

A regulatory cascade involving retinoic acid, Cbfa1, and matrix metalloproteinases is coupled to the development of a process of perichondrial invasion and osteogenic differentiation during bone formation

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Tissue-remodeling processes are largely mediated by members of the matrix metalloproteinase (MMP) family of endopeptidases whose expression is strictly controlled both spatially and temporally. In this article, we have examined the molecular mechanisms that could contribute to modulate the expression of MMPs like collagenase-3 and MT1-MMP during bone formation. We have found that all-trans retinoic acid (RA), which usually downregulates MMPs, strongly induces collagenase-3 expression in cultures of embryonic metatarsal cartilage rudiments and in chondrocytic cells. This effect is dose and time dependent, requires the de novo synthesis of proteins, and is mediated by RAR-RXR heterodimers. Analysis of the signal transduction mechanisms underlying the upregulating effect of RA on collagenase-3 expression demonstrated that this factor acts through a signaling pathway involving p38 mitogen-activated protein kinase. RA treatment of chondrocytic cells also induces the production of MT1-MMP,

a membrane-bound metalloproteinase essential for skeletal formation, which participates in a proteolytic cascade with collagenase-3. The production of these MMPs is concomitant with the development of an RA-induced differentiation program characterized by formation of a mineralized bone matrix, downregulation of chondrocyte markers like type II collagen, and upregulation of osteoblastic markers such as osteocalcin. These effects are attenuated in metatarsal rudiments in which RA induces the invasion of perichondrial osteogenic cells from the perichondrium into the cartilage rudiment. RA treatment also resulted in the upregulation of Cbfa1, a transcription factor responsible for collagenase-3 and osteocalcin induction in osteoblastic cells. The dynamics of Cbfa1, MMPs, and osteocalcin expression is consistent with the fact that these genes could be part of a regulatory cascade initiated by RA and leading to the induction of Cbfa1, which in turn would upregulate the expression of some of their target genes like collagenase-3 and osteocalcin.

Introduction

Proteolytic remodeling of extracellular matrix is an essential event in a variety of physiological processes such as embryonic development, angiogenesis, reproduction, wound healing, and bone formation and remodeling (Werb, 1997). On the other hand, abnormal breakdown of connective tissue components contributes to a large number of pathological

conditions including rheumatoid arthritis, atherosclerosis, and tumor invasion and metastasis (Nagase and Woessner, 1999). A large body of evidence indicates that the matrix metalloproteinases (MMPs)* play a central role in all of these tissue-remodeling processes. These enzymes comprise a family of zinc-dependent endopeptidases that are collectively capable of degrading all protein constituents of the extracel-

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*Abbreviations used in this paper: ERK, extracellular signal-regulated kinase; JNK, Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; RA, all-trans retinoic acid; RCS, rat chondrosarcoma.

lular matrix. At present, the family of human MMPs is composed of more than 20 members that according to structural and functional considerations can be classified into six different families: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs (Uría and López-Otín, 2000). Among all of these proteins, members of the collagenase subgroup are the principal neutral proteases with ability to degrade fibrillar collagens, generating fragments 3/4 and 1/4 the size of the original molecules, which denature rapidly and become susceptible to further degradation by other MMPs. To date, three human collagenases have been identified: fibroblast collagenase (MMP-1), neutrophil collagenase (MMP-8), and the most recently described collagenase-3 (MMP-13) (Freije et al., 1994).

