Parathyroid Hormone-related Protein Enhances Insulin-like Growth Factor-I Expression by Fetal Rat Dermal Fibroblasts*

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Interactions between cells of differing embryonic origins comprise a common theme during tissue development and repair. Often, communication between them can be mediated by soluble growth mediators and in some cases is restricted in focus. That is, some cells respond to, but do not produce, mediators expressed by other cells within the tissue. Because keratinocytes respond to but do not express insulin-like growth factor I (IGF-I), another skin cell population, the dermal fibroblast, may supply this factor. However, keratinocytes express, but do not respond to parathyroid hormone-related protein (PTHrp), which increases cAMP production by dermal fibroblasts. Based on earlier results where inducers of cAMP increase local IGF-I expression in skeletal tissue, we postulated that PTHrp might induce local IGF-I by dermal fibroblasts and provide a source of this factor for keratinocyte activity. Our studies reveal that IGF-I mRNA and protein levels increase in response to PTHrp in vitro, and that this effect is replicated by inducers of cAMP, but not by activators of protein kinase C. Consequently, these factors appear to comprise a paracrine loop within the skin, permitting focused but restricted IGF-I expression to support skin growth, remodeling, or repair.

Insulin-like growth factor-I (IGF-I)* regulates a variety of actions in many somatic tissues (1). In skin, IGF-I induces keratinocyte replication and skin matrix protein synthesis (2). However, keratinocytes do not express detectable amounts of IGF-I and may rely on the circulation, or perhaps expression by other local skin cells, for a supply of this factor (3, 4). In contrast, keratinocytes express another growth regulator, parathyroid hormone-related protein (PTHrp), which shares amino-terminal sequence homology with PTH and acts at least in part through conventional PTH receptors (5, 6). PTHrp was first purified from squamous cell tumors where it was implicated in the paraneoplastic syndrome, humoral hypercalcemia of malignancy. It is a highly conserved and ubiquitous protein with many effects in a broad range of tissues (reviewed in Refs. 6 and 7).

Our earlier studies first demonstrated that IGF-I expression is enhanced by PTH, PTHrp, prostaglandin E2, and other inducers of cyclic adenosine monophosphate (cAMP) in osteoblasts (8–10). Despite the assumption that skin, and dermis in particular, is not a primary target organ for PTH, receptors shared by PTH and PTHrp are clearly present on dermal fibroblasts (11–13) in which fibroenectin synthesis increases after exposure to PTHrp (14). Therefore, we postulated that keratinocyte-derived PTHrp might act as a local regulator in skin in a manner similar to that by circulating PTH in the skeleton. In this study we asked specifically if PTHrp could increase IGF-I production by fetal dermal fibroblasts through a cAMP-dependent pathway. In this way, keratinocytes might support dermal fibroblast function through the action of PTHrp, and as a result dermal fibroblasts could support keratinocyte activity through an increase in IGF-I expression.

EXPERIMENTAL PROCEDURES

Cell Culture—Fibroblasts were isolated from the forehead skin of 22-day-old Sprague-Dawley rat fetuses (Charles River Breeding Laboratories) using procedures approved by the Yale University Animal Care and Use Committee. As in earlier studies (15), skin flaps were incubated for 30 min with collagenase (600 μg/ml preincubated with tosylchloromethyl ketone to inhibit residual clostrinap activity (Worthington Biochemical Co.,)) Cells were collected by centrifugation and plated in Dulbecco's modified culture medium supplemented with 20% Hepes buffer (pH 7.2), 100 μg/ml ascorbic acid, penicillin, and streptomycin (Life Technologies, Inc.) for 3 days. Stock cultures were dispersed with trypsin for 1–2 passages, and plated in 9.6-cm² cultures where virtually all cells displayed typical fibroblast morphology. Cultures were serum-deprived for 24 h before treatment.

