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Transforming Growth Factor-β1 Modulates Chondrocyte Responsiveness to 17β-Estradiol

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This study examined the interrelationship between transforming growth factor-beta1 (TGF-β1) and 17β-estradiol (E_2) in the regulation of growth plate chondrocytes. To determine whether TGF-β1 modulates chondrocyte response to E_2, we used cells isolated from the resting zone (RC) and growth zone (GC) of costochondral cartilage. Confluent, fourth-passage cultures were pretreated with rhTGF-β1 for 24 h, followed by treatment with E_2 for 24 h. The effect of TGF-β1 and E_2 alone, or the sequential combination, were examined by measuring [^{3}H]-thymidine incorporation (proliferation), alkaline phosphatase (AP) specific activity (differentiation), and [^{35}S]-sulfate incorporation (matrix synthesis). TGF-β1 alone increased [^{3}H]-thymidine incorporation in both female and male RC and GC cells, but E_2 affected this parameter only in RC cells, causing a dose-dependent decrease. At the highest concentration of TGF-β1 and E_2, [^{3}H]-thymidine incorporation in female GC cells was the same as seen in untreated control cultures. In male GC cells, [^{3}H]-thymidine incorporation in cultures treated with TGF-β1 and E_2 exhibited a comparable increase, as was seen in cultures treated with TGF-β1 alone. TGF-β1 caused a biphasic stimulation in AP that was maximal at 0.22 ng/mL, in both female and male RC and GC cells. E_2, however, affected only female cells. Whereas the effect of TGF-β1 predominated in RC and GC male cells, the biphasic stimulation caused by E_2, maximal at 10^{-9} *M*, predominated in female RC cells. In female GC cells, however, TGF-β1 caused a synergistic response, resulting in enhanced AP specific activity in cultures pretreated with 0.22 ng/mL of TGF-β1 and 10^{-8} *M* E_2. TGF-β1 alone caused dose-dependent increases in [^{35}S]-sulfate incorporation in female RC and GC cells, as well as in male GC cells, but had no effect on male RC cells. E_2 affected only female cells. TGF-β1 potentiated the effect of E_2 on this parameter, resulting in synergistic increases in the female cells. This is the first demonstration of a gender-specific response to TGF-β1 in chondrocytes. These results suggest that chondrocyte response to a systemic hormone such as E_2 can be modulated by local regulatory agents such as TGF-β1.

Key Words: 17β-estradiol; TGF-β; growth plate; chondrocyte.

Introduction

It is well established that estrogens play a major role in the regulation of mammalian bone growth, development, and remodeling. Many of these hormonal effects are mediated indirectly by other hormones and local factors secreted by cells in response to hormonal stimulation *(1–4)*. However, much of evidence accumulated from both in vivo *(5,6)* and in vitro *(7–9)* studies strongly suggests that sex hormones exert direct effects as well. In cartilage, the direct effects of 17β-estradiol include inhibition of chondrocyte proliferation *(7–9)*, stimulation of protein synthesis and extracellular matrix production *(10–12)*, and cell differentiation *(9,13–15)*. In addition, many of these effects on chondrocytes are gender specific *(8–12,16,17)*, and some are cell maturation dependent *(9,12)*.

It has been traditionally accepted that the direct effects of estrogens are mediated through classic steroid hormone receptor mechanisms. Specific receptors for estrogens have been found in chondrocytes *(18–23)*. However, recent evidence also suggests that some of the direct effects of 17β-estradiol are mediated through membrane-associated nongenomic mechanisms *(14)*.

Transforming growth factor-β1 (TGF-β1) is a major regulator of chondrocyte maturation and differentiation as well *(24–27)*. TGF-β1 is synthesized as a latent complex and is activated in the tissue *(26,28,29)*; hence, part of its regulation of chondrocyte biology is probably via autocrine mechanisms *(25,26,30)*. TGF-β1 has been found to regulate

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chondrocyte proliferation *(25)*, as well as increase collagen *(31)* and proteoglycan *(25,32,33)* production. Recently, it was found that the effect of TGF-β1 on chondrocytes is cell maturation dependent *(27)*. Moreover, TGF-β1 is produced by chondrocytes *(26)*. TGF-β1 stimulates protein kinase C (PKC) activity in chondrocyte cultures after a 6-h exposure to the growth factor, suggesting that some of its effects could be mediated through this signal transduction pathway *(25,27)*.

It is becoming increasingly clear that TGF-β1 has interactive effects with other growth factors known to regulate chondrocyte metabolism, such as fibroblast growth factor, insulin-like growth factor-1, and vitamin D metabolites *(34–37)*. Recently, we found that there is a synergistic enhancement of resting zone chondrocyte (RC) differentiation and PKC activity *(25)* when cells are exposed to rhTGF-β1 and 24,25-$(OH)_2D_3$ together, and that activation of latent TGF-β1 produced by RC and growth zone chondrocytes (GCs) is regulated by 1,25-$(OH)_2D_3$ *(26)*. Furthermore, we found that pretreatment of these chondrocytes with vitamin D metabolites modulates the effects of 17β-estradiol in a hormone-specific and sex- and cell maturation-dependent manner *(38)*.