Collagenase-3 represents a good model to study the molecular mechanisms that modulate the expression of MMPs during normal and pathological conditions. This potent metalloprotease is overexpressed in a growing variety of human pathological processes including malignant tumors (for review see Balbín et al., 1999), inflammatory conditions (Uitto et al., 1998), atherosclerosis (Sukhova et al., 1999), aortic aneurysms (Mao et al., 1999), and destructive joint diseases such as osteoarthritis and rheumatoid arthritis (Lindy et al., 1997). However, in marked contrast with its wide distribution in pathological processes collagenase-3 expression in physiological conditions has only been detected during fetal ossification and postnatal bone remodeling (Stähle-Bäckdahl et al., 1997) and at lower levels in the course of some reproductive processes (Balbín et al., 1996; Dumin et al., 1998). Recent studies have provided information on the mechanisms controlling collagenase-3 expression in both normal and pathological conditions (for review see Balbín et al., 1999). Thus, we and others have reported that Cbfa1, a transcription factor of the *runt* gene family involved in skeletal development, induces the expression of collagenase-3 during bone formation (Jiménez et al., 1999; Porte et al., 1999; Selvamurugan et al., 2000). This gene is also strongly induced by bone-resorbing agents such as parathyroid hormone (PTH) and IL-6 in diverse in vitro systems including osteoblastic cell lines and mouse calvarial osteoblasts (Partridge et al., 1996; Kusano et al., 1998). We have also described that collagenase-3 is expressed within fibroblasts adjacent to invasive breast cancer cells in response to diffusible factors released from the epithelial tumor cells (Uría et al., 1997). Furthermore, it has been reported that IL-1 β and TNF- α may induce collagenase-3 expression in osteoarthritic cartilage (Shlopov et al., 2000), whereas serotonin may be important in the upregulation of this gene during reproductive processes (Dumin et al., 1998). However, a series of factors like PDGF, aFGF, or EGF previously found to play important roles in upregulating expression of other MMPs did not show any effect on collagenase-3 expression by human fibroblasts (Uría et al., 1997; 1998). By contrast, TGF- β assumed to be inhibitory for most MMPs induces collagenase-3 expression in fibroblasts and transformed keratinocytes (Uría et al., 1998; Ravanti et al., 1999a; Johansson et al., 2000). Because these findings suggested that the mechanisms regulating collagenase-3 expression could be distinct from those operating in the control of other MMPs, we have tried to extend our search for factors

that could act as mediators of collagenase-3 expression in normal and pathological conditions.

All-trans retinoic acid (RA) could be a good candidate to act as an inducer of collagenase-3 expression during bone formation or in pathological processes involving bone-forming cells. In fact, retinoids have been found to play important roles in mammalian embryonic limb development and in bone growth and remodeling during fetal and postnatal life (Hofman and Eichele, 1994). However, contrary to this possibility, most previous studies have shown that RA is inhibitory for MMP expression (Lafyatis et al., 1990; Nicholson et al., 1990; Schüle et al., 1991; Schroen and Brinckerhoff, 1996; Benbow et al., 1999). In this work, we provide evidence that collagenase-3 and other MMPs involved in bone formation, such as MT1-MMP (Holmbeck et al., 1999; Zhou et al., 2000), are induced by RA and RA derivatives in embryonic cartilage rudiments and chondrocytic cells. We also analyze the morphological effects resulting from the treatment of these cells with RA and correlate the observed effects with altered patterns of gene expression including those of Cbfa1, MMPs, and osteocalcin genes. Finally, we perform an analysis of the molecular mechanisms and signaling pathways mediating collagenase-3 induction by RA with the finding that RA acts through a signaling pathway involving RAR-RXR heterodimers and is mediated by p38 mitogen-activated protein kinase (MAPK). On the basis of the transcriptional regulation studies presented herein, together with morphological and functional observations, we propose that RA induces a regulatory cascade involving Cbfa1 and MMPs and is coupled to the development of a perichondrial invasion and osteogenic differentiation process that occurs during endochondral ossification.

