsal organ cultures, negatively regulated cartilage growth. Again this regulation was precise and was specific for PC cells; PO cells treated with TGF-β1 showed no such negative regulation.

TGF-β is a 24-kD homodimer that is secreted as an inactive form complexed with latent binding proteins. This complex, in turn, is bound to the extracellular matrix (Taipale *et al.*, 1994; Dallas *et al.*, 1995). Latent TGF-β can be artificially activated by high pH or high temperature, but the physiological mechanism of activation is most likely proteolysis. In the growth plate, several proteases that activate TGF-β have been identified including matrix metalloproteases (MMPs) -2, -3, -9 and -13, all of which are produced by chondrocytes (D'Angelo *et al.*, 2001; Maeda *et al.*, 2002). However, activation of endogenous TGF-β is regulated such that in a tissue only a portion of the total TGF-β becomes active [reviewed in (Annes *et al.*, 2003; Hyytiainen *et al.*, 2004)].

Several studies have suggested that TGF-β signaling lies within the Indian hedgehog (Ihh)/parathyroid hormone related peptide (PTHrP) negative feedback loop, acting downstream of Ihh and upstream of PTHrP (Serra *et al.*, 1999; Pateder *et al.*, 2000; Alvarez *et al.*, 2002). This model suggests that Ihh expressed by prehypertrophic chondrocytes elicits the expression of TGF-β in the PC. This stimulates the expression of PTHrP in the articular cartilage which in turn negatively regulates proliferation and differentiation of the growth plate cartilage.

In the present study, we have investigated the possibility that TGF-β itself, at a specific concentration, might be the secondary factor produced in response to TGF-β1 treatment of PC cells that precisely regulates cartilage growth. Consistent with this hypothesis, we determined using a TGF-β responsive reporter assay that PC cells do respond to treatment with TGF-β by producing active TGF-β and that the concentration of active TGF-β they produce, when added to PC/PO-free tibiotarsal organ cultures, precisely regulates cartilage growth. This suggests a potentially novel mechanism through which PC cells can, in response to TGF-β, produce an amount of active TGF-β that precisely regulates growth of the underlying cartilage.

Results

Exogenous TGF-β promotes the production of active TGF-β from perichondrial cells

As we reported previously (Di Nino *et al.*, 2002), when PC cells are treated with 2 ng/ml TGF-β1 (the only concentration used in that study), they produce conditioned medium that in PC/PO-free cultures precisely regulates cartilage growth (see Introduction). Since TGF-β itself has been reported to be a negative regulator of cartilage growth (Ballock *et al.*, 1993; Serra *et al.*, 1997; Serra *et al.*, 1999; Alvarez *et al.*, 2001), we sought to determine whether (1) a specific concentration of TGF-β was capable of precisely regulating cartilage growth and, (2) if so, whether the PC cells, in response to treatment with exogenous TGF-β1, respond by producing TGF-β at this concentration. Our results, presented next, suggest that both of these hypotheses are correct.

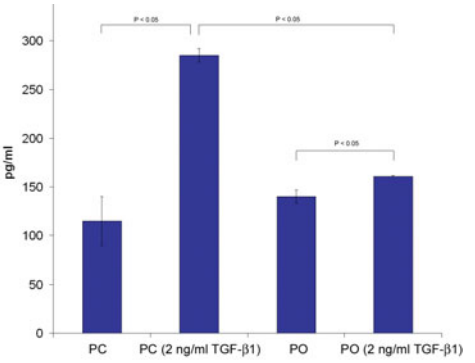

Fig. 1. Quantitation of active TGF-β present in conditioned media. *Concentrations of active TGF-β determined by the PAIL assay in conditioned media produced by perichondrial (PC) and periosteal (PO) cell cultures in response to exogenously added TGF-β1 (2 ng/ml, N = 3). For both PC and PO cell cultures untreated vs. 2 ng/ml TGF-β1 treated, P < 0.05. For 2 ng/ml TGF-β1 treated PC cell culture vs. 2 ng/ml TGF-β1 treated PO cell culture, P < 0.05.*

We first examined whether PC cells, in response to exogenous TGF-β1 treatment, do in turn produce TGF-β and if so whether this is in a dose-dependent manner. For this we employed the PAIL assay, a bioassay that quantitatively and specifically determines active TGF-β. For comparison and as a control, we also tested the response of PO cells, as these, when treated with TGF-β1 do not produce conditioned medium that precisely regulates cartilage growth (Di Nino *et al.*, 2002).