There appears to be a relationship between TGF-β1 and estrogen as well. TGF-β1 secretion by osteoblast cultures is increased in response to estrogens and is depressed in ovariectomized animals *(39–41)*. Moreover, lower doses of TGF-β1 inhibit bone resorption *(42)* and the number of trabecular osteoclasts *(43)*. This suggests that the local effects of TGF-β1 may modulate the effects of steroid hormones such as 17β-estradiol in a manner similar to that described for TGF-β1 and seco-steroids such as the vitamin D metabolites.

To our knowledge, there has been no study regarding the interrelationship between TGF-β1 and estrogen in chondrocytes. Accordingly, the aim of the present study was to examine whether the effects of 17β-estradiol on proliferation, differentiation, and matrix synthesis of growth plate chondrocytes were modulated by pretreatment with TGF-β1 and to determine whether such modulation is sex specific or cell maturation state dependent. To do this, we used a cell culture system in which chondrocytes at two different stages of maturation were isolated from two different zones of costochondral cartilage from male and female rats *(38,44,45)*.

Results

[^{3}H]-Thymidine Incorporation

Recombinant human TGF-β1 (rhTGF-β1) and 17β-estradiol both modulated [^{3}H]-thymidine incorporation by RC cells, but elicited opposite effects (Table 1). Whereas rhTGF-β1 caused an increase, 17β-estradiol caused a decrease in cells from both female and male rats. Pretreatment of the cultures with rhTGF-β1 increased basal levels of [^{3}H]-thymidine incorporation, but it did not change the effect of 17β-estradiol. Female GC cells responded to rhTGF-β1, 17β-estradiol, or rhTGF-β1 followed by 17β-estradiol in a manner similar to that described for RC cells (Fig. 1A). Male GC cells, however, exhibited an increase in [^{3}H]-thymidine incorporation in response to rhTGF-β1, but 17β-estradiol did not counter the stimulatory effect of rhTGF-β1 (Fig. 1B). When male GC cells were pretreated with rhTGF-β1, the expected increase in basal [^{3}H]-thymidine incorporation was observed in control cultures and cultures treated with 17β-estradiol.

Table 1
[^{3}H]-Thymidine Incorporation by Female and Male RCs After Pretreatment with rhTGF-β1 for 24 h Followed by Treatment with 17β-Estradiol[a]

Treatment		cpm/well (× 10^4)	
TGF-β1 (ng/mL)	17β-Estradiol (*M*)	Female RCs	Male RCs
0	0	2.03 ± 017	4.71 ± 0.36
	10^{-10}	2.15 ± 0.20	4.83 ± 0.44
	10^{-9}	1.83 ± 0.21	4.46 ± 0.29
	10^{-8}	1.44 ± 0.07[e]	3.22 ± 0.19[e]
0.22	0	3.10 ± 0.17[c]	5.81 ± 0.27[c]
	10^{-10}	2.75 ± 0.26[c]	5.51 ± 0.25[c]
	10^{-9}	2.71 ± 0.21[c]	3.92 ± 0.62[b]
	10^{-8}	2.59 ± 0.21[b,c]	4.28 ± 0.28[b]
0.88	0	3.23 ± 0.15[c]	6.25 ± 0.25[c]
	10^{-10}	3.25 ± 0.04[c]	6.01 ± 0.46[c]
	10^{-9}	3.12 ± 0.13[c]	5.68 ± 0.33[c,d]
	10^{-8}	2.67 ± 0.16[b,c]	4.83 ± 0.40[b]

[a]Confluent, fourth-passage RCs from male and female rats were pretreated for 24 h with control media, or media containing 0.22 or 0.88 ng/mL of rhTGFβ-1. At the end of pretreatment, the media were replaced with fresh control media or media containing 10^{-10} to 10^{-8}*M* 17β-estradiol, the incubation was continued for another 24 h, and [^{3}H]-thymidine incorporation by the cells was measured. Values are the mean ± SEM of six cultures. Data are from one of three replicate experiments. [b]$p < 0.05$: pretreatment with TGF-β1 followed by treatment with 17β-estradiol vs pretreatment with TGF-β1 followed by treatment with control media.[c]$p < 0.05$: TGF-β1 pretreatment vs control. [d]$p < 0.05$: pretreatment with 0.22 vs 0.88 ng/mL TGF-β1. [e]$p < 0.05$: pretreated with media followed by treatment with 17β-estradiol vs. control.

Alkaline Phosphatase Specific Activity

The effect of rhTGF-β1 on alkaline phosphatase (AP) specific activity was independent of gender or cell maturation state. For both male and female RCs (Fig. 2) or GCs (Fig. 3), rhTGF-β1 caused a biphasic increase in enzyme activity that was significant at 0.22 ng/mL. By contrast, the effects of 17β-estradiol were gender specific, causing increases