Results

Collagenase-3 and MT1-MMP are induced by retinoic acid in chondrocytic cells

To study the putative effect of RA on the expression of collagenase-3 during bone formation, we first performed in situ hybridization experiments on embryonic metatarsal rudiments. As can be seen in Fig. 1, a and c, relatively low levels of collagenase-3 transcripts were found in cartilage from control samples. Labeling was restricted to some hypertrophic chondrocytes and cells localized in the perichondrium, but it was not detected in proliferating or in resting chondrocytes. By contrast, a high level of collagenase-3 expression was found in rudiments treated with 10^{-7} M RA for 7 d (Fig. 1, b and d). In these samples, expression of collagenase-3 was located mainly at an abnormally extended perichondrium, being especially marked in those zones in which it seems to invade into the underlying cartilage. In most cases, positive signal was found in spindle-shaped cells having oval nuclei and small size (Fig. 1 d). As in control animals, chondrocytes expressing collagenase-3 were low in number and restricted to the hypertrophic zone. The overexpression of collagenase-3 induced by RA was coupled to a series of morphological alterations in the metatarsal rudiments (Fig. 1, e and f). Thus, the longitudinal growth was reduced (control length, 3.1 ± 0.18 mm, $n = 4$; treatment with

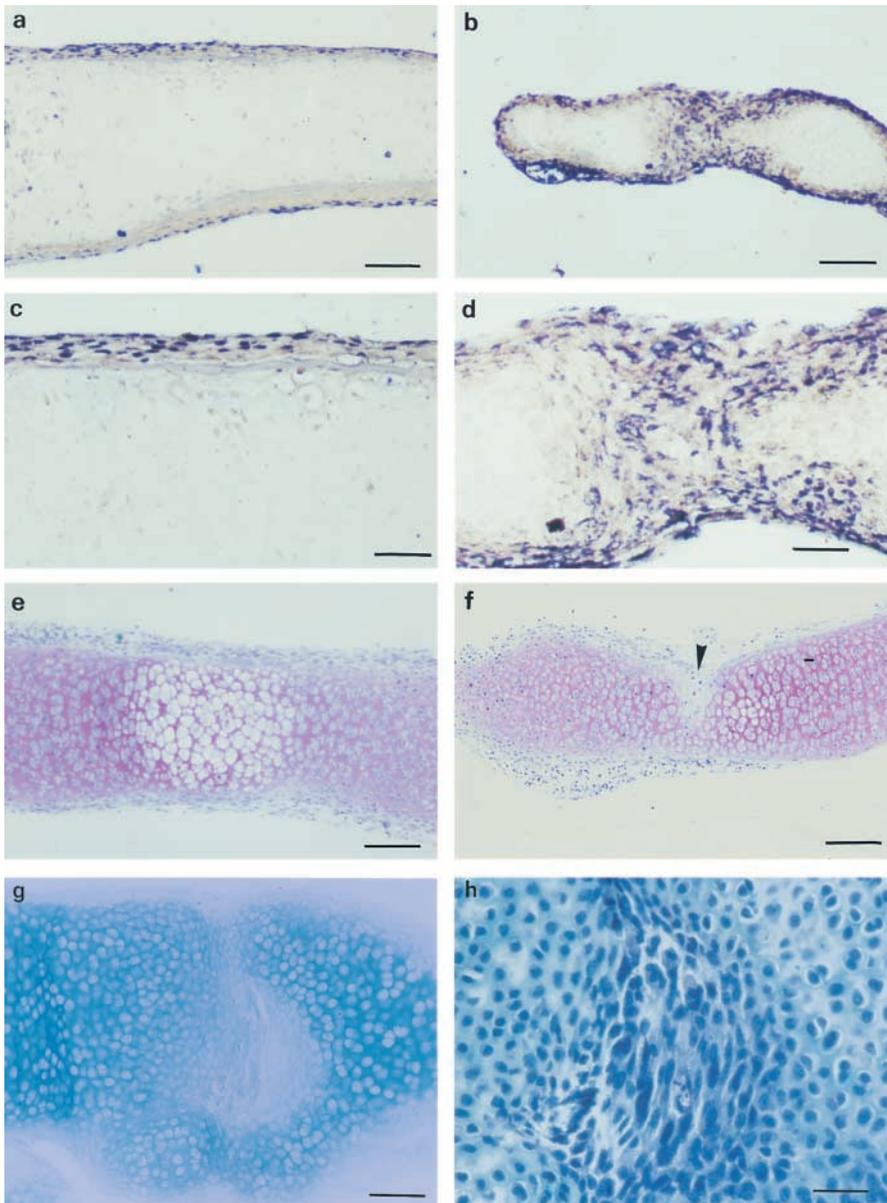


Figure 1. Collagenase-3 expression in metatarsal bone rudiments. Paraffin sections from embryonic metatarsal rudiments untreated (a and c) or treated with RA (b and d) were hybridized with a labeled antisense riboprobe for collagenase-3. In untreated cultures, collagenase-3 expression was relatively low, being restricted to a low number of cells located mainly in the perichondrium. (c) Higher magnification of a peripheric area showing that most collagenase-3-positive cells were small sized and flat shaped. Metatarsals treated with RA (b) showed higher levels of collagenase-3 expression. Positive signal was increased in zones in which the perichondrium