For these studies, PC (and PO) cell cultures were treated with various concentrations of active TGF-β1. The conditions employed were those devised previously for producing the conditioned medium used for examining the regulation of cartilage growth in PC/PO-free organ cultures (Di Nino *et al.*, 2001). This consisted of growing the cells in serum-containing medium to which exogenous TGF-β1 was added and then, when the cultures reached confluency (in 4 to 5 days), removing this medium, washing the cells and adding serum-free medium (without TGF-β1). 18 hours later this "conditioned medium" was harvested and assayed for active TGF-β (by the PAIL assay).

When the PC cultures were treated with 2 ng/ml exogenous TGF-β (i.e. the concentration previously shown to effect the production of conditioned medium that precisely regulates cartilage growth in PC/PO-free organ cultures [see (Di Nino *et al.*, 2002) and below]), the conditioned medium produced by these cultures contained TGF-β at a concentration of 285 pg/ml [Fig. 1, PC (2 ng/ml TGF-β1)]. The conditioned medium from untreated, control PC cultures contained TGF-β at a concentration of 115 pg/ml (Fig. 1, PC). So this treatment with exogenous TGF-β1 effected approximately a 2.5-fold increase in the subsequent production of TGF-β by the PC cells which, from data presented below, seems to be a threshold response.

PO cells, in contrast, showed little response in their production of TGF-β to treatment with exogenous TGF-β1 (Fig. 1). Although the conditioned medium from untreated PO cell cultures did

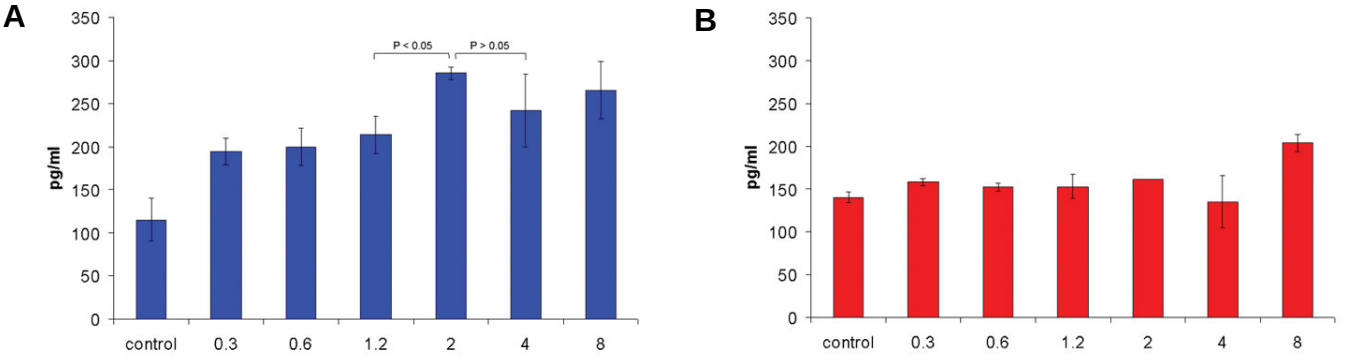

Fig. 2. Quantitation of active TGF-β produced in response to increasing amounts of TGF-β1. *Concentration of active TGF-β determined by the PAIL assay in conditioned medium of* **(A)** *perichondrial (PC) and* **(B)** *periosteal (PO) cell cultures, treated with either vehicle (control) or 0.3, 0.6, 1.2, 2, 4 and 8 ng/ml TGF-β1 (N = 3). For all PC cell culture treatments, analysis of variance between groups (ANOVA) F = 4.92, P < 0.05. For PC cell cultures treated with 1.2 vs. 2 ng/ml TGF-β1, Student's* t*-test P < 0.05. For PC cell cultures treated with 2 vs. 4 ng/ml TGF-β1, Student's* t*-test P > 0.05. For PO cell culture treatments, ANOVA F = 2.86, P = 0.05. For PO cell cultures treated with 4 vs. 8 ng/ml TGF-β1, Student's* t*-test P > 0.1.*

contain active TGF-β at a concentration similar to that of untreated PC cell cultures (140 pg/ml for the PO cells versus 115 pg/ml for the PC cells), the PO cells showed only a slight yet significant response to the treatment with 2 ng/ml exogenous TGF-β1, producing conditioned medium containing 161 pg/ml active factor.