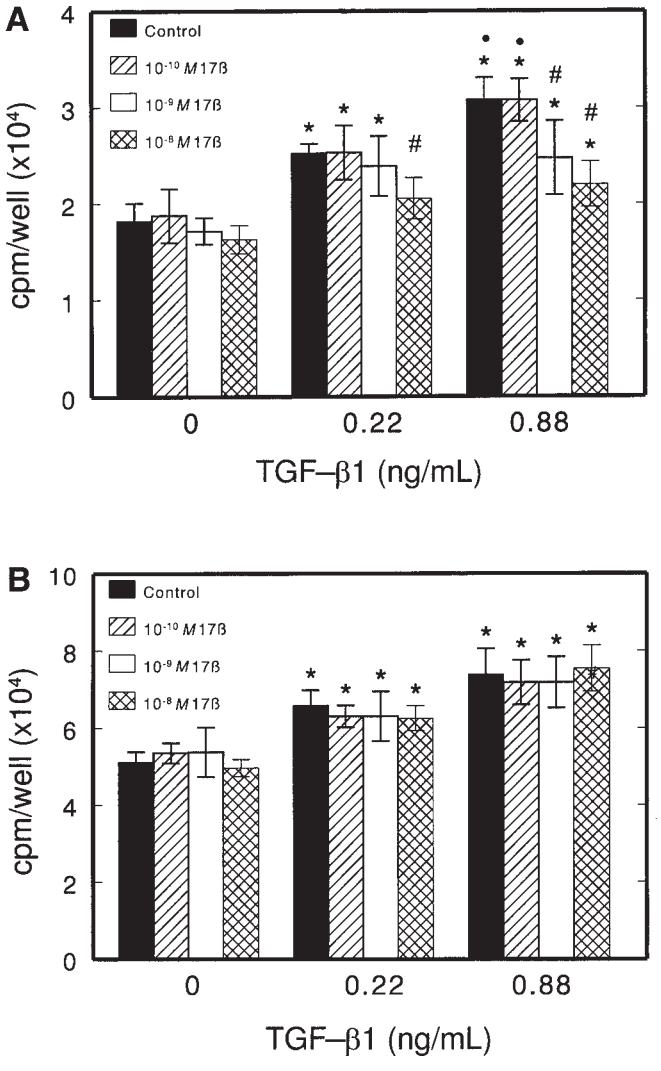

Fig. 1. [^{3}H]-Thymidine incorporation by female and male growth zone chondrocytes after pretreatment with rhTGF-β1 for 24 h followed by treatment with 17β-estradiol. Confluent, fourth-passage GCs from female **(A)** and male **(B)** rats were pretreated for 24 h with control media or media containing 0.22 or 0.88 ng/mL of rhTGF-β1. At the end of pretreatment, the media were replaced with fresh control media or media containing 10^{-10} to $10^{-8}M$ 17β-estradiol; the incubation was continued for another 24 h, and [^{3}H]-thymidine incorporation by the cells was determined as described in Materials and Methods. Values are the mean ± SEM of six cultures. Data are from one of three separate experiments, all with comparable results. *,$p < 0.05$: rhTGF-β1 pretreatment vs control; $^\#$,$p < 0.05$: rhTGF-β1 pretreatment followed by treatment with 17β-estradiol vs rhTGF-β1 pretreatment followed by treatment with control media; $^\bullet$,$p < 0.05$: 0.88 vs 0.22 ng/mL of rhTGF-β1 pretreatment.