invaded into the underlying cartilage (d). (e-h) Metatarsal sections untreated (e) and treated with RA (f-h) and stained with toluidine blue (e, f, and h) and Alcian blue (g). A clear boundary between proliferating and hypertrophic zones was observed in untreated metatarsals (e). RA-treated cultures showed a partial inhibition of cellular enlargement during chondrocytic hypertrophy resulting in ill-defined boundaries between proliferating and hypertrophic zones (f). Invasion of cartilage by cells from the perichondrium also increased in RA-treated cultures (f, arrowhead; g and h). Weakly Alcian blue-stained intrachondral cells invading from the perichondrium are observed in g. (h) Higher magnification of intrachondral cells showed in g and stained with toluidine blue. These cells are elongated and have basophilic cytoplasm. Bars: (a, e, and f) 100 μ m; (b) 160 μ m; (c, d, and g) 40 μ m; (h) 25 μ m.

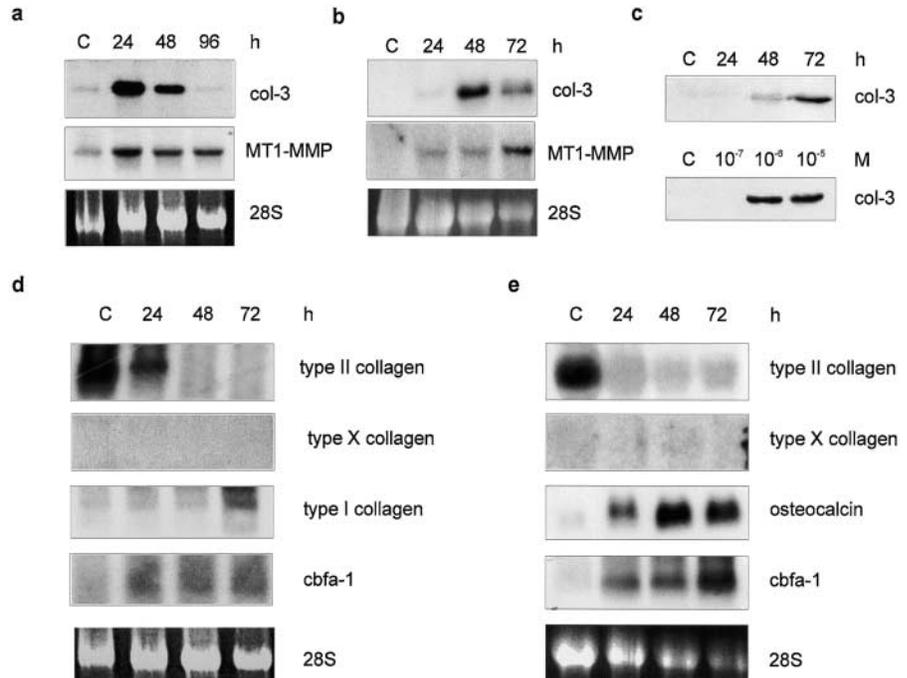
10^{-8} RA, 2.7 ± 0.17 mm, $n = 4$, $p < 0.05$; treatment with 10^{-7} RA, 2.2 ± 0.14 mm, $n = 4$, $p < 0.01$). Histologically, the RA-treated metatarsal rudiments showed a decrease in the degree of structural anisotropy. As a result, boundaries between resting and proliferating zones appeared poorly defined and could hardly be recognized in these samples (Fig. 1 f). Likewise, RA treatment resulted in a partial inhibition of cellular enlargement during chondrocytic hypertrophy. In this way, hypertrophic-like chondrocytes showing mitotic figures were sometimes observed in RA-treated cartilages. Additionally, the perichondrium in RA-treated cultures appeared bigger and more irregular than in controls. Whereas the perichondrium in controls was only two to three layers thick (Fig. 1 e), the perichondrium in RA-treated cultures contained five to nine layers of cells, often invading into the underlying cartilage (Fig. 1 f). Proteoglycan content, as estimated by cytochemical staining with Alcian blue, was lower in RA-treated cartilages. This effect was especially evident in zones where the perichondrium expands and gives rise to

