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DEMONSTRATION OF RECEPTORS FOR EPIDERMAL GROWTH FACTOR ON CULTURED RABBIT CHONDROCYTES AND REGULATION OF THEIR EXPRESSION BY VARIOUS GROWTH AND DIFFERENTIATION FACTORS

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SUMMARY: Epidermal growth factor (EGF) receptors were demonstrated on cultured rabbit costal chondrocytes. After crosslinking, the receptors on the cells with ^{125}I-EGF, one major band of 170 KDa was separated by SDS-PAGE. Scatchard analysis demonstrated two classes of EGF receptors with Kd values of 0.3 nM and 1.6 nM. The numbers of high and low affinity receptors were 3,000 and 10,000 per cell, respectively. EGF receptors on chondrocytes were increased by treatment with retinoic acid and interleukin-1ß, which inhibited proteoglycan synthesis. On the other hand, parathyroid hormone and dibutyryl cyclic AMP, which stimulated proteoglycan synthesis, decreased the number of EGF receptors. Treatments with these agents did not change the affinity of the receptors. These findings suggest that the number of EGF receptors is a negative marker of chondrocyte differentiation.

Epidermal growth factor (EGF) has been shown to be physiologic important in many cells (1). For instance, this growth factor stimulates the proliferation of not only epithelial cells but also mesenchymal cells (1). It also has stimulatory or inhibitory effects on the differentiations of various types of cells (1). EGF has been shown to stimulate the proliferation of osteoblastic cells and inhibit bone formation and type I collagen synthesis by the cells (2,3). We have also shown that EGF stimulates DNA synthesis and inhibits proteoglycan synthesis, a differentiated phenotype of chondrocytes, in rabbit costal chondrocytes in culture (4). Previous findings indicate that the effects of EGF are mediated by EGF receptors, which have been demonstrated on various kinds of cells (1). However, there is no report on EGF receptors on chondrocytes.

Growth cartilage cells actively synthesize proteoglycans, typical markers of chondrocytes, when they mature during the process of endochondral ossification (5). Chondrocytes isolated from growth cartilage also synthesize proteoglycans in culture (6-12). This phenotype of chondrocytes is modulated by various factors. For example, EGF (4), retinoic acid (RA) (6-8) and interleukin-1ß (IL-1ß) (9) inhibit

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proteoglycan synthesis in cultured chondrocytes. On the other hand, PTH (6,10,11), cyclic AMP analogues (7,10), and insulin-like growth factor (IGF) (12,13) stimulate their expression by chondrocytes.

In this study, using cultured rabbit growth cartilage cells, we demonstrated the presence of EGF receptors on chondrocytes for the first time. We also observed a relationship between expression of EGF receptors and the differentiated phenotype of chondrocytes.

MATERIALS AND METHODS

Materials: Epidermal growth factor (EGF) and ^{125}I-labelled EGF (^{125}I-EGF) were obtained from Collaborative Research (Bedford, MA) and Amersham Japan (Tokyo). Retinoic acid (RA) and dibutyryl cyclic AMP (DBcAMP) were purchased from Sigma (St. Louis, MO). Bovine parathyroid hormone-(1-34) (bPTH) was obtained from Peninsula Laboratories (Belmont, CA). Insulin-like growth factor-I (IGF-I) was kindly provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). Interleukin-1ß (IL-1ß) was a gift from Otsuka Pharmaceutical Co. (Osaka, Japan). Other materials used were commercial products of the highest grade available.

Cell culture: Chondrocytes were isolated from growth cartilage of the ribs of young male New Zealand rabbits weighing 300-500 g as described previously (6,10). The isolated cells were plated at a density of 3.5 x 10^4 cells/well in 16-mm multiwell plates (Corning, NY), and cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) contaning 10% fetal bovine serum (GIBCO, Grand Island, NY) before used for experiments.

Binding studies: Binding assay was performed by a modification of the method of Carpenter *et al*.(14). Briefly, cell layers were washed three times with phosphate-buffered saline (PBS), incubated with binding buffer [Dulbecco's modified minimum essential medium (DMEM) (Nissui Pharmaceutical Co.) containing 15 mM HEPES (pH 7.2) and 0.1% bovine serum albumin (BSA)] for 15 min at 4 °C, and then incubated with ^{125}I-EGF and other components. The final volume of the incubation mixture was 300 µl. After incubation, the cell layers were washed six times with chilled 0.8 M NaCl containing 0.1% BSA and solubilized in 1.0 N NaOH. The radioactivity in the solution was measured in a Gamma counter.