To determine whether this response by PC cell cultures was specific for an input of 2 ng/ml TGF-β1, or whether it represented a threshold level, cultures were treated with concentrations of exogenous TGF-β1 ranging from 0 to 8 ng/ml and their subsequent production of TGF-β was measured by the PAIL assay. The results (Fig. 2) showed that in response to treatments with increasing amounts of exogenous TGF-β1, the active TGF-β produced by the PC cells increased from a baseline level of 115 pg/ml in control medium, to a threshold of 285 to 265 pg/ml in cultures treated with 2 - 8 ng/ml (Fig. 2A). Statistically, there was no difference between the amounts of active TGF-β produced by the PC cells in response to the 2 ng/ml TGF-β1 and higher treatments. Thus ~285 pg/ml TGF-β seems to be the maximum response of PC cells to treatment with exogenous TGF-β1 (i.e. it plateaus at the concentration that effects precise regulation of cartilage growth, as described below).

For comparison, cultures of PO cells (Fig. 2B) showed no response to any concentration of exogenous TGF-β1 – except possibly with the highest concentration tested (8 ng/ml) – at which a slight increase was detected.

Precise regulation of cartilage growth by TGF-β1

We next examined whether active TGF-β at the concentration produced by the PC cells in response to TGF-β1 treatment (~285 pg/ml) would effect precise regulation of cartilage growth. To test this, we examined the effect of different concentrations of active

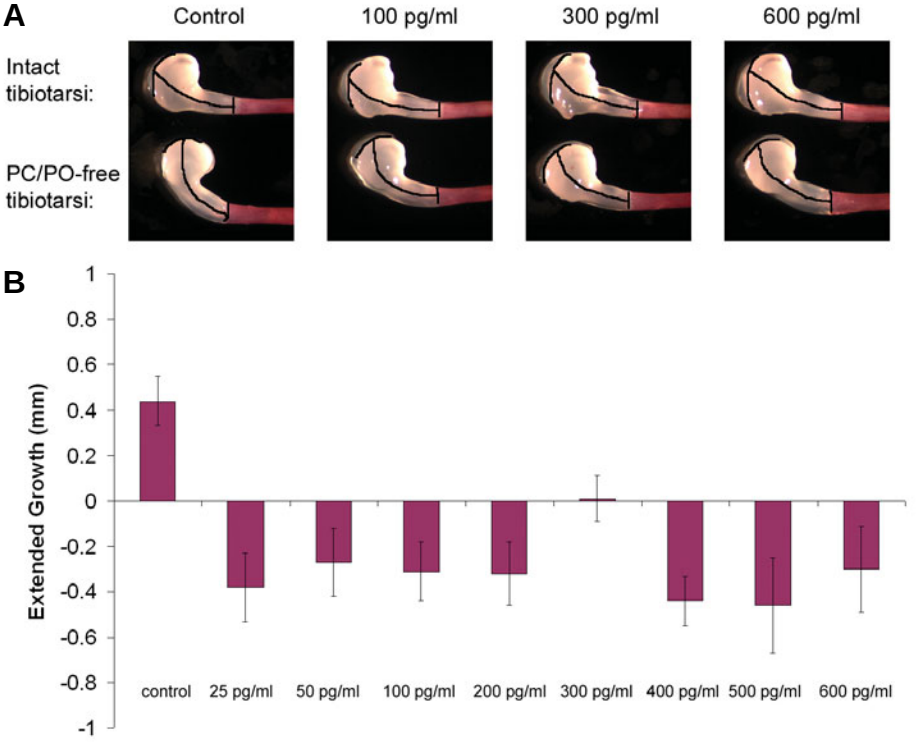

Fig. 3. Effect of various concentrations of TGF-β1 on cartilage growth. (A) *Photographs of the ends of pairs of tibiotarsi (one intact and one PC/PO-free) grown in control medium or with the PC/PO-free tibiotarsus in different concentrations of TGF-β1 (stained with alizarin red to visualize bone). The black lines along the midline of the cartilage were used to measure the lengths.* **(B)** *Bar graph showing the extended growth (see Results) of tibiotarsal pairs grown in control medium (N = 28), or TGF-β1 at concentrations of 25 pg/ml (N = 9), 50 pg/ml (N = 9), 100 pg/ml (N = 8), 200 pg/ml (N = 8), 300 pg/ml (N = 7), 400 pg/ml (N = 12), 500 pg/ml (N = 7) and 600 pg/ml (N = 8). For all organ culture pairs except those treated with 300 pg/ml TGF-β1, the differences between the intact vs. the PC/PO-free, P < 0.05.*

TGF-β1 on cartilage growth, including one (300 pg/ml) which is essentially the same as that produced by the TGF-β1 treated PC cell cultures. As an assay we used the PC/PO-free tibiotarsal organ culture system employed in our previous studies (Di Nino *et al.*, 2001; Di Nino *et al.*, 2002).

