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Expression of prepro-enkephalin in human articular chondrocytes is linked to cell proliferation

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This study shows that cultured human articular chondrocytes express high levels of 1.4 kb prepro-enkephalin mRNA. Chondrocytes store met-enkephalin intracellularly and secrete this neuropeptide in mature as well as in precursor form. Gene expression is inducible by serum factors. High levels of prepro-enkephalin mRNA are detected in proliferating chondrocytes but not in confluent, contact-inhibited cells. Phorbol myristate acetate and dibutyryl cyclic AMP, but not dexamethasone, increase levels of prepro-enkephalin mRNA. Furthermore, transforming growth factor β (TGFβ) and platelet derived growth factor (PDGF) upregulate gene expression, whereas retinoic acid, which inhibits chondrocyte proliferation, suppresses both basal and induced gene expression. Using *in situ* hybridization it is shown that only 1–3% of primary chondrocytes express prepro-enkephalin mRNA, whereas 52 ± 12% of subcultured cells are strongly positive. Analysis of DNA synthesis, by autoradiography of incorporated [^{3}H]thymidine, shows that these numbers correspond to the percentage of cells in S-phase of the cell cycle. In cultures of primary chondrocytes TGFβ promotes the formation of cartilage nodules and stimulates proliferation of adherent cells. This is associated with high levels of prepro-enkephalin mRNA in proliferating cells but not in contact-inhibited cells in cartilage nodules. In contrast, formation of cartilage nodules, proliferation and the expression of enkephalin are suppressed by interleukin-1β. In summary, expression of prepro-enkephalin in human articular chondrocytes is differentially controlled by cartilage regulatory factors and closely associated with cell proliferation.

Key words: chondrocytes/enkephalins/growth factors/retinoic acid/proliferation

Introduction

Opioid peptides are encoded by three genes, the pro-opio-melanocortin (POMC) gene, the pro-dynorphin gene and the prepro-enkephalin gene. They all give rise to precursor proteins, which are processed in a tissue-specific manner into smaller biologically active peptides (Lynch and Snyder, 1986; Udenfriend and Kilpatrick, 1984). Met-enkephalin consists of five amino acids and is derived from pro-enkephalin A. It has been identified in brain, spinal cord, sympathetic ganglia and adrenal medulla (Eiden, 1987).

In addition to its neuromodulatory functions, many reports have described effects of enkephalins on immune and inflammatory cells. Enkephalins stimulate T-cell migration (Heagy *et al.*, 1990), augment lymphocyte proliferation (Hucklebridge *et al.*, 1990) and inhibit primary and secondary antibody synthesis (Johnson *et al.*, 1982; Munn and Lum, 1989). They also stimulate nonspecific effector functions such as chemotaxis (Van Epps *et al.*, 1984) and superoxide production (Foris *et al.*, 1984). These findings identified enkephalins as a group of mediators that coordinate interactions between the nervous and immune systems.

A new perspective on the role of enkephalins in cell communication was based on the findings that enkephalins are produced by non-neuronal cells. This has first been observed in lymphocytes (Zurawski *et al.*, 1986) and subsequently been extended to bone marrow derived cells (Martin *et al.*, 1987; Vindrola *et al.*, 1990). In these tissues, prepro-enkephalin is expressed and the propeptide is processed in a tissue-specific manner into smaller, biologically active peptides (Vindrola *et al.*, 1990; Kuis *et al.*, 1991). More recently, enkephalins have also been detected in glial cells (Shinoda *et al.*, 1989; Hauser *et al.*, 1990; Melner *et al.*, 1990; Spruce *et al.*, 1990), a finding that was originally attributed to the fact that these cells share many characteristics with neurons. However, *in vitro* studies with opioid antagonists suggested that enkephalins may not only have opioid function, but also exert regulatory effects on cell proliferation and cell differentiation in neural tissues (Zagon and McLaughlin, 1987; Hauser *et al.*, 1990; Verbeeck *et al.*, 1990). Little is known about prepro-enkephalin expression and its regulation in mesoderm derived cells (Springhorn and Claycomb, 1989; Rosen *et al.*, 1990b). This study identifies articular chondrocytes as a source of met-enkephalin and shows that prepro-enkephalin gene expression is controlled by factors known to regulate chondrocyte function.

Results

Expression of prepro-enkephalin mRNA by articular chondrocytes

Cytoplasmic RNA from human articular chondrocytes was analyzed by Northern blotting for the presence of prepro-enkephalin mRNA. A strong hybridization signal at 1.4 kb was detected in all samples prepared from cells which were subcultured in serum-containing media. To evaluate the effect of serum, chondrocyte cultures were expanded in the presence of serum and transferred to serum-free media for 24 h prior to RNA isolation. Under these conditions, mRNA levels were reduced. However, complete suppression was not observed even when the cells were starved of serum for 72 h. Addition of serum to serum-starved cells resulted

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in an increase in prepro-enkephalin mRNA (Figure 1A). These results show that human articular chondrocytes express the prepro-enkephalin gene and that it is induced by serum factors.

To study the effect of cell density, we examined prepro-enkephalin expression in subconfluent, proliferating and in confluent, contact-inhibited cultures. Figure 1B shows that prepro-enkephalin mRNA was absent in confluent cells (lane 1). However, upon subculture of replicate flasks at 50% and 15% confluency (lanes 2 and 3), chondrocytes expressed this gene again. This finding suggests that prepro-enkephalin expression may be a function of proliferating chondrocytes.

Prepro-enkephalin gene expression in primary versus subcultured chondrocytes

Chondrocytes grown in monolyer cultures change their morphology from round cells, that are surrounded by pericellular matrix, to adherent, elongated, spindle-shaped cells (Figure 2). Associated changes in functional characteristics include a reduction in collagen type II and proteoglycan synthesis and an increase in collagen type I production. Northern blot analysis of freshly isolated articular chondrocytes did not show any hybridization signal for prepro-enkephalin mRNA. This could have been due to complete absence of prepro-enkephalin mRNA, or to its expression in only a very small percentage of cells. To answer this question, gene expression was analyzed at a single cell level by *in situ* hybridization. Figure 2 shows that a few primary chondrocytes (1–3%) spontaneously expressed prepro-enkephalin mRNA and that subculture (passage 3) resulted in an increase in positive cells up to 64%. Thus, prepro-enkephalin gene expression occurs not only in subcultured cells, but also in primary, differentiated chondrocytes. However, the percentage of positive cells and the amount of mRNA per cell increase upon subculture.

With the results presented so far, we have determined cellular aspects and culture conditions that affect prepro-enkephalin expression. With this information, it was possible to study the effect of exogenous regulatory factors such as cytokines, growth factors and other agents on prepro-enkephalin gene expression and to address the question of which factors are responsible for the distinct patterns in prepro-enkephalin expression between primary and subcultured chondrocytes.