Covalent attachment of ^{125}I-EGF to receptors: Crosslinking of ^{125}I-EGF to cultured chondrocytes was carried out by the method of Neufeld *et al*.(15). Briefly, after 2 h incubation with the radioligand, cell layers were washed five times with cold PBS and incubated with 0.15 mM disuccinimidyl suberate (DSS) in PBS at room temperature. The reaction was terminated by addition of quenching buffer (10 mM Tris-HCl, pH 7.5, 200 mM glycine, and 2 mM EDTA) for 2 min at room temperature. The quenching buffer was aspirated and the cells were scraped into sample buffer for SDS-PAGE [14 mM Tris-HCl (pH 6.8), 10%(v/v) glycerol, 3% (w/v) SDS and 180 mM 2-mercaptoethanol], denatured by heat treatment for 5 min at 100 °C and subjected to SDS-PAGE (16) and autoradiography.

Other methods: Proteoglycan synthesis was monitored by determining incorporation of [^{35}S]sulfate into cetylpyridinium chloride-precipitable materials (9). DNA synthesis was monitored by determining incorporation of [^{3}H]thymidine into acid precipitable materials as described (17).

RESULTS

When confluent cultures of rabbit costal chondrocytes were incubated with ^{125}I-EGF at 4°C, the total binding of EGF increased to a plateau after 2 h (Fig. 1). In

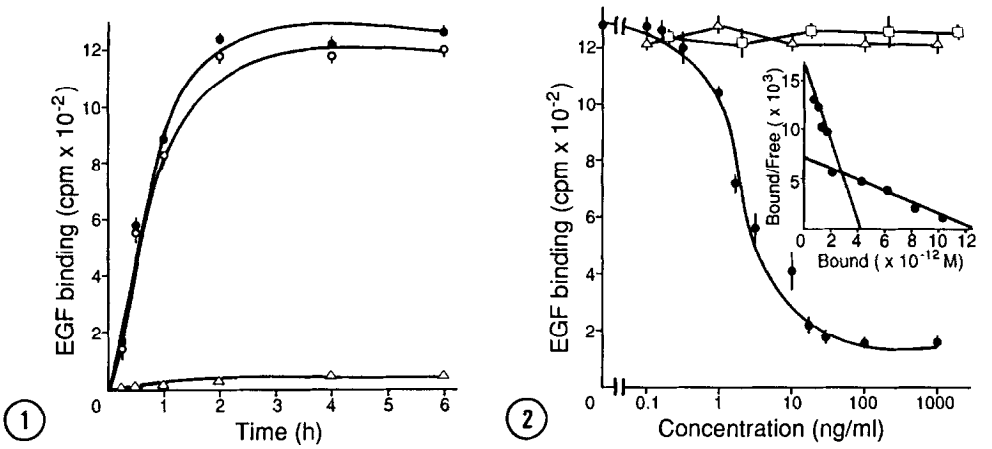

Fig. 1. Time course of ^{125}I-EGF binding to cultured rabbit costal chondrocytes. The cells were incubated with the radioligand (1 x 10^5 cpm: 2.48 x 10^{-10} M) in the absence or presence of EGF (1.64 x 10^{-7} M), and the radioactivity bound to the cells was counted at the indicated times. Points and bars are means and S.D., respectively. Total binding (●), specific binding (○),and nonspecific binding (△).

Fig. 2. Competitive inhibition of ^{125}I-EGF binding by unlabeled EGF or unrelated peptides. Cells were incubated with ^{125}I-EGF (1 x 10^5cpm: 2.48 x10^{-10} M) in the presence of the indicated concentrations of EGF (●), PTH (□) and IGF-I (△). Inset: Scatchard plot.

contrast, nonspecific binding, determined by simultaneous addition of 660-fold excess of unlabeled EGF, reached equilibrium in 1 h. The maximal nonspecific binding was only 2.7% of the maximal total binding, indicating that specific binding in the steady state was about 97.3% of the total binding.