In this system, tibiotarsi from the same 12-day chicken embryo are cultured and analyzed as pairs, thus eliminating the potential variability in the growth characteristics we and others (Lovitch and Christianson, 1997) have observed when tibiotarsi from embryos are pooled. For each pair, the PC and PO of one tibiotarsus is removed (PC/PO-free) and the other is left intact. Both tibiotarsi were cultured in serum-free medium, with the medium of the PC/PO-free cultures containing exogenously added TGF-β1 at concentrations ranging from 25 to 600 pg/ml (Fig. 3B). After 3 days in culture, the growth of the cartilages in each tibiotarsal pair was measured and compared.

When both tibiotarsi were cultured in control, serum-free medium (i.e. without TGF-β1), the cartilage of the PC/PO-free tibiotarsus grew longer than the one that was left intact [i.e. they exhibited abnormally long, "extended growth", as defined in (Di Nino *et al.*, 2001)] (Fig. 3 A,B, control). When, however, the medium of the PC/PO-free cultures was supplemented with exogenous TGF-β1, this abnormal, "extended growth" was eliminated, confirming the ability of TGF-β to effect negative regulation. However, at only one concentration was the regulation "precise", with the treated cultures growing to the same length as the intact cultures (i.e. the extended growth was "0"). That was 300 pg/ml (Fig. 3 A,B), which is essentially the same as the 285 pg/ml produced by the PC cell cultures in response to their treatment with 2 ng/ml TGF-β1. At all other concentrations - either greater than or less than 300 pg/ml - the treated PC/PO-free tibiotarsi had an extended growth less than "0" (i.e. its cartilage was abnormally shorter than the intact, contralateral tibiotarsus) resulting in a non-monotonic dose response (Fig. 3 A,B). Thus, TGF-β1 can effect precise regulation of cartilage growth, but only at the specific concentration that is produced by PC cells in response to their treatment with TGF-β1.

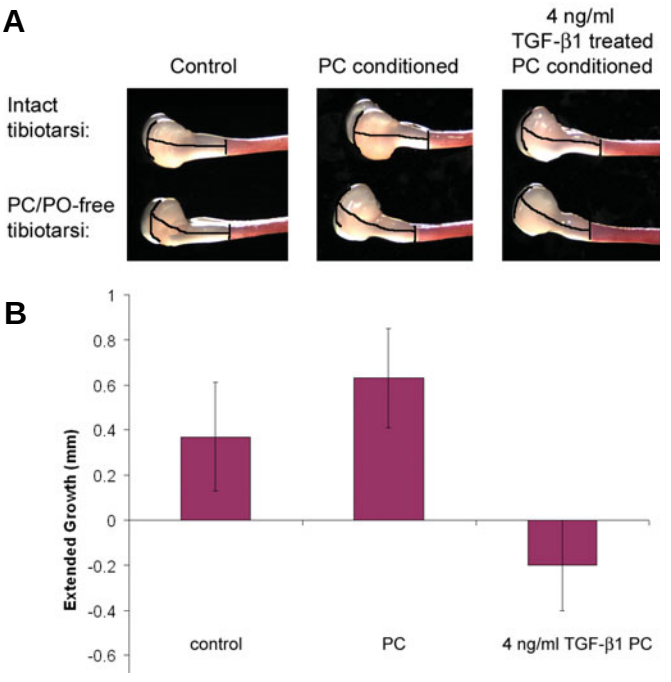

Fig. 4. Effect of conditioned medium from perichondrial (PC) cell cultures treated with 4 ng/ml TGF-β1 on cartilage growth. (A) *Photographs of pairs of tibiotarsi in which the intact tibiotarsus of each pair was grown in control medium and the PC/PO-free tibiotarsus was grown in either control medium, conditioned medium from PC cell cultures grown in control medium, or conditioned medium from PC cell cultures treated with 4 ng/ml TGF-β1.* **(B)** *Bar graph showing the extended growth (see Results) in tibiotarsal pairs grown in control medium (N = 6), conditioned medium from PC cell cultures grown in control medium (N = 6), or conditioned medium from PC cell cultures treated with 4 ng/ml TGF-β1 (N = 5). For all organ cultures of intact vs. PC/PO-free except those grown in conditioned medium from PC cell cultures treated with 4 ng/ml TGF-β1, P < 0.05. The extended growth observed in 4 ng/ml TGF-β1 PC compared to 0 (no growth), P > 0.50*

Perichondrium controls the amount of TGF-β available to regulate cartilage growth