Effects of second messengers, dexamethasone and retinoic acid on prepro-enkephalin gene expression

The regulatory sequences in the promoter region of the prepro-enkephalin gene have been analyzed (Comb *et al.*, 1986, 1988; Hyman *et al.*, 1988; Rattner *et al.*, 1991) and it has been shown that gene expression is inducible by activators of the protein kinase A (PKA) and protein kinase C (PKC) signal transduction pathways (Comb *et al.*, 1986; Kley, 1988). Furthermore, glucocorticoids are known inducers of prepro-enkephalin expression in neuronal cells (Yoshikawa and Sabol, 1986; Inturrisi *et al.*, 1988). To determine which factors can regulate prepro-enkephalin gene expression in chondrocytes, subcultured cells were starved of serum for 24 h and then activated with phorbol myristate acetate (PMA; 5 ng/ml), dibutyryl cyclic AMP (cAMP; 250 μg/ml) or dexamethasone (1 μg/ml). Analysis by Northern blotting (Figure 3) showed that PMA and cAMP, but not dexamethasone, caused a rapid and prolonged stimulation of gene expression, causing an increase in

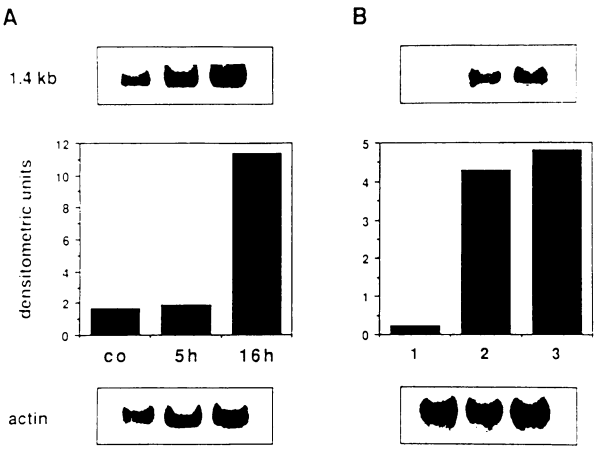


Fig. 1. Effect of serum and cell density on prepro-enkephalin gene expression. **A.** Subcultured chondrocytes (P3) were starved of serum for 48 h. Culture medium was replaced by fresh medium containing 10% FBS and cells were harvested after 5 and 16 h respectively. RNA was extracted and analyzed by Northern blotting for the presence of prepro-enkephalin mRNA (top) and β-actin mRNA (bottom). The hybridization signals were quantified by densitometry. **B.** Subcultured chondrocytes (P2) were grown to confluency and harvested (1). Replicate flasks were subcultured at 1:2 or 1:6 dilutions (2 and 3 respectively) for three additional days. RNA was extracted and analyzed as described above.

mRNA after 3 h (PMA) and 6 h (cAMP) respectively, and lasting for at least 23 h. Thus, expression of this gene in chondrocytes is responsive to second messengers acting via both PKA and PKC signal transduction pathways. However, it differs from neuroendocrine cells with respect to the lack of a response to glucocorticoids. This latter finding could suggest that glucocorticoids alone are insufficient to induce gene expression and require an additional stimulus such as in neuronal cells where dexamethasone acts in synergy with cAMP (Yoshikawa and Sabol, 1986). To answer this question, subcultured chondrocytes were grown in the presence of 10% serum (Figure 4) which, as expected, resulted in a high level of prepro-enkephalin expression (lane 1). Under these conditions, cAMP (lane 3) caused only a minimal increase in mRNA levels. However, dexamethasone alone (lane 4) as well as in combination with cAMP (lane 6) did not detectably alter prepro-enkephalin mRNA levels. Subsequent hybridization of the same filter with a probe for monocyte chemoattractant protein 1 (MCP-1), a pro-inflammatory cytokine, showed that dexamethasone strongly decreased serum-induced as well as cAMP-induced expression of this gene. Taken together, these results show that under conditions where glucocorticoids are operative in chondrocytes they do not exert a measurable effect on prepro-enkephalin expression whether applied as a single agent or in combination with cAMP.

Retinoic acid (RA) is a transcriptional regulator of a large number of genes. It upregulates and activates intracellular RA receptors (RAR α,β,γ) which bind to DNA sequences containing RAR binding motifs or interact with the DNA binding activity of AP-1 (Nicholson *et al.*, 1990; Schule *et al.*, 1991). In the case of the prepro-enkephalin gene it is thought that PKA and PKC pathways converge to activate an AP-1 like element in its promoter region (Comb *et al.*, 1986). Furthermore, gene expression has been shown to be induced by activation of *jun/fos* (Sonnenberg *et al.*, 1989). These data suggested that the prepro-enkephalin gene might