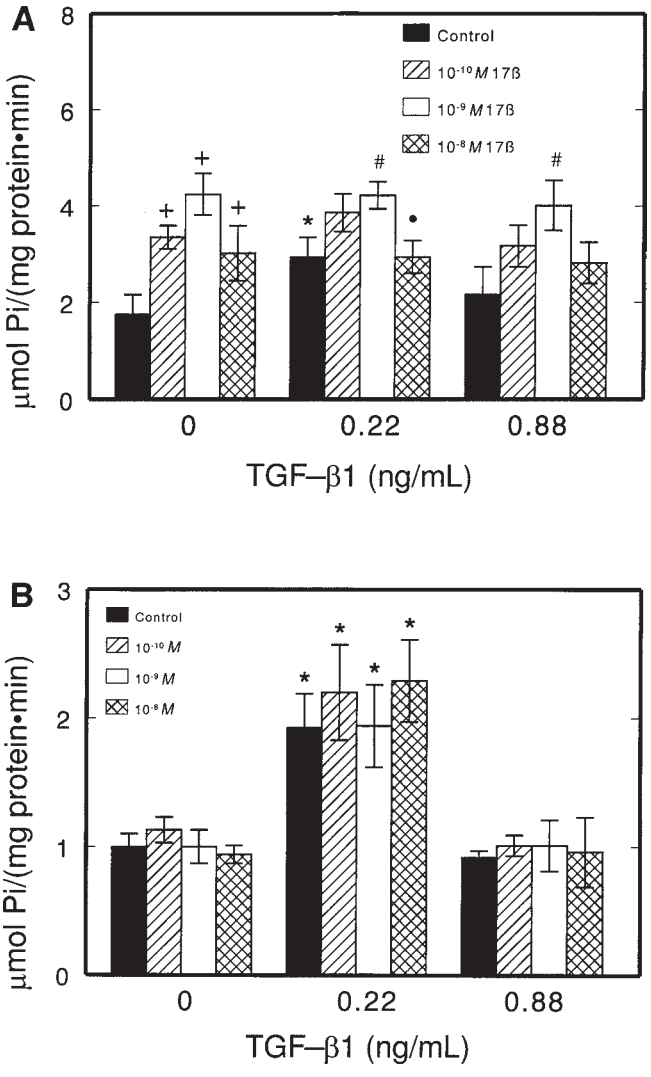

Fig. 2. AP specific activity in the cell layer of female **(A)** and male **(B)** RC cultures after pretreatment with rhTGF-β1 for 24 h followed by treatment with 17β-estradiol. Confluent, fourth-passage RCs from male and female rats were pretreated with control media or media containing 0.22 or 0.88 ng/mL rhTGF-β1 for 24 h. At the end of pretreatment, the media were replaced with fresh control media or media containing 10^{-10} to $10^{-8}M$ 17β-estradiol, the incubation was continued for another 24 h, and AP specific activity in the cell layer was determined as described in Materials and Methods. Values are the mean ± SEM of six cultures. Data are from one of three separate experiments, all with comparable results. *,$p < 0.05$: rhTGF-β1 pretreatment vs control; $^\#$,$p < 0.05$: TGF-β1 pretreatment followed by treatment with 17β-estradiol vs TGF-β1 pretreatment followed by treatment with control media; $^\bullet$,$p < 0.05$: treatment with 10^{-8} vs $10^{-9}M$ 17β-estradiol, after pretreatment with the same dose of rhTGF-β1; $^+$,$p < 0.05$: treatment with 17β-estradiol vs control.

only in female chondrocytes. RC cells exhibited maximal stimulation at $10^{-9}M$ 17β-estradiol (Fig. 2A), whereas maximal activity was noted in GC cells at $10^{-8}M$ 17β-estradiol (Fig. 3A).

In cultures pretreated with rhTGF-β1, the gender-specific effects of 17β-estradiol were retained. In male cells, only the effects of rhTGF-β1 were evident (Figs. 2B and 3B). In female cells, the interrelationship between 17β-estradiol and TGF-β1 was cell maturation dependent. In female RC cells, the dose-dependent effects of 17β-estradiol predominated, even though basal activity in cultures pretreated with 0.22 ng/mL rhTGF-β1 was higher (Fig. 2A). By contrast, rhTGF-β1 potentiated the effects of 17β-estra-

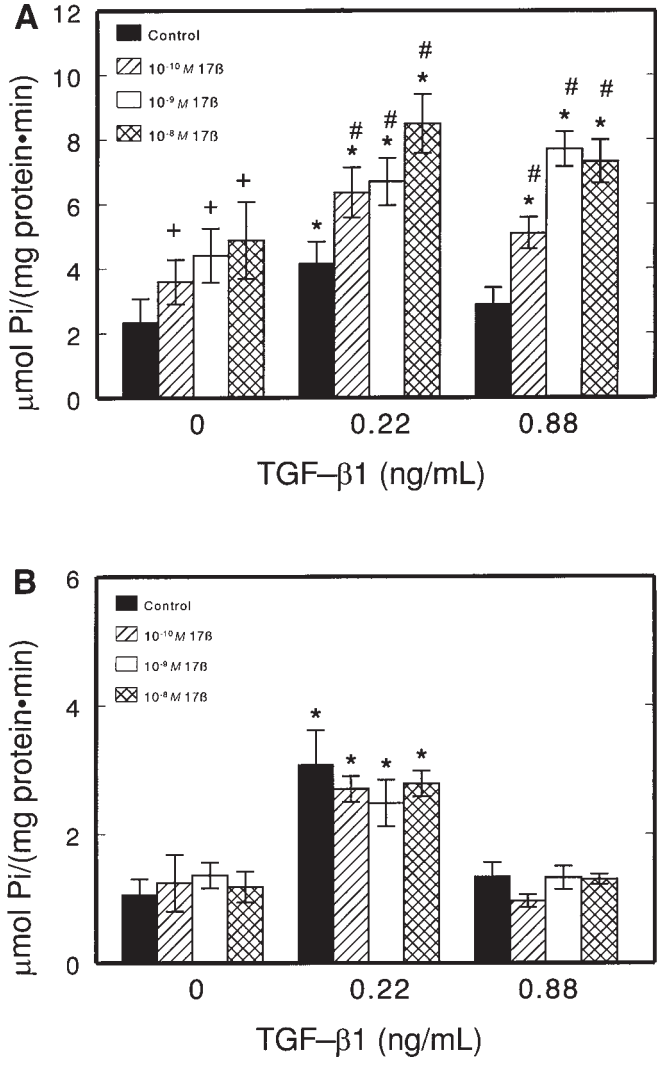

Fig. 3. AP specific activity in the cell layer of female **(A)** and male **(B)** GC cultures after pretreatment with rhTGF-β1 for 24 h followed by treatment with 17β-estradiol. Confluent, fourth-passage GCs from male and female rats were pretreated with control media or media containing 0.22 or 0.88 ng/mL rhTGF-β1 for 24 h. At the end of pretreatment, the media were replaced with fresh control media or media containing 10^{-10} to $10^{-8}M$ 17β-estradiol, the incubation continued for another 24 h, and AP specific activity in the cell layer was determined as described in Materials and Methods. Values are the mean ± SEM of six cultures. Data are from one of three separate experiments, all with comparable results. *,$p < 0.05$: TGF-β1 pretreatment vs control. $^{\#}$,$p < 0.05$: TGF-β1 pretreatment followed by treatment with 17β-estradiol vs TGF-β1 pretreatment followed by treatment with control media; $^{+}$,$p < 0.05$: treatment with 17β-estradiol vs control.