If in the conditioned medium from the PC cell cultures treated with 2 ng/ml TGF-β it is the ~285 pg/ml TGF-β that effects the precise regulation of cartilage growth, and, if this value of ~285 pg/ml TGF-β does represent a threshold for the production of active TGF-β – as the results using the PAIL assay suggest – then treatment of the PC cells with an even higher concentration of TGF-β1 (e.g., 4 ng/ml) should still produce a conditioned medium that effects precise regulation of growth. The data are consistent with this prediction. When PC cell cultures were treated with 4 ng/ml TGF-β1 and the resulting conditioned medium – which, by the PAIL assay, contained 280 pg/ml TGF-β – was tested for its regulation of cartilage growth in PC/PO-free organ cultures, the growth was not significantly different from the treated PC/PO-free cultures to the intact (i.e. the regulation was precise, see Figure 4). The difference in the growth between the PC/PO-free cultures to the intact when treated with 4 ng/ml TGF-β1 was also not significantly different from 0, or no growth (Fig. 4B). This is in contrast to the growth in the PC/PO-free cultures when treated with conditioned medium from untreated PC cell cultures which stimulated growth even more so than when the PC/PO-free cultures were treated with control, serum-free medium (Fig. 4)[1]. These results, when taken together, suggest that even in the presence of excess amounts of exogenous TGF-β1 (i.e. 2 ng/ml or higher), PC cells can regulate the amount of active TGF-β they produce and release into their conditioned medium and this is at a concentration that precisely regulates cartilage growth.

Lastly, as 300 pg/ml TGF-β1 is the only concentration that effects precise regulation and as this is the threshold level of active factor produced by the PC cells in response to TGF-β1, we examined whether this response of PC cells reflects their maximum synthesis, maximum activation, or both. To determine this, PC and PO cell cultures were treated with TGF-β1 at the same concentrations as used above (0 to 8 ng/ml) and

[1] As previously published, PC cells produce calcitonin, which stimulates cartilage growth (Di Nino and Linsenmayer, 2003).