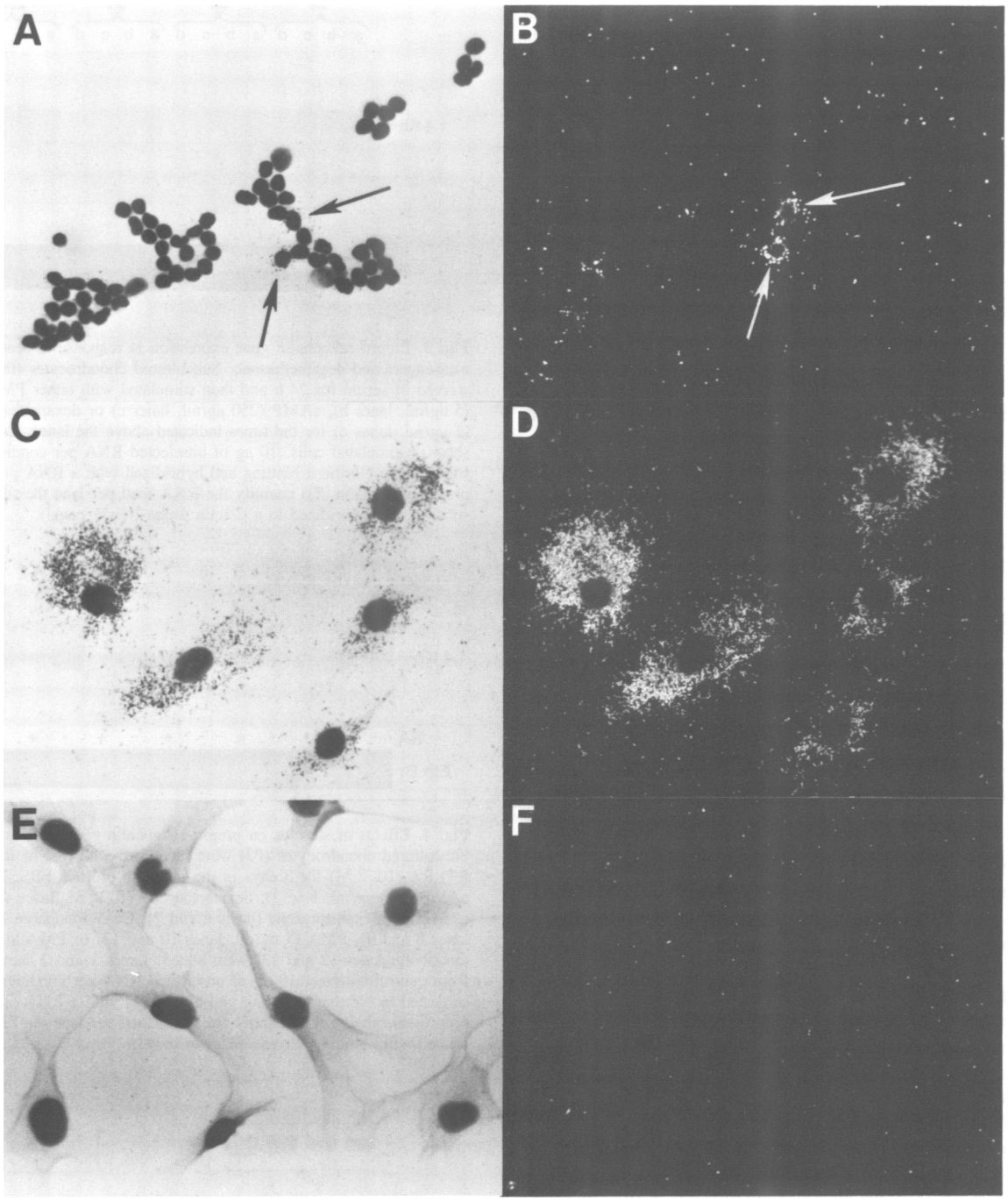


Fig. 2. Prepro-enkephalin gene expression in primary versus subcultured cells. Primary chondrocytes (A and B) and subcultured cells (C–F) were analyzed by *in situ* hybridization for the presence of prepro-enkephalin mRNA. 1–3% of primary chondrocytes expressed prepro-enkephalin (marked with arrows), while subculture in the presence of 10% FBS resulted in an increased number of positive cells (up to 64%) and a marked increase in positivity per cell (granules covering cells). To confirm specificity of the hybridization signal, replicate slides were hybridized to sense probe (E and F).

be responsive to RA. The next set of experiments tested whether RA can modulate the effects of cAMP and PMA. Figure 4 shows that preincubation of subcultured cells with RA (10^{-6} M) for 3 days indeed strongly decreased mRNA levels in response to cAMP, PMA, Ca^{2+}-ionophore (10^{-6} M) and the combination of PMA plus Ca^{2+}-ionophore (lanes 5,7,9,11 and 13). In addition, RA also reduced the serum-induced gene expression (lane 2). These results suggest an interaction of RA with AP-1 dependent activation of the prepro-enkephalin promoter and identify RA as a potent inhibitor of prepro-enkephalin expression.

Effects of growth factors and IL-1 on prepro-enkephalin mRNA

Growth factors and cytokines are important regulators of chondrocyte function. As some of the observations presented above suggested a correlation between prepro-enkephalin expression and chondrocyte proliferation, we analyzed the effects of growth factors such as transforming growth factor β (TGFβ) and platelet derived growth factor (PDGF) on prepro-enkephalin mRNA levels. Figure 5 shows that both factors upregulated prepro-enkephalin mRNA, leading to an increase after 6 h (PDGF) and 5 h (TGFβ) of stimulation. In

contrast, interleukin-1β (IL-1) moderately decreased prepro-enkephalin mRNA levels in subcultured cells (not shown).

We then compared the effects of TGFβ with those of IL-1 on primary chondrocytes at a single cell level by *in situ* hybridization. While TGFβ acts as an anabolic factor, promoting proliferation and formation of cartilage nodules, IL-1 opposes many of these effects, functioning primarily as a catabolic factor (reviewed in Le and Vilcek, 1987). Primary chondrocytes were cultured for nine days in the presence of TGFβ or IL-1. Figure 6a shows the effects of TGFβ or IL-1 on cell morphology (A, C and E) and on prepro-enkephalin expression (B, D and F). In primary chondrocyte cultures, TGFβ had two qualitatively distinct effects. It enhanced the formation and density of cartilage nodules (dense cell clusters in C), but it also promoted the attachment and proliferation of cells that have adhered to the culture plate. In control cultures, chondrocytes showed a heterogeneous pattern of cells with small and large nuclei, whereas IL-1 treatment resulted in a rather homogeneous population of smaller cells. Prepro-enkephalin mRNA was present in a low percentage of cells in control cultures (A and B). No positive cells were found after treatment with IL-1 (E and F), however, TGFβ strongly upregulated prepro-enkephalin mRNA levels and resulted in a high percentage of positive cells in the adherent and proliferating population (C and D). Interestingly, prepro-enkephalin mRNA was rare in the cells forming cartilage nodules (~0.5%). These findings show that the prepro-enkephalin gene is under differential control by factors which are known to antagonize in the regulation of other chondrocyte functions and they provide further support for the notion that prepro-enkephalin expression is associated with proliferation.

Cell cycle and prepro-enkephalin expression

To examine the link between cell proliferation and prepro-enkephalin gene expression more directly, chondrocytes were labeled with [^{3}H]thymidine. Autoradiography revealed that only 3–5% of primary chondrocytes entered S-phase during the first 24 h after isolation from cartilage. Subsequently, the percentage of proliferating cells increased slowly and reached ~35% in subcultured cells (not shown). These numbers resemble the percentage of primary cells expressing prepro-enkephalin mRNA but are lower than the percentage of prepro-enkephalin expressing subcultured chondrocytes. As TGFβ and IL-1 had opposing effects on prepro-enkephalin expression, we studied whether similar effects could be observed for proliferation. Primary chondrocytes cultured in the presence of TGFβ or IL-1 for nine days were exposed to [^{3}H]thymidine and then *in situ* hybridization was performed as outlined in the previous section. Figure 6b confirmed that in primary chondrocytes, cells entering S-phase were rare (A and B). IL-1 treatment resulted in a decline in the number of proliferating cells (E and F). In contrast, TGFβ strongly increased the number of cells synthesizing DNA in the adherent cell population (C and D). Again chondrocytes localized in nodules remained negative and even after 48 h of exposure to [^{3}H]thymidine only a few cells became positive indicating that these represent cells predominantly in G_0 or early G_1 phase of the cell cycle. Figure 6b also shows that ~70% of prepro-enkephalin expressing cells did not synthesize DNA whilst 30% were double positive for prepro-enkephalin mRNA and thymidine incorporation (see arrows). In addi-