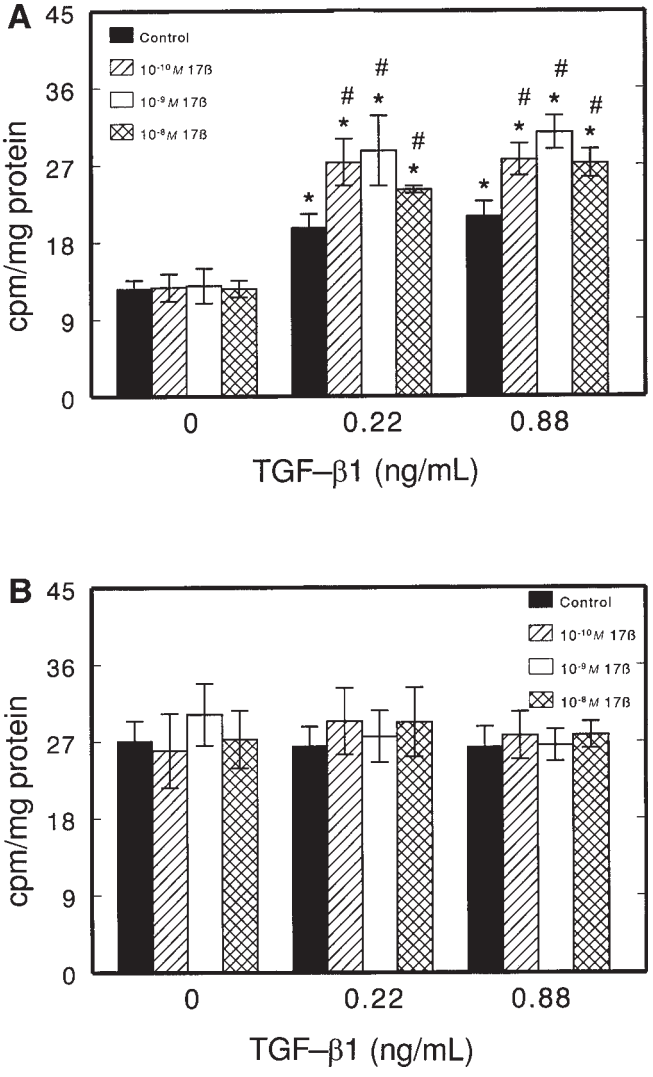

Fig. 4. [^{35}S]-Sulfate incorporation in the cell layer of female **(A)** and male **(B)** RC cultures after pretreatment with rhTGF-β1 for 24 h followed by treatment with 17β-estradiol. Confluent, fourth-passage RCs from male and female rats were pretreated with control media or media containing 0.22 or 0.88 ng/mL rhTGF-β1 for 24 h. At the end of pretreatment, the media were replaced with fresh control media or media containing 10^{-10} to $10^{-8}M$ 17β-estradiol, the incubation was continued for another 24 h, and [^{35}S]-sulfate incorporation into the cell layer was measured as described in Materials and Methods. Values are the mean ±SEM of six cultures. Data are from one of three separate experiments, all with comparable results. *,$p < 0.05$: rhTGF-β1 pretreatment vs control; $^{\#}$,$p < 0.05$: rhTGF-β1 pretreatment followed by treatment with 17β-estradiol vs rhTGF-β1 pretreatment followed by treatment with control media.

diol in female GC cells (Fig. 3A), resulting in synergistic increases in enzyme activity in cultures pretreated with both 0.22 and 0.88 ng/mL.

[^{35}S]-Sulfate Incorporation

Our results show, for the first time, that the effects of rhTGF-β1 on [^{35}S]-sulfate incorporation are not only cell maturation dependent, but also sex specific (Figs. 4 and 5).

As shown previously, rhTGF-β1 had no effect on [^{35}S]-sulfate incorporation in male RCs (Fig. 4B), but caused a dose-dependent increase in male GCs, significant at 0.88 ng/mL (Fig. 5B). Both RCs and GCs from female rats exhibited dose-dependent increases in [^{35}S]-sulfate incorporation; in RC cells, the effect was significant at 0.22 ng/mL (Fig. 4A), and in GC cells, the effect was significant at 0.88 ng/mL (Fig. 5A).