cup-shaped depressions protruding into cartilage. An abrupt transition was evident between the weakly Alcian blue-stained intrachondral cells invading from the perichondrium and the strongly stained surrounding chondrocytes (Fig. 1 g). Such intrachondral cells were larger in size than surface perichondrial cells, presented basophilic cytoplasm (Fig. 1 h), and were histochemically positive for calcification (unpublished data). The characteristics of perichondrium-derived cells in RA-treated rudiments partially resembled those of osteoblasts and clearly differed from those of untreated bones where a sequential differentiation process from perichondrial cells to chondrocytes was observed.

To further study the effect of RA on the expression of collagenase-3, we first used primary chondrocyte cultures. Cells were treated with 10^{-6} M RA, and total RNA was obtained at different times and analyzed by Northern blot using a specific collagenase-3 probe. As shown in Fig. 2 a, RA induced the accumulation of a 2.9-kb mRNA transcript corresponding to collagenase-3, the maximal effect being reached

Figure 2. Effect of RA on expression of collagenase-3 and different chondrocytic and osteoblastic markers.

(a) Primary chondrocytes were exposed to 10^{-6} M RA for the times shown, and then total RNA was extracted and analyzed by Northern blot with collagenase-3 and MT1-MMP cDNA probes. 28S rRNA stained with ethidium bromide is shown as loading control. (b) RCS cells were incubated with 10^{-6} M RA for the times shown, and total RNA was then analyzed as in a. (c) RCS cells were treated with 10^{-6} M RA for the times indicated, and proteins in conditioned media were analyzed by Western blot using an antibody against collagenase-3. Cells were also treated for 72 h with different RA concentrations, and collagenase-3 protein secreted to media was detected. Primary chondrocytes (d) or RCS cells (e) were induced with 10^{-6} M RA for the times shown, and total RNA was analyzed by Northern blot with specific probes for the indicated



between 24 and 48 h and declining at longer times of incubation. Similar results were obtained when rat chondrosarcoma (RCS) cells were used as the experimental model. The main advantage of using this cell line derives from the fact that its chondrocyte phenotype is extremely stable in standard tissue culture conditions compared with the unstable phenotype exhibited by other cells from the same lineage (Mukhopadhyay et al., 1995). RCS cells treated with 10^{-6} M RA also showed a marked induction of collagenase-3 expression (Fig. 2 b). In addition, a dose-response analysis showed that as little as 10^{-7} M RA induced a detectable expression of collagenase-3 mRNA, whereas incubation of the cells with 10^{-5} M induced a maximal accumulation of this mRNA (unpublished data). To determine if the inducing effect of RA on collagenase-3 mRNA levels was also reflected at the protein level, we performed Western blot analysis with conditioned medium from RCS cells treated with RA. As can be seen in Fig. 2 c, a band immunoreactive against collagenase-3 monoclonal antibodies was detected in medium from cells treated with 10^{-6} M RA for 48 h. This band was absent in medium obtained from control untreated cells. A time course analysis demonstrated that the maximum level of collagenase-3 protein was detected in cells treated with RA for 72 h (Fig. 2 c).