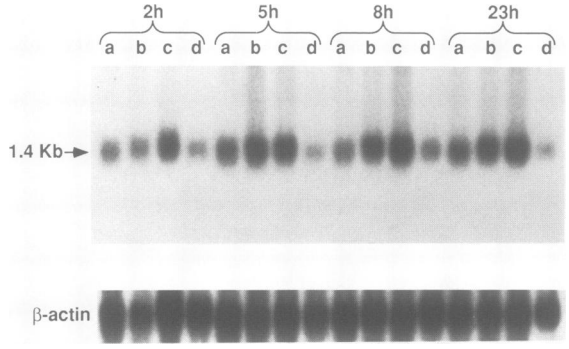


Fig. 3. Prepro-enkephalin gene expression in response to second messengers and dexamethasone. Subcultured chondrocytes (P6) were starved of serum for 24 h and then stimulated with either PMA (5 ng/ml, lanes b), cAMP (250 μg/ml, lanes c) or dexamethasone (1 μg/ml, lanes d) for the times indicated above the lanes. Lanes a show unstimulated cells. 10 μg of unselected RNA per condition was subjected to Northern blotting and hybridized with a RNA probe for prepro-enkephalin. To quantify the RNA load per lane the filter was subsequently hybridized to a β-actin probe (lower panel).

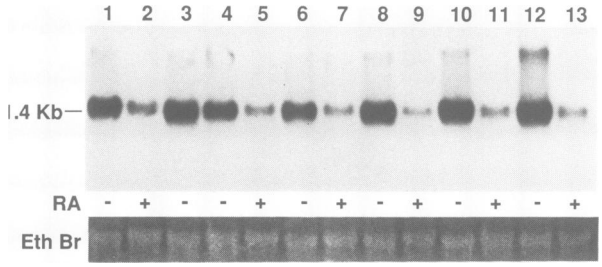


Fig. 4. Effects of steroids on prepro-enkephalin gene expression. Subcultured chondrocytes (P3) were incubated with (+) or without (−) RA (10^{-6} M) for 3 days in the presence of 10% FBS. Then cAMP (250 μg/ml, lane 3), dexamethasone (10^{-7} M, lanes 4 and 5), cAMP and dexamethasone (lanes 6 and 7), Ca^{2+} ionophore (10^{-6} M, lanes 8 and 9), PMA (5 ng/ml, lanes 10 and 11) or PMA and Ca^{2+}-ionophore (lanes 12 and 13) were added. Lanes 1 and 2 show RNA from control cultures. 10 μg of unselected RNA per condition was subjected to Northern blotting and hybridized with a RNA probe for prepro-enkephalin. To quantify the RNA load per lane the filter was stained with ethidium bromide (lower panel).

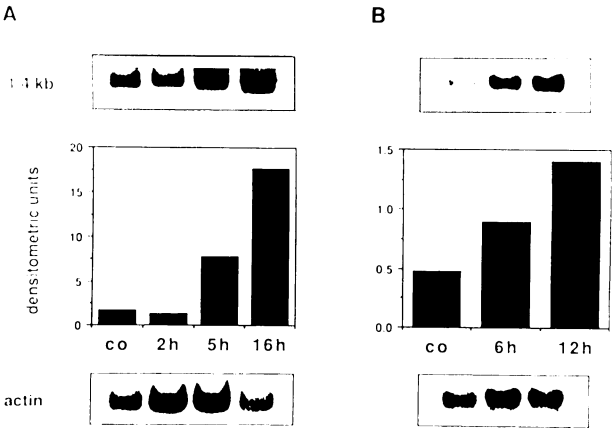


Fig. 5. TGFβ and PDGF induce prepro-enkephalin gene expression. Subcultured chondrocytes (P4) were starved of serum for 48 h and then stimulated with TGFβ (**A**, 10 ng/ml) or PDGF (**B**, 10 ng/ml). Total RNA was extracted and subjected to Northern blotting. After hybridization with a probe for prepro-enkephalin (top panel) and thereafter with a probe for β-actin (bottom panel), the hybridization signals were quantified by densitometry.

tion, cells in M-phase never contained prepro-enkephalin mRNA (not shown). Collectively, these findings indicate that prepro-enkephalin is probably expressed in late G_1/S phase of the cell cycle, in cells that are committed to or have initiated DNA synthesis.

Chondrocytes contain and secrete met-enkephalin

The results presented above suggested that chondrocytes may secrete enkephalins. Opioids are small peptides which are cleaved from larger precursor molecules, a complex process which has been shown to be cell-type specific. To answer the question whether chondrocytes translate and fully process the precursor protein pro-enkephalin A, immunocytochemistry with an antibody recognizing the mature pentapeptide met-enkephalin was performed. This showed a heterogeneous pattern of strongly positive and completely negative cells, resembling the findings with *in situ* hybridization (not shown). The amount of processed and secreted met-enkephalin was then measured with a radioimmunoassay (RIA) specific for met-enkephalin. Figure 7 shows that chondrocytes spontaneously secreted up to 63 pg/ml met-enkephalin during 48 h culture. Stimulation with TGFβ or PDGF resulted in a 10- and 11-fold increase, respectively, whilst cells in the presence of IL-1 reduced met-enkephalin production by ~50%. To compare the amount of fully processed met-enkephalin to the amount of unprocessed, i.e. cryptic, met-enkephalin, aliquots of conditioned media were treated with trypsin and carboxypeptidase to liberate mature pentapeptides from precursor forms. Enzymatic digestion revealed that up to 6250 pg/ml of met-enkephalin was present in precursor form under control conditions and that TGFβ resulted in an increase up to 14 580 pg/ml. These results show that chondrocytes translate prepro-enkephalin and are capable of fully processing the precursors and that opioids are also secreted in an incompletely processed form.

Discussion

This study shows that human articular chondrocytes express the prepro-enkephalin gene and process the propeptide into mature met-enkephalin. Gene expression is induced by activators of the PKA and PKC signal transduction pathways and it is blocked by RA. The peptide regulatory factors TGFβ, PDGF and IL-1 differentially modulate mRNA levels and peptide production. Chondrocyte expression of prepro-enkephalin is a function of cells that are induced to proliferate.