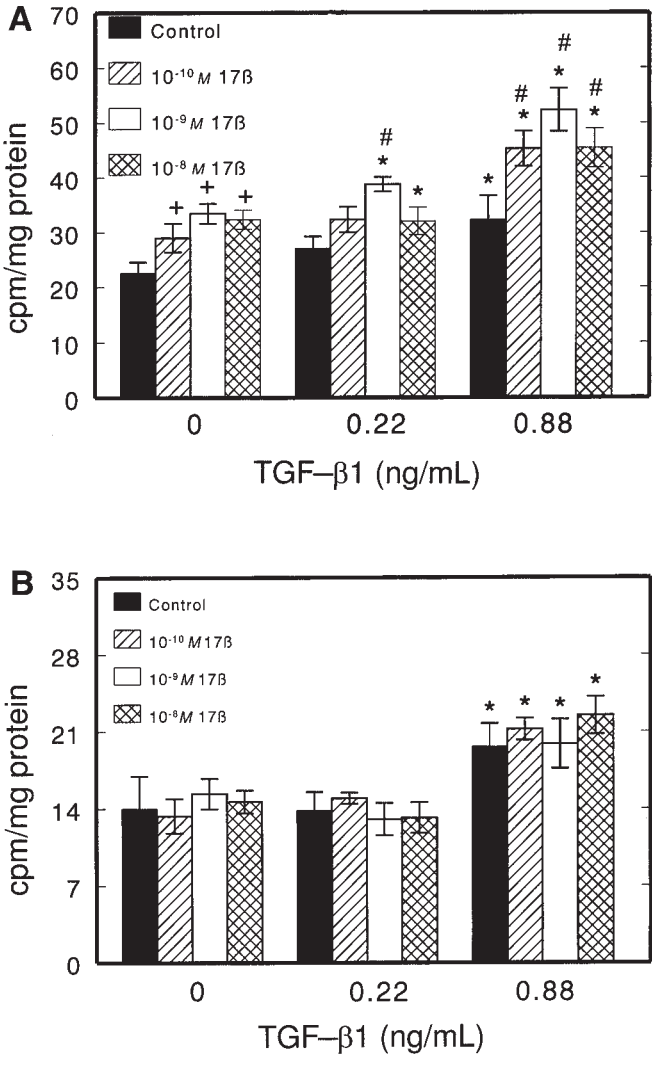

Fig. 5. [^{35}S]-Sulfate incorporation in the cell layer of female **(A)** and male **(B)** GC cultures after pretreatment with rhTGF-β1 for 24 h followed by treatment with 17β-estradiol. Confluent, fourth-passage GCs from male and female rats were pretreated with control media or media containing 0.22 or 0.88 ng/mL of rhTGF-β1 for 24 h. At the end of pretreatment, the media were replaced with fresh control media or media containing 10^{-10} to 10^{-8}*M* 17β-estradiol, the incubation was continued for another 24 h, and [^{35}S]-sulfate incorporation into the cell layer was measured as described in Materials and Methods. Values are the mean ± SEM of six cultures. Data are from one of three separate experiments, all with comparable results. *,$p < 0.05$: rhTGF-β1 pretreatment vs control; $^{\#}$,$p < 0.05$: rhTGF-β1 pretreatment followed by treatment with 17β-estradiol vs rhTGF-β1 pretreatment followed by treatment with control media; $^{+}$,$p < 0.05$: treatment with 17β-estradiol vs control.

17β-Estradiol had no effect on [^{35}S]-sulfate incorporation in male chondrocyte cultures, regardless of maturation state (Figs. 4B and 5B). When male cells were pretreated with rhTGF-β1, the effect of the growth factor predominated. Thus, an increase was noted only in male GC cells pretreated with 0.88 ng/mL of TGF-β1 whether or not the cells were subsequently exposed to 17β-estradiol.

In female cells, 17β-estradiol caused a dose-dependent increase in [^{35}S]-sulfate incorporation only in GC cells (Fig. 5A). The effect was maximal at 10^{-9} *M*. Even when GC cells were pretreated with 0.22 ng/mL of rhTGF-β1, the effect of 17β-estradiol was comparable to that seen in the absence of the growth factor. However, at 0.88 ng/mL of rhTGF-β1, the response of female GC cells to 17β-estradiol was potentiated, and at 10^{-9} *M* 17β-estradiol, the increase was synergistic. The synergistic effect of rhTGF-β1 with 17β-estradiol was evident in RC cells at 0.22 ng/mL of rhTGF-β1 (Fig. 4A).

Discussion

The results of this study, summarized in Table 2, support the hypothesis that TGF-β1 modulates cellular response to 17β-estradiol. TGF-β1 potentiated the stimulatory effect of 17β-estradiol on AP specific activity in GC cells, resulting in synergistic increases at 0.22 and 0.88 ng/mL of growth factor. Pretreatment with rhTGF-β1 also potentiated the effects of 17β-estradiol on proteoglycan production in both RC and GC cultures. The fact that rhTGF-β1 pretreatment caused a synergistic increase in proteoglycan production in RC cells was particularly remarkable because in the absence of pretreatment, 17β-estradiol had no effect on [^{35}S]-sulfate incorporation in cultures of these chondrocytes *(12)*.

The effect of TGF-β1 is dependent on the maturation state of the chondrocytes. Whereas 17β-estradiol-dependent AP activity was stimulated by TGF-β1 in GC cells, synergistic increases in enzyme activity were not noted in RC cell cultures. Both TGF-β1 and 17β-estradiol cause increases in matrix vesicle AP specific activity *(9,46)*. Although RC chondrocytes produce matrix vesicles enriched in AP, this phenotypic characteristic is expressed, to a greater extent, in matrix vesicles produced by GC cells *(44,47)*. The effect of TGF-β1 alone is slightly greater in RC cells *(46)*; however, the effect of 17β-estradiol is much greater in matrix vesicles produced by GC cells *(9)*. This suggests that the synergistic effect of TGF-β1 on 17β-estradiol-dependent AP is targeted to these extracellular organelles, supporting the interpretation that endochondral differentiation is stimulated.

The effect of TGF-β1 was dose dependent. Although peak increases in AP were observed at 0.22 ng/mL rhTGF-β1, the greatest synergistic effects were noted in cultures of GCs treated with 0.88 ng/mL of rhTGF-β1. This was even more evident with respect to proteoglycan sulfation. The synergistic effect of TGF-β1 on proteoglycan production in RC cell cultures was sensitive to the lower concentration of growth factor, with no further enhancement of this effect at the higher concentration.