Reagents—PTHrp (1–34) was obtained from Bachem Bioscience, Inc. Isobutylmethylxanthine, phosphol 12-myristate 13-acetate (PMA), forskolin, isoproterenol, prostaglandin E2, 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB) were obtained from Sigma. Radioisotopes were from NEN Life Science Products.

cAMP Assay—Serum-deprived cultures were incubated for 5 min with 0.5 mM isobutylmethylxanthine to inhibit endogenous phosphodiesterase activity and then treated with vehicle or test agent in medium supplemented with the inhibitor. Medium was aspirated and cell layers were extracted with 90% n-propanol. Extracts were dried and resublimed in 0.05 N sodium acetate, and cAMP levels were measured by radioimmunoassay (RIA) with a commercial kit (Biomedical Technologies, Inc., Stoughton, MA) using data from the linear portion of the standard curve (5, 9).

IGF-I Assay—Serum-deprived cultures were incubated with vehicle or test agent, and conditioned medium was collected and extracted with 2.5 M acetic acid and 95% ethanol to release IGF-I from IGF-binding proteins, as described previously (8, 9). Relative IGF-I levels were measured by RIA with a commercial kit (Nichols Institute) using data from the linear portion of a curve generated with human recombinant IGF-I as standard.

RNA Analysis—Total RNA was extracted with acid guanidine-phenothiocyanate (16), precipitated with isopropanol, and heat denatured in 2.2 M formaldehyde, 12.5 M formamide at 60 °C. Equal amounts of RNA (20 μg/lane) were fractionated on 1.5% agarose, 2.2 M formaldehyde gels

23498   This paper is available on line at http://www.jbc.org

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PTHp Enhances IGF-I Synthesis by Fibroblasts

**RESULTS**

**cAMP Levels**—PTHp caused rapid, time-, and dose-dependent increases in cAMP accumulation in the dermal fibroblast cultures. At 10–30 nM PTHrp, there was a maximal 8-fold to 10-fold increase in cAMP that occurred within 1–5 min of treatment and regressed by 15 min. Basal cAMP levels were reduced in the absence of isobutylmethylxanthine, suggesting an active conventional phosphodiesterase system for normal cAMP turnover in these cells. Treatment with the phorbol ester PMA had no effect on cAMP levels, whereas forskolin was stimulatory (Fig. 1). In parallel cultures, cAMP was similarly increased by treatment with two other positive control reagents, isoproterenol (7.5 ± 0.5-fold) and prostaglandin E2 (12.4 ± 3.4-fold).

**IGF-I Expression**—PTHp also induced time- and dose-dependent increases in IGF-I expression. A 1.7–2-fold increase in steady state IGF-I transcript levels (Fig. 3, panel a) was combined with a 3.4-fold increase in steady state IGF-I transcript levels (Fig. 3, panel c), and iIGF-I secretion, whereas PMA did not (Fig. 2a). As shown in Fig. 3, dermal fibroblasts exhibit a complex IGF-I transcript pattern with predominate bands at 6.5, 4.1, 1.7, and 0.9 kb, analogous to that previously found in fetal rat bone cells (8–11).

6 h of exposure of dermal fibroblasts to PTHrp caused a 2-fold increase in steady state IGF-I transcript levels (Fig. 3, left panel), which remained elevated for 12 h and resided by 24 h (data not shown). Consistent with their effects on cAMP and iIGF-I polypeptide, forskolin increased steady state IGF-I transcripts, and PMA had no effect. Next, the cultures were treated for 6 h with control medium or with PTHrp to induce IGF-I mRNA and then supplemented with DRB for the next 24 h to suppress new mRNA transcription (18, 22). IGF-I transcripts decreased throughout the 24 h period of DRB treatment in control cultures. In contrast, the stimulatory effect of PTHrp persisted for the next 6 h and was still moderately elevated.
even 24 h after DRB exposure, indicating that PTHrp enhanced IGF-I mRNA stability.

Unlike their effects on IGF-I expression, PTHrp and forskolin did not enhance DNA synthesis, collagen synthesis, or general noncollagen protein synthesis by dermal fibroblasts, whereas PMA increased each of these biochemical activities (Fig. 4). Therefore, the effects of the cAMP stimulators on IGF-I expression appeared selective, and the fibroblasts were able to respond to PMA in other ways.