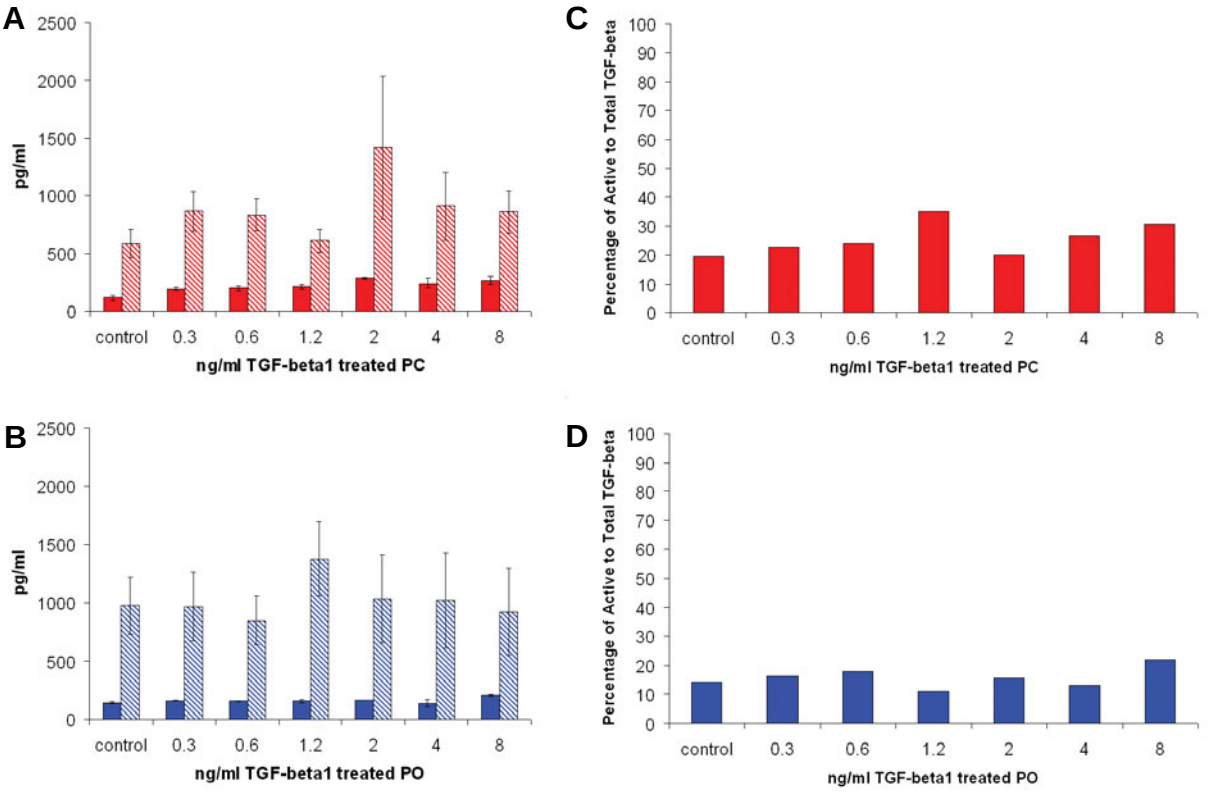

Fig. 5. Quantitation of active and total TGF-β produced in response to increasing concentrations of TGF-β1. *Concentrations of active (solid bars) and total (active plus latent) (hatched bars) TGF-β determined by the PAIL assay in conditioned media from* **(A)** *perichondrial (PC) cell cultures and* **(B)** *periosteal (PO) cell cultures treated with increasing concentrations of TGF-β1 (N = 3). For total TGF-β in all PC cell culture treatments, ANOVA F = 0.96 P = 0.48. For total TGF-β in all PO cell culture treatments, ANOVA F = 0.25 P = 0.95. Percentage of active compared to total TGF-β in PC* **(C)** *and PO* **(D)** *cell cultures treated with increasing amounts of TGF-β1.*

medium from these cultures were assayed for both active and total TGF-β (by the PAIL assay), with the "total" (active + latent) activity being determined following heat-activation of the latent form. As can be seen in Figure 5A, at all concentrations of TGF-β1 treatment, the majority of the factor in the PC cell conditioned media was the latent form, as was also true for the untreated control cultures. Also, with the different concentrations of exogenously added TGF-β1, the amount of total TGF-β produced by the cells (hatched bars) did fluctuate somewhat. Nonetheless, statistically, there was no difference in the amount of total TGF-β produced by the PC (or the PO, Fig. 5B) cell cultures with treatments of increasing concentrations of TGF-β1. However, it was the active factor (solid bars) that showed a progressive dose response increase, finally plateauing at 285 pg/ml. The percentage of active compared to total TGF-β did not fluctuate much in PC cell cultures treated with increasing concentrations of TGF-β1 (Fig. 5C). This suggested that the predominant regulation in producing the concentration of active TGF-β required to effect precise regulation of cartilage growth is by activation of the latent factor, rather than by net synthesis.

The PO cells showed no such dose response to TGF-β1 treatment (Fig. 5B). Although like the PC cells, the PO cells produced considerable latent TGF-β1, the amount of active factor was small and did not increase in response to increasing exogenous TGF-β1 treatment. Neither did the percent of active compared to total TGF-β differ much in PO cells treated with increasing concentrations of TGF-β1 (Fig. 5D), although the percent of active TGF-β was generally lower in all the treated PO cell cultures compared to the PC cell cultures (Fig. 5 C,D).

Discussion

Our previous studies (Di Nino *et al.*, 2002) suggested that the PC can regulate cartilage growth through several different mechanisms, one of which involves the TGF-β1- stimulated production of a secondary, soluble negative regulator. When cultures of PC cells were treated with 2 ng/ml TGF-β1 they produced conditioned medium that precisely regulated cartilage growth. This result was specific to PC cells, as the conditioned medium from TGF-β1-treated PO cells elicited no such response. Because TGF-β is itself a known negative regulator of cartilage growth (Ballock *et al.*, 1993; Serra *et al.*, 1997; Serra *et al.*, 1999; Alvarez *et al.*, 2001), in the present study we examined whether TGF-β itself might be the secondary factor produced by PC cells in response to their treatment with exogenous TGF-β1. If so, the concentration of TGF-β in the conditioned medium should be the amount of active TGF-β that precisely regulates cartilage growth and the data suggest that this is true.

In our previous study (Di Nino *et al.*, 2002), the concentration

of TGF-β1 used to treat cultures of PC cells was 2 ng/ml. In the present study, we observed that choosing this concentration had serendipitously been the lowest concentration of TGF-β1 treatment of PC cells that caused them to produce active TGF-β in the amount that precisely regulates cartilage growth (~300 pg/ml). Although 2 ng/ml seems to be a high concentration of TGF-β when compared with those normally employed for the treatment of cell cultures (Iwasaki *et al.*, 1993; Beier *et al.*, 2001), Gibson *et al.* (2001) recently reported that hypertrophic chondrocytes in culture produce TGF-β at a concentration of 10 ng/ml and that 20% of this is active (i.e. 2 ng/ml). It remains to be determined whether during long bone development hypertrophic chondrocytes are the source of the TGF-β that effects the production of the TGF-β by the PC cells. This, however, seems feasible, as the target chondrocytes for the PC-produced TGF-β are most likely the proliferating and maturing chondrocytes, as TGF-β reduces both the proliferation and maturation of the cartilage in the PC/PO-free tibiotarsal cultures (Di Nino *et al.*, 2002).