We observed that both primary as well as subcultured human articular chondrocytes produced 1.4 kb prepro-enkephalin mRNA. The first series of studies examined the regulatory factors and mechanisms involved with its expression. Serum was identified as a major inducer of this neuropeptide gene in chondrocytes. Subconfluent cells that proliferated in the presence of serum contained prepro-enkephalin mRNA which was markedly reduced after serum starvation. PDGF and TGFβ are anabolic modulators of connective tissue metabolism and contribute to stimulatory effects of serum. Both factors induced prepro-enkephalin expression in serum-free cultures. IL-1 is a very potent activator of chondrocytes. It induces synthesis of arachidonic acid metabolites, several proinflammatory cytokines such as IL-8 (Lotz *et al.*, 1992), MCP-1 (Villiger,P.M., Terkeltaub,R. and Lotz,M., submitted) and proteases, and it inhibits glucosaminoglycan synthesis. Based on these functions, IL-1 is considered to be a major cartilage catabolic factor ('catabolin') and is involved with the destruction of this tissue in arthritis. IL-1 was found to inhibit the expression of the prepro-enkephalin gene. This was seen by Northern blot analysis and *in situ* hybridization at the single cell level.

To define more details of the signal transduction pathways involved with the regulation of prepro-enkephalin in chondrocytes, we examined second messenger agonists, dexamethasone and RA. The prepro-enkephalin gene contains an AP-1-like consensus sequence which confers PMA and cAMP responsiveness (Comb *et al.*, 1986) and an NF-$\varkappa$B site which regulates expression in lymphocytes (Rattner *et al.*, 1991). In chondrocytes, prepro-enkephalin mRNA was induced in response to the second messenger agonists PMA and cAMP. Gene expression is thus inducible by activators of PKA and PKC signal transduction pathways. It has also been shown that glucocorticoids induce prepro-enkephalin mRNA in neuronal cells and that they act synergistically with cAMP (Yoshikawa and Sabol, 1986). In contrast to cells of neural origin, dexamethasone had no effect on mRNA levels in chondrocytes whether applied as a single agent or in combination with cAMP. However, dexamethasone strongly inhibited serum and cAMP-induced expression of a proinflammatory cytokine (MCP-1) in identical cultures where it did not influence prepro-enkephalin mRNA.

Retinoic acid has important effects in development, limb regeneration (Giguere *et al.*, 1989) and in the regulation of mature cell types (Amos and Lotan, 1990). Intracellular RA receptors (RAR α,β,γ) and their DNA binding domains were recently identified. RA stimulates synthesis and activates its own receptors which bind to the RAR consensus DNA sequences in target genes. Alternatively, RARs regulate gene expression by interaction with other DNA binding proteins such as AP-1 (Nicholson *et al.*, 1990; Schule *et al.*, 1990). We found that preincubation of chondrocytes with RA almost completely blocked the induction of prepro-enkephalin by activators of the PKA and PKC signal transduction pathways and strongly reduced even serum-induced gene expression. A recent study directly demonstrated *jun/fos* mediated prepro-enkephalin gene expression (Sonnenberg *et al.*, 1989). This, together with the present results on the induction of prepro-enkephalin, emphasizes the importance of the AP-1 pathway which is probably also the target for RA mediated inhibition. Since the human prepro-enkephalin promoter does not contain a RAR-like consensus sequence (unpublished), the inhibition is conceivably due to interaction of RAR with the fos−jun protein complex (Schule *et al.*, 1991).

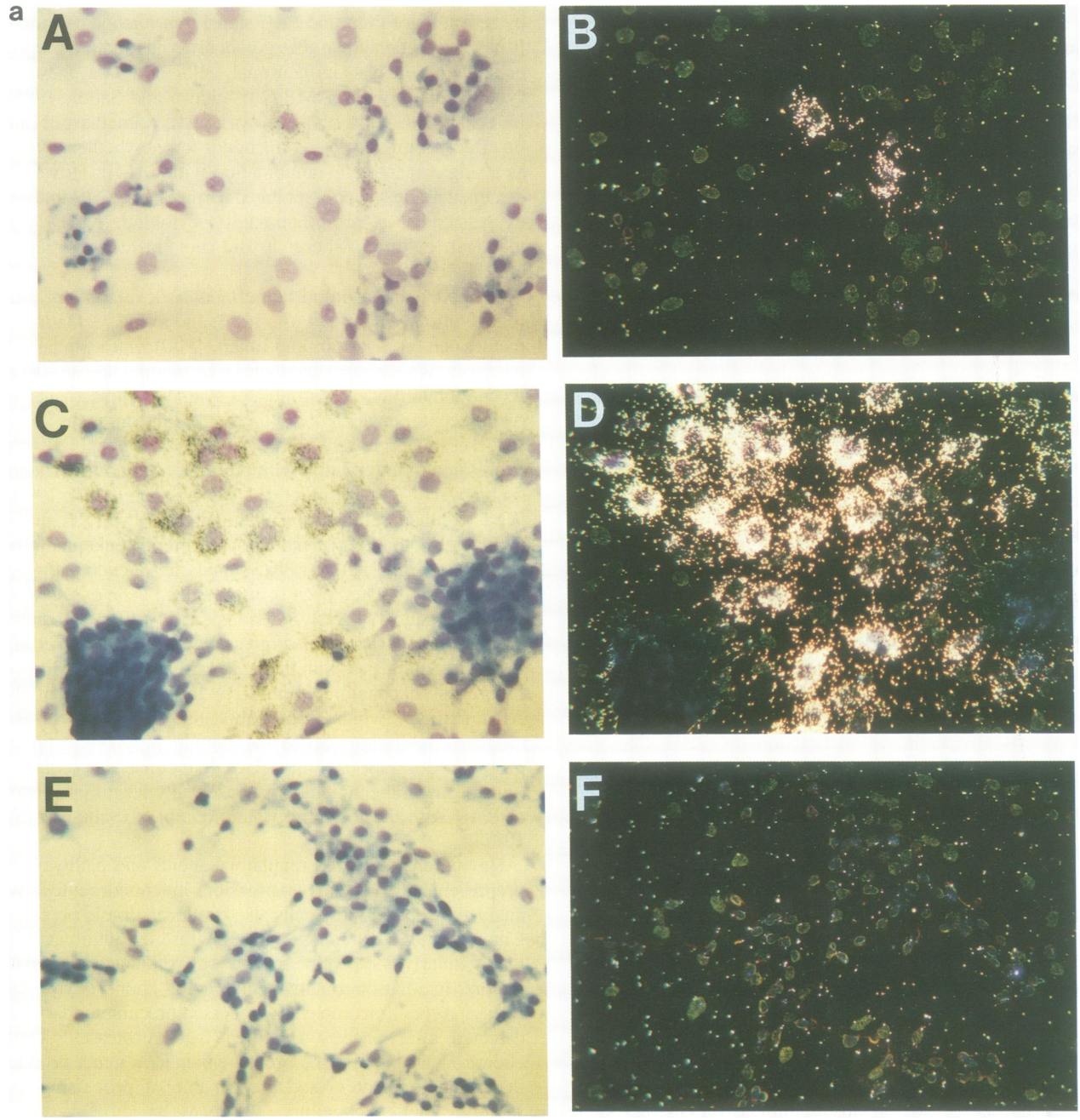
Having characterized regulatory agents and pathways of prepro-enkephalin gene expression in chondrocytes, we analyzed whether these cells also produced opioid peptides. Chondrocytes stored met-enkephalin immunoreactivity intracellularly and peptide release was stimulated by growth factors. In addition to native enkephalin, chondrocytes also release cryptic, incompletely processed precursors that were detected after *in vitro* treatment with proteases. Thus, chondrocytes express the prepro-enkephalin gene, produce the precursor protein and are capable of processing and secreting met-enkephalin