We next examined the possibility that other MMPs could also be a target of the upregulatory effect of RA in chondrocytic cells. We focused our interest on MT1-MMP, a membrane metalloproteinase essential for bone formation (Holmbeck et al., 1999; Zhou et al., 2000) and proposed to be part of a proteolytic cascade involving collagenase-3 (Knäuper et al., 1996). As illustrated in Fig. 2, a and b, MT1-MMP expression was increased by RA treatment both in RCS cells and primary chondrocyte cultures, although in these cells the upregulatory effect was lower. Further studies also revealed that other MMPs, such as gelatinase B (MMP-9) potentially involved in bone formation, are not significantly

upregulated by retinoids in chondrocytic cells (unpublished data).

Collagenase-3 induction by retinoic acid is coupled to an osteogenic differentiation process mediated by Cbfa1

After treatment of cell cultures with RA, a clear time-dependent change in cell morphology was found. Both primary chondrocytes and RCS cells shifted from rounded polygonal shape to a very distinct flattened and more stellate shape, being such changes correlated with a decrease of proteoglycan content (unpublished data). In addition, an increase in calcium deposition was observed in RCS cells and primary cultures of chondrocytes. Previous studies have provided opposing results on the role that RA exerts on chondrocytic differentiation. Some works have shown that RA induces maturation and mineralization of chondrocytes (Iwamoto et al., 1994; Cancedda et al., 1995; Koyama et al., 1999), whereas other groups have reported that RA exerts an inhibitory effect on chondrocyte function (Ballock et al., 1994; De Luca et al., 2000). To analyze the molecular alterations associated with these morphological changes, we examined the putative occurrence of variations in the expression levels of different genes that could be associated with chondrocytic differentiation. Northern blot analysis revealed that type II collagen expression was strikingly downregulated after RA treatment of primary chondrocytes and RCS cells (Fig. 2, d and e). The loss of this cartilage-specific collagen suggested that chondrocytic cells had differentiated toward a mature hypertrophic chondrocyte or, alternatively, had dedifferentiated toward a fibroblastic phenotype. Hybridization of the blots with a probe for type X collagen showed the absence of detectable mRNA transcripts of this gene whose expression is characteristic of hypertrophic chondrocytes. However, hybridization of the same blots with probes for osteoblastic