Fig. 2 shows results for competitive binding assay in the steady state. Addition of EGF decreased the binding of ^{125}I-EGF dose-dependently; the inhibition was observable at a concentration of 0.5 ng/ml and complete at a concentration of 100 ng/ml. On the other hand, neither PTH nor IGF-I inhibited the binding of ^{125}I-EGF. Scatchard analysis of the data indicated the presence of two classes of binding sites on rabbit costal chondrocytes. The apparent dissociation constants (Kd) of the high and low affinity binding sites were 0.3 nM and 1.6 nM and the numbers of these high and low affinity binding sites (B_{max}) were about 3 x 10^3 sites/cell and 1 x 10^4 sites/cell, respectively.

For characterization of the physicochemical properties of EGF receptors, ^{125}I-EGF was covalently attached to rabbit chondrocytes by treatment with DSS, and the complex of labeled hormone and receptors was separated by SDS-PAGE under reducing conditions. An autoradiogram of the gel revealed one major band with an apparent mol wt of 180,000. This band disappeared almost completely on addition of 660-fold excess of unlabeled EGF (Fig. 3).

For examination of the relationship between the EGF receptor and proteoglycan synthesis, chondrocytes were cultured in the presence of RA, IL-1ß, PTH and DBcAMP for 72 h and then proteoglycan synthesis and ^{125}I-EGF binding were

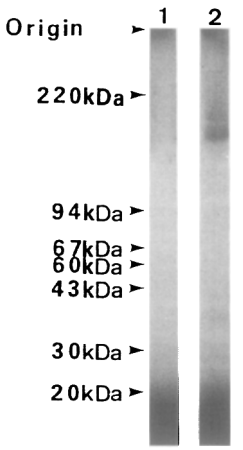

Fig. 3. Crosslinking of EGF receptors with ^{125}I-EGF. Rabbit costal chondrocytes were incubated with the ^{125}I-EGF (1 x 10^6 cpm) in the presence (lane 1) or absence (lane 2) of unlabeled EGF (1μg/ml). After crosslinking the cells were rinsed and solubilized in SDS-PAGE sample buffer with 180 mM 2-mercaptoethanol.

determined. As shown in Table 1, RA and IL-1ß which decreased proteoglycan synthesis, increased the number of EGF receptors. On the other hand, PTH and DBcAMP, which increased proteoglycan synthesis, decreased the number of EGF receptors. In both cases, there was no significant change in the Kd value of EGF receptors.

EGF stimulates DNA synthesis in rabbit costal chondrocytes in culture (4). To determine whether the decrease in the number of EGF receptors affects the

Table 1. Effects of RA, IL-1ß, PTH and DBcAMP on number of EGF receptors and proteoglycan synthesis

Treatment	Number of receptors		Proteoglycan synthesis
	low affinity (Kd=1.6 nM)	high affinity (Kd=0.3 nM)	(% of control)
	($x10^{-3}$ sites/cell)		
Control	10.0	3.0	100[a]
RA (0.2 μM)	18.0	5.4	26
IL-1ß (100 ng/ml)	16.0	4.8	29
PTH (0.1 μM)	7.7	2.3	141
DBcAMP (0.5 mM)	6.2	1.9	143

Rabbit costal chondrocytes were inoculated at a density of $3x10^4$ cells into 16-mm diameter multiwell plates in MEM containing 10% FBS. From day 6, they were cultured with RA, IL-1ß, PTH or DBcAMP for 3 days. On day 9, competitive inhibition analysis of EGF binding was carried out, and the receptor number and Kd value were calculated by Scatchard analyses. Proteoglycan synthesis was monitored by determining incorporation of [^{35}S]sulfate into cetylpyridinium chloride-precipitable materials. The control value of incorporation of $^{35}SO_4^{2-}$ into proteoglycan was 33,100 ± 1,800 dpm /10^5 cells.

[a] Values are means for three wells which differed by less than 10% from the means.