This is the first demonstration that TGF-β1 regulates growth plate chondrocytes in a gender-specific manner. As noted previously, neither AP specific activity nor proteoglycan sulfation is sensitive to 17β-estradiol in chondrocytes from male rats *(12)*, even though these cells possess traditional nuclear

Table 2
Comparison of Effects of TGF-β1, 17β-Estradiol, and TGF-β1 + 17β-Estradiol on Proliferation ([^{3}H]-thymidine incorporation), Differentiation (AP specific activity), and Matrix Synthesis ([^{35}S]-sulfate incorporation) in Cultures of Female or Male Resting Zone (RC) or Growth Zone (GC) Costochondral Cartilage Cells

		[^{3}H]-Thymidine incorporation				AP activity				[^{35}S]-Sulfate incorporation			
		Female		Male		Female		Male		Female		Male	
Treatment		RC	GC	RC	GC	RC	GC	RC	GC	RC	GC	RC	GC
TGF-β1 (ng/mL)	0.22	↑	↑	↑	↑	↑	↑	↑	↑	↑	—	—	—
	0.88	↑	↑	↑	↑	—	—	—	—	↑	↑	—	↑
17β-Estradiol ($10^{-8}M$)		↓	—	↓	—	↑	↑	—	—	—	↑	—	—
TGF-β1 + 17β-estradiol ($10^{-8}M$)	0.22	↑	↑	↓	—	↑	↑↑	↑	↑	↑↑	↑	—	—
	0.88	↑	↑	↓	—	↑	↑↑	—	—	↑↑	↑↑	—	↑

[α]↑, increase; ↑↑, strong increase; ↓, decrease; —, no change or effect.

receptors for this hormone *(18)*. By contrast, both RC and GCs from male rats respond to TGF-β1 with a dose-dependent increase in AP specific activity, and male GCs also exhibit a dose-dependent increase in proteoglycan production *(46)*. Pretreatment with the growth factor resulted only in the expected response to TGF-β1, regardless of the concentration of 17β-estradiol used.

In addition to modulating cell response to 17β-estradiol via gender-specific mechanisms, TGF-β1 also exerts direct effects on the cells that are gender specific. Our previous studies examining the direct effects of TGF-β1 on rat costochondral chondrocytes in culture *(26,46,48,49)* always used cells isolated from male rats. The present study confirmed these published studies, showing that the effect of TGF-β1 was cell maturation dependent, since proteoglycan production was stimulated in GC cells at 0.88 ng/mL, but no effect was noted in RC cells at either concentration of growth factor. In contrast, proteoglycan production was stimulated at both concentrations of TGF-β1 in cultures of both RC and GC cells from female rats. Thus, the response of female chondrocytes is gender-specific but not cell maturation state specific.

Proliferation of chondrocytes from female and male rats was sensitive to both TGF-β1 and 17β-estradiol. Whereas TGF-β1 elicited a dose-dependent increase in [^{3}H]-thymidine incorporation, 17β-estradiol caused a dose-dependent decrease. Pretreatment with rhTGF-β1 caused an increase in basal DNA synthesis, but did not alter the dose-dependent decrease owing to 17β-estradiol. As a result, [^{3}H]-thymidine incorporation was higher in cultures pretreated with 0.88 ng/mL rhTGF-β1 and 10^{-8} *M* 17β-estradiol than in control cultures exposed to the steroid hormone, but the actual effect of the hormone was not changed.

Although this is the first study to examine the interrelationship between TGF-β1 and 17β-estradiol in chondrocytes, there have been some studies demonstrating a relationship between these two factors in the regulation of bone. Estrogens increase synthesis of TGF-β1 by osteoblast cultures *(40)* and TGF-β3 gene expression in rat bone *(50)*. Conversely, TGF-β1 production is depressed in ovariectomized animals *(41)*. Bone resorption is also affected *(51)*. The direct action of 17β-estradiol on osteoclasts, resulting in lower resorption activity, is probably mediated by autocrine/paracrine production and activation of TGF-β *(52)*.

The synergistic response elicited by sequential exposure of the cells to TGF-β1 and 17β-estradiol suggests that sensitization or induction has occurred. These cellular responses may be mediated through genomic or nongenomic mechanisms, or both *(26)*. One of the genomic mechanisms may be upregulation of genes for 17β-estradiol receptors. Alternatively, enzymes that modulate the 17β-estradiol receptor protein post translation may be affected, thereby increasing the binding capacity or affinity of 17β-estradiol receptors in the cell, augmenting the 17β-estradiol effect. This may involve changes in gene expression or modulation of enzyme activity via nongenomic signaling pathways.

We have previously shown the existence of nuclear receptors for 17β-estradiol in GCs and RCs isolated from the costochondral cartilage of male or female rats *(18)*. At present, there are no data in the literature indicating whether or not TGF-β1 regulates the 17β-estradiol receptor in rat costochondral chondrocytes. However, it has been reported recently that TGF-β1 regulates estrogen receptor expression in the MCF-7 breast cancer cell line *(52)*. Certainly within the time course used in the present study, there was ample time for gene expression to have been modulated by either TGF-β1 or 17β-estradiol, or both.