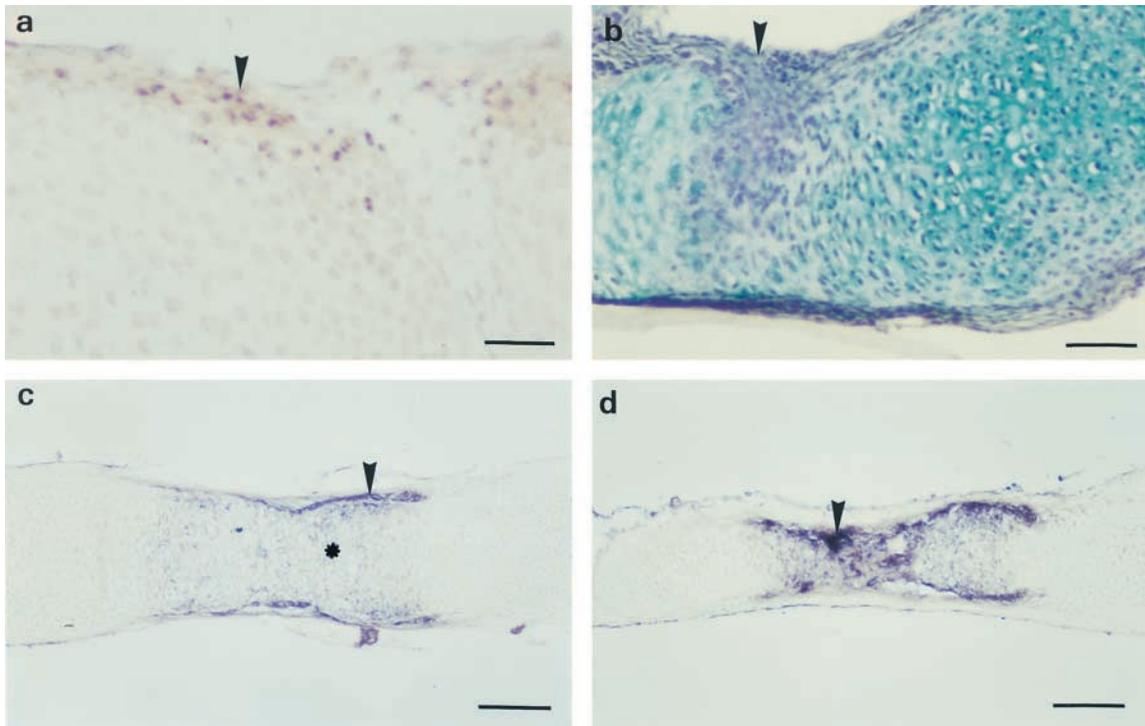


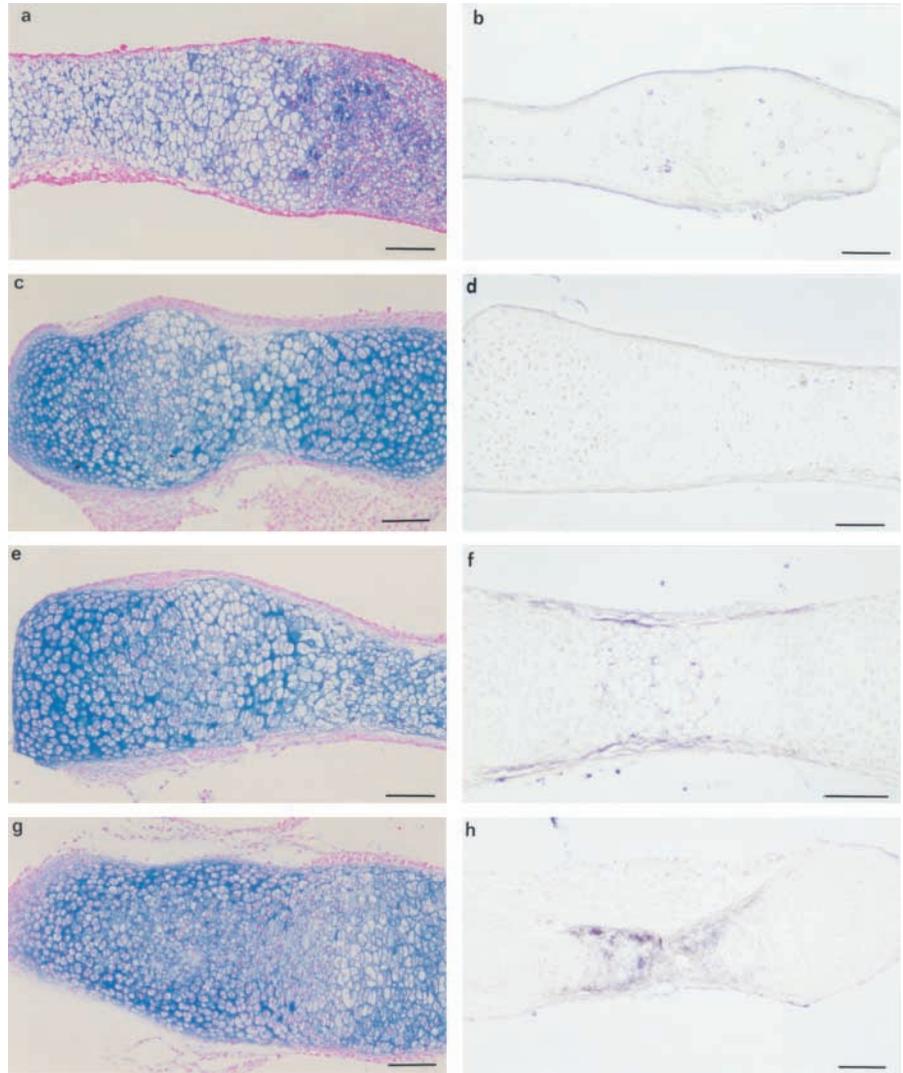
Figure 3. Osteocalcin and Cbfa1 expression in metatarsal bone rudiments treated with RA. Osteocalcin mRNA was found in RA-treated rudiments in a low number of cells located in zones in which perichondrium appeared extended into the underlying cartilage (a). Cells showing positive signal (arrowhead) were small sized and spindle shaped and partially overlapped those positive for collagenase-3. Osteocalcin expression was correlated with a decrease of proteoglycans as demonstrated by cytochemical detection with Alcian blue on paraffin sections (b, arrowhead). Cbfa1 expression was observed in both untreated rudiments (c) and treated with RA (d). In untreated cultures, Cbfa1 expression was low, being restricted to cells of the perichondrium (arrowhead) and some hypertrophic chondrocytes (asterisk). By contrast, metatarsals treated with RA (d) showed higher levels of Cbfa1 expression that partially overlapped that of collagenase-3. Positive signal was especially evident in central (diaphyseal) zones in which the perichondrium was expanded and typical chondrocytes were scarce (arrowhead). Bars: (a and b) 250 μm ; (c and d) 67 μm .