Table 2. Effects of EGF on DNA synthesis in chondrocytes pretreated with RA, IL-1ß, PTH or DBcAMP

Treatment	[^{3}H]Thymidine incorp. (dpm/10^4 cells)		Increase
	PBS	EGF	(% of control)
Control	259 ± 13	584 ± 41	100
RA (0.2 μM)	326 ± 27	1083 ± 68	233
IL-1ß (100 ng/ml)	312 ± 44	848 ± 43	165
PTH (0.1 μM)	350 ± 6	528 ± 18	55
DBcAMP (0.5 mM)	241 ± 19	417 ± 37	54

Rabbit costal chondrocytes were inoculated at a density of $3x10^4$ cells into 16-mm diameter multiwell plates in MEM containing 10% FBS. From day 6, they were then cultured with RA, IL-1ß, PTH or DBcAMP for 3 days. On day 9, the cell layers were incubated in a mixture of DMEM and Ham's-F12 (Nissui Pharmaceutical Co.) (v/v;1:1) containing EGF (100 ng/ml). After 22 h, they were incubated in a mixture of DMEM and Ham's F 12 containing 5 μCi/ml of [^{3}H]thymidine for 2 h.

responsiveness to EGF, we next examined the effect of EGF on DNA synthesis in chondrocytes that had been treated with RA for 72 h. As shown in Table 2, pretreatment with RA potentiated the stimulation of DNA synthesis in chondocytes. Like RA, IL-1ß, which increased the number of EGF receptors, potentiated EGF-stimulated DNA synthesis (Table 2). On the other hand, PTH and DBcAMP, which decreased the number of EGF receptors, suppressed EGF-stimulated DNA synthesis (Table 2).

DISCUSSION

In the present study, we demonstrated the presence of EGF receptors on cultured rabbit costal chondrocytes. The binding of ^{125}I-EGF was inhibited by the addition of unlabeled EGF, but not by the addition of unlabelled PTH or IGF-I (Fig. 2). These findings indicate that the binding of labeled EGF to chondrocytes is specific. Scatchard analysis demonstrated the presence of two classes of EGF receptors on rabbit costal chondrocytes; their Kd values were 0.3 nM and 1.6 nM and their numbers per cell were 3,000 and 10,000, respectively (Fig. 2). EGF receptors on other types of cells are also reported to consist of low-affinity receptors and high-affinity receptors (1). The low-affinity receptors are monomers of EGF receptors while the high affinity receptors, which are believed to be involved in mediation of biological activity (18,19), are dimers and oligomers (19,20). A crosslinking study revealed a single band of EGF-receptor complex with a molecular weight of 180 kDa (Fig. 3). This indicates that the molecular weight of EGF receptors on rabbit costal chondrocytes is about 170 kDa. The molecular weight of EGF receptors on various other cells is about 170 kDa (18,20). Thus the EGF receptors on chondrocytes seem to be similar to those on other cells (18,21).

RA and IL-1ß, which inhibited proteoglycan synthesis, increased the levels of two classes of EGF receptors. On the other hand, PTH and DBcAMP, which increased proteoglycan synthesis, decreased their levels. In these cases, there was no significant change in the Kd value of EGF receptors (Table 1). Similar effects of retinoids and PTH have been reported. For example, there are reports that retinoids increase the binding of EGF to various mouse fibroblasts and epidermal cell lines (22) and PTH down regulates EGF receptors in clonal osteoblastic mouse calvarial cells (23). However, DBcAMP induces EGF receptor expression in pheochromocytoma cells (24). Moreover, the promoter element of the EGF receptor gene is reported to have a DBcAMP responsive element (25). Therefore, decrease in the number of EGF receptors by DBcAMP may be observed only in chondrocytes (Table 1). On the other hand, EGF receptors are permanently lost during terminal differentiation of skeletal muscle cells (26). PTH and DBcAMP promote the differentiation of chondrocytes (6,7,10,11) and decrease their EGF receptors (Table 1), so decrease in the number of EGF receptors during differentiation may be a universal phenomenon. Anyway, the results suggest that the number of EGF receptors is a negative marker of at least chondrocyte differentiation.

Previously, we reported that PTH stimulates proteoglycan synthesis in rabbit costal chondrocytes in culture (10,11). Concerning the relationship between chondrocyte differentiation and expression of hormone receptors, we recently reported that increase in the number of PTH receptors on chondrocytes is closely related to expression of the differentiated phenotype of chondrocytes and that the number of these receptors is a good marker of the differentiated phenotype of chondrocytes (27). The findings that the numbers of receptors of both PTH and EGF are changed by various growth and differentiation factors suggest that these factors regulate cartilage growth and endochondral ossification *via* mutual control of the expressions of their receptors.

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