To assess the significance of met-enkephalin production

by chondrocytes, we analyzed functional characteristics of chondrocytes that are associated with prepro-enkephalin expression. Expression of this gene is not a mere function of chondrocyte activation but is part of a more specific program in chondrocyte responses. This notion is based on the induction of its expression by PDGF and TGFβ and its inhibition by IL-1. All three factors stimulate secretory functions of chondrocytes but TGFβ and IL-1 induce distinct sets of genes, the former resulting in the formation of extracellular matrix, the latter causing its degradation. Thus, in the context of secretory responses, prepro-enkephalin expression appears to be part of the anabolic program in chondrocytes.

This is further supported by multiple findings indicating that expression of this gene is associated with chondrocyte proliferation. It was expressed in subconfluent cells proliferating in the presence of serum but not in non-proliferating cells in confluent and contact-inhibited cultures. IL-1 inhibited chondrocyte proliferation and prepro-enkephalin expression, but both chondrocyte responses were stimulated by TGFβ and PDGF.

To confirm this potential link between expression of this neuropeptide gene and cell proliferation, double-labeling studies for [^{3}H]thymidine incorporation and prepro-enkephalin mRNA expression were performed. These experiments clearly showed that TGFβ treatment of primary chondrocytes not only induced expression of prepro-enkephalin mRNA but also resulted in a parallel increase in the number of proliferating cells. On the other hand, IL-1 treatment decreased proliferation and opioid gene expression to the same degree. Interestingly, TGFβ also led to formation of dense cell clusters which are referred to as cartilage