Recently, we have shown that both TGF-β1 *(25)* and 17β-estradiol *(53)* mediate some of their effects on chondrocyte

differentiation through regulation of PKC activity. The effects of 17β-estradiol on PKC are seen only in female cells, which may explain the synergistic effects of TGF-β1 and 17β-estradiol on AP specific activity in GCs from female rats. However, this does not explain why this synergy did not appear in RCs from female rats. Proteoglycan production is also sensitive to PKC regulation, suggesting that modulation of PKC by TGF-β1 and 17β-estradiol may also play a role in the synergistic effects of these factors on proteoglycan production in GC and RC cells from female rats.

Another mechanism through which TGF-β1 may mediate its regulation on 17β-estradiol is the second messenger, prostaglandin E_2(PGE_2). It has been shown that pretreatment with PGE_2 regulates responsivness to 17β-estradiol in epiphyseal cartilage *(54)*, and it was found that addition of TGF-β1 to articular cartilage cultures stimulated the production *(55)* and release *(56)* of PGE_2. PGE_2 causes an increase in AP specific activity in both RCs and GCs, as well as an increase in proteoglycan production *(57)*.

The results of the present study indicate that TGF-β1 modulates the effects of 17β-estradiol on costochondral chondrocytes from female rats in vitro in a gender– and cell maturation–specific manner. The exact mechanisms through which TGF-β1 mediates its effects on 17β-estradiol regulation are not clear. Two important questions are raised: (1) Does TGF-β1 regulate 17β-estradiol receptors in these chondrocytes? and (2) Does it mediate its effect on 17β-estradiol regulation through genomic or nongenomic mechanisms or through both? Resolution of these questions is presently under way.

Materials and Methods

Chondrocyte Cultures

The costochondral cartilage cell culture system used in this study has been described previously *(44)*. Chondrocytes were isolated from resting zone and growth zone cartilages from 125-g male or female Sprague Dawley rats and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 μg/mL of vitamin C, and 1% penicillin-streptomycin in an atmosphere of 5% CO_2 and 100% humidity at 37°C. Fourth-passage cells were used for the experiments because our previous studies demonstrated that the chondrocytes retained their differential phenotype and vitamin D metabolite–specific responses at this passage *(45,58)*.

Experimental Design

Fourth-passage RCs or GCs were grown to confluence, at which time the media were replaced and the cells treated for 24 h with media containing 0.22–0.88 ng/mL of rhTGF-β1 or vehicle alone (control). After removing these media, the cultures were incubated with 10^{-10} *M* to 10^{-8} M 17β-estradiol or vehicle alone (control) for 24 h. The cells were then harvested and assayed as described below. The hormone vehicle was ethanol diluted 1:5000 (v/v) with DMEM before addition to the culture media to minimize any toxic effects.

[^{3}H]-Thymidine Incorporation

The effect of TGF-β1 pretreatment on 17β-estradiol-dependent changes in chondrocyte proliferation was determined by measuring [^{3}H]-thymidine incorporation by quiescent RC or GC cells *(9)*. Chondrocytes were grown to confluence in 6-mm-diameter microwells, and 50 μL of [^{3}H]-thymidine (NEN-DuPont, Boston, MA) were added 4 h before harvest so that the final concentration in the medium was 2 μCi/mL. At harvest, the cell layers were washed twice with cold phosphate-buffered saline and twice with 5% trichloroacetic acid (TCA) and then treated with saturated TCA for 30 min. TCA-precipitable material was dissolved in 0.2 mL of 1% sodium dodecyl sulfate, and the radioactivity was measured by scintillation spectroscopy.

Alkaline Phosphatase Specific Activity

To assess the effect of TGF-β1 pretreatment on chondrocyte differentiation in response to 17β-estradiol, we measured alkaline phosphatase (AP) specific activity using lysates of the cell layers *(45,59)*. AP (orthophosphoric monoester phosphohydrolase, alkaline; EC 3.1.3.1) specific activity was measured as a function of *para*-nitrophenol release from *para*-nitrophenylphosphate at pH 10.2, as previously described *(60)*.

Matrix Synthesis

To determine the effect of TGF-β1 pretreatment on extracellular matrix synthesis in response to 17β-estradiol, we examined [^{35}S]-sulfate incorporation as an indicator of proteoglycan production *(61)*. Fourth-passage RC or GC cells were grown to confluence in 24-well culture plates (Corning, NY), and 50 μL of a solution containing 18μCi/mL [^{35}S]-sulfate (NEN-DuPont) and 0.814 m*M* carrier sulfate was added 4 h before harvest. The amount of [^{35}S]-sulfate incorporation into the cell layer was determined by liquid scintillation spectrometry and calculated as disintegrations per minute/milligram of protein.

Statistical Analysis

The data presented are from one of three separate sets of experiments, all with comparable results. For each variable in each independent experiment, six individual cultures were performed and examined. Data are presented as the mean ± SEM of these six cultures. Statistical significance was determined by comparing each value to the control using Bonferroni's modification of the student's *t*-test, with $p < 0.05$ considered significant.

Because these experiments used primary cultures and not cell lines and were conducted at different times, variability in the exact onset of confluence, cell culture media, and assay conditions contributed to differences in basal values for many parameters. It is tempting to cite

differences in basal conditions as contributing to the results. However, the only way to accurately compare data among experiments (or as a function of gender) is to compare treatment/control ratios. When we did this to assess the validity of our observations, basal conditions were eliminated as a variable.

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