Dose-dependent effects of TGF-β

Numerous studies have shown that cellular responses of skeletal tissues to TGF-β treatment occur in a dose-dependent manner including, for example, (1) the proliferation and collagen synthesis of rabbit PC cells (Dounchis *et al.*, 1997), (2) the production of uronic acid by chicken PO cells (Iwasaki *et al.*, 1995) and (3) the proliferation and differentiation of chondrocytes (Pateder *et al.*, 2000; Qi and Scully, 2003). Similarly, we have observed that PC cells respond to treatment with exogenous TGF-β1 by their production of active TGF-β - with a threshold level of ~300 pg/ml reached when treated with 2 ng/ml or greater TGF-β1. This threshold level of TGF-β was reached after the TGF-β1 treated PC cell cultures had been maintained for 18 hours in serum-free medium. Although we cannot rule out the possibility that the amount of active TGF-β would increase with time beyond 18 hours, we chose to study this time point as it was the time found previously (Di Nino *et al.*, 2001) to be the most critical for collecting conditioned medium from cell cultures that resulted in the precise regulation of cartilage growth in the tibiotarsal organ culture system.

We also observed that organ cultures of PC/PO-free tibiotarsi respond to treatments with TGF-β1 in a dose-dependent, non-monotonic manner. While the threshold production of TGF-β (~300 pg/ml) by PC cells in response to TGF-β1 treatment negatively regulates cartilage growth in a precise manner, treatments with concentrations of TGF-β1 either above or below 300 pg/ml resulted in abnormally small cartilages, as compared to the intact tibiotarsi. This type of non-monotonic dose response has been observed in other studies, including the effect of TGF-β on the metastatic potential of mammary adenocarinoma cells (Welch *et al.*, 1990). Although the mechanism(s) responsible for this type of dose response is still speculative, it has been proposed (Kohn and Melnick, 2002) that this may reflect a lack of receptor saturation or an alteration in the affinity of coreceptors/activators for the ligand. In reality, however, we currently have no explanation for this response in the TGF-β-mediated regulation of cartilage growth, as observed here.

Although we did not directly determine TGF-β to be a component of the TGF-β1 treated PC cell conditioned medium, the response of the PAIL assay suggests that TGF-β is present. Our efforts to detect TGF-β from the conditioned medium by western blot analysis were unsuccessful, as we and others (M.N. Nurminskaya, personal communication) have not found a TGF-β-specific antibody that cross-reacts with the chicken protein. In an effort to block the effect of the TGF-β present in the conditioned medium, we treated the conditioned medium with a neutralizing antibody to TGF-β, but discovered that the antibody also did not cross-react with chicken TGF-β (data not shown). Nonetheless, the fact that we were able to demonstrate that treatment of tibiotarsal organ cultures with exogenous TGF-β1 at the same concentration detected from the conditioned medium that precisely regulated cartilage growth (300 pg/ml) was itself able to precisely regulate cartilage growth indicates that TGF-β was indeed present in the conditioned medium. We cannot exclude the possibility, however, that the effect of TGF-β on cartilage growth may be indirect such that in the presence of 300 pg/ml TGF-β1 another, secondary molecule(s) is either expressed or activated which then itself regulates the rate of cartilage growth.

Activation of perichondrium-produced TGF-β

We observed that in response to TGF-β1 treatment PC cells produce a threshold level of active TGF-β (~285 pg/ml) no matter how much total (active plus latent) TGF-β they produce. The mechanism through which this precise amount of active TGF-β is generated from the latent pool is unknown, but one possibility is that TGF-β behaves in an autocrine fashion to regulate its own activation - as has been reported for its translational expression (Kim *et al.*, 1990). Another possibility is that PC cells produce TGF-β scavengers such as alpha-2-macroglobulin (Fabrizi *et al.*, 1999) which could decrease the amount of TGF-β available to the cartilage, but this remains to be tested.

Although we cannot eliminate the possibility that some of the active TGF-β detected is a result of carryover from that used to treat the cultures, for three reasons any appreciable carryover seems unlikely. First, the form of TGF-β1 used to treat the cultures with was the active form and the major form of TGF-β detected in the conditioned media was the latent form and therefore cannot result from carry over. Second, the conditioned media from untreated, control cell cultures also contained an appreciable amount of active TGF-β and this could not result from carry over. Third and perhaps most significant, a threshold level of active factor is reached, no matter how much active factor is used for the treatments.