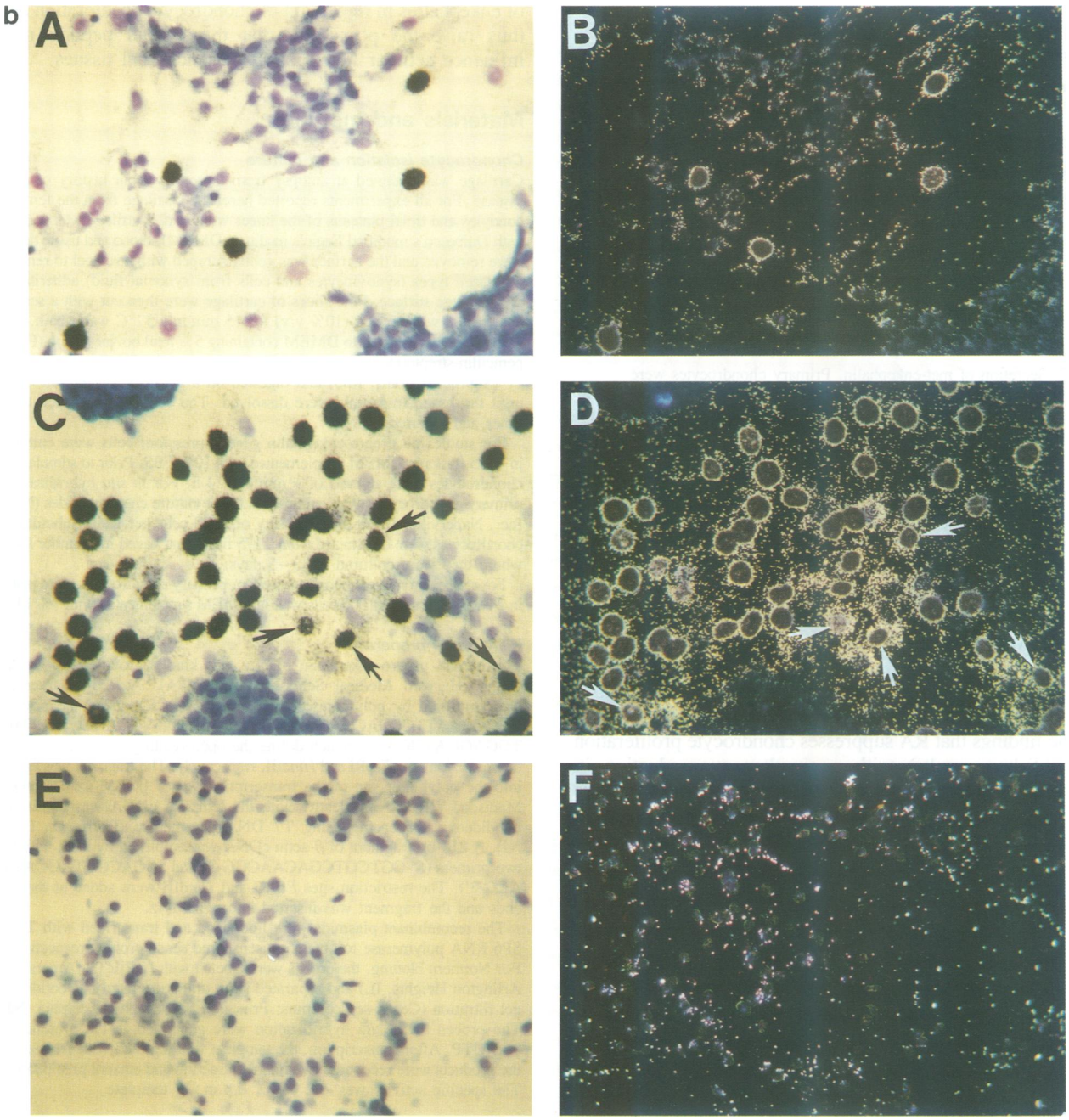


Fig. 6. Panel a. TGFβ and IL-1 differentially activate chondrocytes. Primary chondrocytes were cultured on tissue culture chamber slides in the presence of 10% serum alone (A and B) or in combination with TGFβ (C and D; 10 ng/ml) or IL-1β (E and F; 10 ng/ml) for 9 days. Prepro-enkephalin gene expression was analyzed by *in situ* hybridization. TGFβ enhances the formation and density of cartilage nodules (dense cell clusters), but it also promotes the attachment and proliferation of cells that have adhered to the culture plate and express high amounts of prepro-enkephalin mRNA (cells covered with granules). In control cultures chondrocytes show a heterogeneous pattern of cells with small and large nuclei and few prepro-enkephalin expressing cells are present, whereas IL-1 treatment results in a population of smaller cells and a complete absence of prepro-enkephalin mRNA. **Panel b.** Correlation of cell proliferation and prepro-enkephalin expression. Primary chondrocytes were treated as outlined in (a) but with addition of [^{3}H]thymidine for the last 24 h of culture in order to label cells in S-phase of the cell cycle. Compared to control condition (A and B), TGFβ strongly induces proliferation which is typically found in areas of high prepro-enkephalin expression (C and D), whilst IL-1 reduces both cell proliferation and prepro-enkephalin expression. Cells simultaneously positive for DNA synthesis and prepro-enkephalin mRNA are marked with arrows.

nodules. Proliferating cells were very rare in these clusters, indicating that these chondrocytes are contact-inhibited and differentiated matrix producing cells. Prepro-enkephalin expression was similarly rare in cartilage nodules. These findings provide insight into the regulation of prepro-enkephalin expression during functionally distinct phases of chondrocyte activation. Under physiological conditions, adult articular cartilage consists of highly differentiated cells which produce extracellular matrix but rarely divide. Cartilage nodules represent an *in vitro* equivalent for this. Immediately after isolation, chondrocytes are surrounded by pericellular matrix and only a few cells enter S-phase. In primary cultures cells become adherent and begin to proliferate. This process increases in subculture and at all stages the number

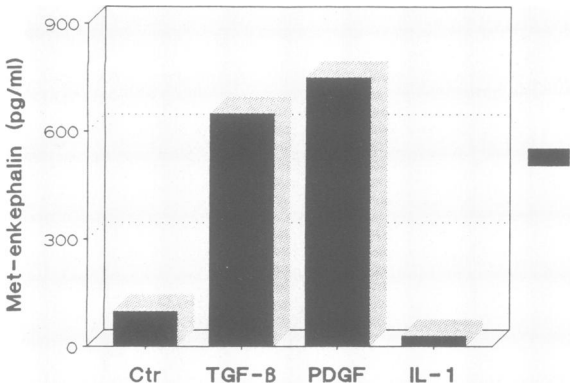


Fig. 7. Secretion of met-enkephalin. Primary chondrocytes were cultured for 48 h in the presence of TGFβ, PGF or IL-1 (all stimuli at 10 ng/ml) and conditioned media were analyzed with a RIA specific for met-enkephalin.

of prepro-enkephalin expressing cells is closely linked to the number of proliferating cells. This interpretation is consistent with the findings on met-enkephalin expression *in vivo*. Met-enkephalin immunoreactivity was present in peri-chondrial cell layers, where chondrocytes show a high rate of proliferation, but was absent in mature cartilage which contains only a very low number of proliferating cells (not shown).

The concept that prepro-enkephalin expression reflects a particular state of chondrocyte activation is also supported by the findings that RA suppresses chondrocyte proliferation (unpublished results) with a simultaneous reduction in prepro-enkephalin expression. Consistent with this are reports on prepro-enkephalin expression at certain stages of organogenesis (Keshet *et al.*, 1989; Polakiewicz and Rosen, 1990), in rapidly proliferating granulosa cells of the ovaries (Rosen *et al.*, 1990a) or in immature spermatocytes of the testes (Kilpatrick and Millette, 1986). *In vivo* conditions that can be associated with prepro-enkephalin expression by articular chondrocytes are organogenesis or in the mature organism, situations of cartilage remodeling such as in arthritis or in responses to trauma (Cuevas *et al.*, 1988).

In summary, the present findings of met-enkephalin production by articular chondrocytes have several implications. This neuropeptide was originally described as an opioid, exerting analgesic effects in nervous tissues. Recently, opioid receptors have also been identified on peripheral nerve endings (Stein *et al.*, 1990). These findings thus provide the basis for an interaction between peripheral organs and central nervous system and indicate that met-enkephalin, synthesized by articular chondrocytes, could represent a peripheral mechanism of pain modulation. A second aspect concerns the immuno-modulatory properties of opioids. Articular chondrocytes are mesoderm derived cells which are involved in inflammatory processes in the joint in part through the production of regulatory factors. These include proinflammatory cytokines, such as IL-1 (Towle *et al.*, 1987), IL-6 (Guerne *et al.*, 1990) or IL-8 (Lotz *et al.*, 1992) and TGFβ (Villiger,P.M. and Lotz,M., submitted). Met-enkephalin has effects on leukocyte chemotaxis, antibody production and T-cell proliferation, and based on the present results thus represents a new chondrocyte derived modulator of immune and inflammatory responses. Furthermore, the present results provide a basis for a role of enkephalins in regulation of chondrocyte proliferation and thus raise the possibility that this opioid peptide may influence cellular functions in mesenchymal tissues.

Materials and methods

Chondrocyte isolation and culture

Cartilage was obtained at autopsy from donors without history of joint disease. For all experiments reported here, the cartilage from the femoral condyles and tibial plateaus of the knees was used. Cartilage was washed with Dulbecco's modified Eagle's medium (DMEM), blood and tissue debris were removed and the surface was gently scraped with a scalpel to remove other cell types (synoviocytes and cells from synovial fluid) adhering to the cartilage surface. Fine slices of cartilage were then cut with a scalpel and treated with trypsin (10% v/v) for 15 min in a 37°C water bath. The tissues were transferred to DMEM containing 5% fetal bovine serum (FBS), penicillin-streptomycin-fungizone and 2 mg/ml clostridial collagenase type IV (Sigma, St Louis, MO) and digested on a gyratory shaker for ~3 h until the tissue fragments were dissolved. The cells were washed three times and cultured.

For studies on prepro-enkephalin gene expression, cells were cultured in T175 flasks in DMEM supplemented with 10% FBS. Prior to stimulation, chondrocytes were starved of serum for 24 h. For *in situ* hybridization, primary chondrocytes were cultured on tissue culture chamber slides (Nunc Inc., Naperville, IL) at a cell density of 5000 cells per well. Subcultured chondrocytes were trypsinized from T175 flasks and plated in chamber slides where they were cultured for 3–5 days.

For studies on met-enkephalin secretion, primary chondrocytes were plated in 24-well plates at 20 000 cells/well in DMEM supplemented with 1% FBS.