**IGF-I Promoter Studies—**Studies with a probe that distinguishes the use of two separate gene promoters for IGF-I transcription (18) revealed that only promoter 1 drove IGF-I expression in fetal rat dermal fibroblasts. Several larger protected bands of 202–243 nucleotides in length, corresponding to the use of promoter 2 in rat liver, were not evident with RNA from control or treated dermal fibroblasts. A prominent smaller protected band of 143 nucleotides, corresponding to transcripts initiated from promoter 1, was enhanced by PTHrp and forskolin, but not by PMA (Fig. 5). To locate the minimal region of promoter 1 required for IGF-I expression in the dermal fibroblast cultures, they were transfected with various IGF-I promoter/reporter plasmid constructs truncated from the 5' or 3' ends (21). Negligible reporter gene was expressed using the 4.3-kb fragment of IGF-I promoter DNA, and maximal activity occurred with a 5' truncated fragment of 1.7 kb (Fig. 6a). An important enhancer element occurs within exon 1 at nucleotides +202 to +209 (23). In agreement with this, when 3' sequences from exon 1 encompassing this site were removed from the active 1.7 kb upstream promoter sequence, basal promoter activity in dermal fibroblasts was reduced (Fig. 6b). Therefore, basal IGF-I promoter activity in fetal rat dermal fibroblast cultures is fully consistent with its expression in fetal rat osteoblasts (21). However, in striking contrast to the changes that occur in osteoblasts, no stimulator of cAMP enhanced the activity of any promoter construct that we tested. In this context, we examined the maximally active 1.7-kb constructs truncated from the 5' and 3' ends to eliminate possible suppressor sequences and compared effects by PTHrp with dibutyryl cAMP, isoproterenol, and PMA, and invariably found no significant variations (Fig. 6, panels a-c).

**DISCUSSION**

Interactions between epithelial and mesenchymal cells are common during development, remodeling, repair, and perhaps the aging of many tissues. Often these interactions are dependent on the effects of local or systemic growth factors. Paracrine factors appear to influence skin cell metabolism (24), and of these IGF-I is thought to have an integral role (2, 25–27).

![FIG. 3. PTHrp increases IGF-I transcript levels in fetal rat dermal fibroblasts.](image)

![FIG. 4. Stimulatory effects of PTHrp on IGF-I expression are not related to increases in replication or general protein synthesis by fetal rat dermal fibroblasts.](image)

![FIG. 5. PTHrp enhances the amount of IGF-I mRNA that is expressed through promoter 1 in fetal rat dermal fibroblasts.](image)
fail to respond to these hormones and accordingly exhibit undetectable levels of mRNA for the conventional PTH/PTHrp receptor that is coupled to cAMP generation (34, 35). We initially reported that all inducers of cAMP, including PTH and PTHrp, increase IGF-I expression by differentiated osteoblasts (8–10). For these reasons, we postulated that keratinocyte-derived PTHrp might promote IGF-I expression by skin fibroblasts and in this way complete a paracrine loop within skin and support keratinocyte metabolism. A model for these interactions is shown in Fig. 7.

Our current studies confirm the potent effect of PTHrp on cAMP production in dermal fibroblasts and now demonstrate its ability to enhance IGF-I secretion and steady state mRNA levels by these cells. In addition, IGF-I expression was also enhanced by other cAMP stimulators but not by PMA, indicating protein kinase A-dependent mechanisms. This differs from the protein kinase C-dependent permissive effect of growth hormone in liver cells that may respond similarly to cAMP (36). We found an early and transient increase in IGF-I mRNA in PTHrp-induced dermal fibroblasts. IGF-I mRNA levels remained high in PTHrp treated cultures in the presence of DRB, consistent with mRNA stabilization. However, we saw no change in IGF-I promoter activity in control and treated dermal fibroblasts. In these animals the dermis and the epidermis are disrupted and there is abnormal development of hair follicles (38). Our current studies suggest that increases in paracrine interactions may account in part for these effects, and that imbalances may occur when normal regulatory processes in skin are subverted by local overexpression of PTHrp. Further studies to determine the mechanisms by which these factors are expressed and necessarily suppressed under normal conditions are needed.

2 C. Ji, T. L. McCarthy, and M. Centrella, unpublished observations.

3 T. L. McCarthy, J. Shin, S. Casinghino, and M. Centrella, unpublished observations.
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