It has also been reported that the expression of TGF-β mRNA is increased in the PC of mouse long bone rudiments in response to treatment with Shh, a functional substitute for Ihh (Alvarez *et al.*, 2002). However, since the levels of (active) protein were not examined in that study, we cannot compare whether the amount of active TGF-β produced by the PC cells in response to treatment with TGF-β1 would be the same as that which would result from treatment with Shh - especially as the mechanism responsible for activating the specific amount of TGF-β that is required to precisely regulate cartilage growth remains unknown.

Materials and Methods

Perichondrial and periosteal cell cultures and collection of conditioned media

Cultures of PC and PO cells were generated as described previously (Di Nino *et al.*, 2001; Di Nino *et al.*, 2002). Briefly, the tissues were

dissected from 12-day chicken (*Gallus gallus*) embryo tibiotarsi, minced with #15 scalpel blades and allowed to attach as small pieces on 60 mm tissue culture plates (Falcon) in medium [DMEM (Gibco) containing 10% fetal calf serum (Hyclone) and penicillin and streptomycin (Gibco)]. Cultures were maintained at 37°C in 7% CO_2 for 7 days, after which time they were passaged by trypsinization [0.25% trypsin (Gibco) for 3 minutes at 37°C] and plated into 60 mm tissue culture dishes (Falcon). Cells were grown to confluency (4 to 5 days) in serum-containing medium. The medium was changed daily with the TGF-β1 treated cultures receiving exogenous TGF-β1 [(R&D Systems) in 4 mM HCl + 1% BSA]. Once the cells reached confluency, the medium was removed, the cells were rinsed with Hanks buffered saline (Gibco) and switched to serum-free DMEM. After 18 hours, conditioned medium was collected and used immediately or stored at 4°C.

Determination of levels of TGF-β in conditioned medium

The concentrations of active and total TGF-β in the conditioned media from the PC and PO cell cultures were determined using the PAIL assay. For this assay, mink lung epithelial cells (Abe *et al.*, 1994) stably transfected with a TGF-β-responsive plasminogen activator inhibitor-1 (PAI-1) promoter construct linked to a luciferase reporter gene (PAIL cells) were grown in 24 well tissue culture plates (BD Biosciences) in 0.5ml of the PC or PO conditioned medium (in triplicate) for 20 hours. To determine total TGF-β (active plus latent), the conditioned media were heat activated (85°C for 12 minutes) prior to addition to the PAIL cells. The cells were then washed with phosphate buffered saline and the cell lysates were assayed for luciferase activity using the Promega Luciferase Assay System (Promega) and an Optocomp I luminometer (GEM Instruments). Standard curves were generated using commercially available active TGF-β1. Analysis of variance between groups (ANOVA) was performed on all groups; Student's *t*-tests were performed on select pairs.

Tibiotarsal organ cultures

Tibiotarsi were cultured as described previously (Di Nino *et al.*, 2001; Di Nino *et al.*, 2002). Briefly, 12-day embryonic chicken tibiotarsi were dissected with the skin, muscle and connective tissues removed, with the PC/PO remaining intact (intact cultures) or removed (PC/PO-free cultures). Pairs of tibiotarsi consisting of one intact tibiotarsus and one PC/PO-free tibiotarsus were grown in organ culture dishes (Falcon) on a piece of Millipore filter (Millipore HTTP4700) supported on a stainless steel mesh grid (Mesh Grid Co.) for 3 days (37°C; 7% CO2) with medium changed daily. Intact cultures were grown in serum-free DMEM whereas PC/PO-free cultures were grown in either serum-free DMEM with or without TGF-β1 [in 4 mM HCl + 1% BSA] or in serum-free conditioned medium produced by PC cell cultures.

Analysis of organ cultures

Tibiotarsal organ cultures were analyzed as described previously (Di Nino *et al.*, 2001; Di Nino *et al.*, 2002). Briefly, to measure the length of the cartilagenous ends of the tibiotarsi the border between the cartilage and the sleeve of diaphyseal bone had to be visualized. For this the bone of the tibiotarsi was stained with 0.1% Alizarin red S (Fisher) in phosphate buffered saline at room temperature for 7 minutes (to facilitate penetration of the stain the PC and PO were removed from the intact tibiotarsi before staining). Then the tibiotarsi were photographed with an RT-Spot camera (Spot Diagnostics) attached to a Leica dissecting microscope. Then from the photographs, the cartilage of each tibiotarsus was measured along the midline from the articular surface to the bony collar (see Fig. 3A) using the ImagePro program (Media Cybernetics) and the average lengths were calculated. Standard deviations were calculated and paired *t*-tests were performed.

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