RNA probe preparation

The human prepro-enkephalin cDNA was kindly provided by Dr M. Comb (Harvard Medical School, Boston, MA). A 926 bp fragment was amplified by polymerase chain reaction (PCR), using the primers (5′-GCGAATTCCGACGAGTCGTGTCT-3′) and (5′-GCAAGCTTCCAC-TGGAGGATGGA-3′) which define the open reading frame and contain the restriction sites *Eco*RI and *Hin*dIII, respectively. The fragment was ligated into the polylinker site of the transcription vector pGEM-4z (Promega, Madison, WI) and its identity with the template cDNA was confirmed by dideoxy sequencing using T7 DNA polymerase (Pharmacia LKB, NJ). A 216 bp fragment of β-actin cDNA was amplified by PCR with the two primers (5′-CGTCGTCGACAACGG-3′ and 5′-GACCGTAGCACT-ACC-3′). The restriction sites *Eco*RI and *Hin*dIII were added at their 5′ ends and the fragment was inserted into pGEM-4z.

The recombinant plasmids were linearized and transcribed with T7 or SP6 RNA polymerase to obtain antisense and sense probes, respectively. For Northern blotting, the probes were labeled with [^{32}P]UTP (Amersham, Arlington Heights, IL) and separated from unincorporated nucleotides by gel filtration (Centri-sep columns; Princeton Separations, Adelphia, NJ). The probes for *in situ* hybridization were generated in the presence of [^{35}S]UTP. After transcription, the template was digested with DNase and the products were recovered by phenol extraction and ethanol precipitation. The specific activity was ~2 × 10^8 d.p.m./μg template.

Northern blotting

Chondrocytes were lysed in the T175 flasks with 4 M guanidinium thiocyanate and RNA was extracted using the single step guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Unselected RNA (10–25 μg) was subjected to formaldehyde gel electrophoresis, blotted onto nylon filters and cross-linked by exposure to UV light for 5 min on each side. The filters were prehybridized in 50% formamide, 6 × SSC, 0.5% SDS, 0.1% Tween 20 and 100 μg tRNA/ml for 15 min at 65°C. Hybridization was performed overnight in fresh hybridization solution plus 1 × 10^6 c.p.m./ml of probe at 65°C and followed by washes in 1 × SSC, 0.1% SDS at room temperature (2 × 30 min) and in 0.1 × SSC, 0.1% SDS at 65°C (2 × 30 min). The damp filters were exposed to Kodak XAR film at −70°C for 2–24 h. Equal loading of RNA per lane was assessed by ethidium bromide stain (in experiments where RA was used, as RA downregulates expression of β-actin) and hybridization to a β-actin probe. For quantification the hybridization signals were measured by scanning densitometry (Densitometer, Model 1650, Bio-Rad, Richmond, CA).

In situ hybridization

Chondrocytes were cultured on tissue culture chamber slides as described above. After appropriate incubation times, the cells were fixed in freshly

prepared 4% paraformaldehyde in PBS for 4 min at 20°C, dipped in dH_2O and stored in 70% ethanol at 4°C until use. Hybridization was performed as described elsewhere (Villiger *et al.*, 1991). In brief, cells were rehydrated in 2 × SSC, acetylated in 0.1 M triethanolamine with 0.25% acetic anhydride and incubated in glycine buffer (0.1 M glycine, 0.2 M Tris−HCl, pH 7.4). Then they were covered with a prehybridization solution consisting of 50% formamide and 3 × SSC. Freshly prepared probe (10^5 c.p.m./μl) was heated at 80°C for 1 min and the hybridization mixture (50% formamide, 3 × SSC, 500 μg/ml yeast tRNA, 1 mg/ml single-stranded calf thymus DNA, 2 mg/ml BSA, 10 mM DTT, 1% PEG) was added. The prehybridization mixture was drained off the slides and replaced by hybridization mixture. The specimens were covered immediately with coverslips and sealed with rubber cement to avoid dehydration. Hybridization was carried out overnight at 50°C. After hybridization the slides were washed twice in 2 × SSC with 50% formamide at 50°C for 15 min and then incubated with RNase solution (1 mM EDTA, 20 mM Tris−HCl, pH 7.5, 50 μg/ml RNase A) at 37°C for 30 min. This treatment was followed by two 15 min washes in 2 × SSC at 55°C and by three 15 min washes in 2 × SSC at room temperature. The cells were dehydrated in ethanol, air-dried and dipped into Kodak NTB nuclear track emulsion (Eastman Kodak, Rochester, NY) for autoradiography. After exposure for three to six days at 4°C, the slides were developed, fixed and counterstained with Giemsa.

Cells were considered positive when there were >20 granules over nucleus and cytoplasm. A replicate slide was hybridized with sense probe to serve as a negative control for each condition.

DNA synthesis and autoradiography

Freshly isolated chondrocytes were cultured on tissue chamber slides in the absence or presence of TGFβ or IL-1 for nine days. For the last 24 h of culture 1 μCi of [^{3}H]thymidine was added per chamber. Slides were then dried, fixed in 4% paraformaldehyde and stored in 70% ethanol at 4°C until use. Autoradiography was performed as described above and replicate slides were subjected to *in situ* hybridization to assess the number of double positive cells.

Radioimmunoassay

Met-enkephalin in chondrocyte conditioned media was quantified by RIA (Incstar, Stillwater, MN) as reported previously (Kuis *et al.*, 1991). The cross-reactivity of the antibody used in this assay with other products of the prepro-enkephalin gene or other neuropeptides is <3%, except for peptide F (22%). In brief, culture supernatants (1 ml aliquots) were acidified and extracted with ODS silica elements (Immunonuclear Corporation). The columns were washed with 4% acetic acid, eluted with 99.8% methanol and the eluates were lyophilized. The samples were then tested in the met-enkephalin RIA. Met-enkephalin concentrations in the culture supernatants were calculated on the basis of a standard curve using synthetic met-enkephalin (10 pg−2.5 ng) that was included in each assay. In some experiments, the dried eluates were resuspended in Tris buffer with 0.2% BSA and digested with TPCK treated trypsin (1 μg trypsin/200 μl) for 16 h at 37°C, followed by treatment with carboxypeptidase B (0.1 μg/200 μl) for 2 h at 37°C. The enzymes were inactivated by incubation for 20 min at 90°C and the samples were then tested in the met-enkephalin RIA.

Reagents

TGFβ and PDGF were purchased from R&D Systems (Minneapolis, MN). IL-1 was obtained from Amgen (Thousand Oaks, CA). Dexamethasone, RA, Ca^{2+} ionophore (A-23187), cAMP and PMA were purchased from Sigma. [^{3}H]Thymidine was otained from ICN (Irvine, CA).

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