markers provided positive results. Thus, type I collagen positive signal was observed in RA-treated primary chondrocytes (Fig. 2 d). Similarly, osteocalcin mRNA was markedly induced in RA-treated RCS cells (Fig. 2 e), although the effect on primary cultures was virtually undetectable (unpublished data). These differences in the expression patterns of both types of RA-treated cells indicate that their response to retinoids is not identical, although they share several common RA-induced morphological and molecular alterations suggestive of their differentiation toward osteoblastic-like cells. In relation to this, it is remarkable the finding that matrix molecules, including osteocalcin, are only expressed in some populations of morphologically indistinguishable osteoblasts depending on variations in maturational status or in the microenvironment in which they are present (Candelieri et al., 2001). Consistent with this, *in situ* hybridization experiments on metatarsal rudiments revealed that osteocalcin transcripts were detected clearly in some cells from rudiments treated with 10^{-7} M RA for 7 d (Fig. 3 a) but not in control samples. In these RA-treated rudiments, expression of osteocalcin significantly overlapped that of collagenase-3, being found in a relatively low number of small spindle-shaped cells located in zones where the perichondrium appeared to protrude into the underlying cartilage (Fig. 3 a). Osteocalcin expression was negatively correlated with proteoglycan content (Fig. 3 b).

We next tried to evaluate the possibility that the effect of RA could be mediated by protein factors involved in bone cells maturation. In this regard, it is remarkable that Cbfa1, a transcription factor involved in the maturation of chondrocytes and osteoblasts, is an *in vivo* inducer of both osteocalcin and collagenase-3 expression in bone cells (Ducy et al., 1997; Jiménez et al., 1999; Porte et al., 1999; Selvamurugan et al., 2000). From these considerations, we examined the possibility that RA could modulate Cbfa1 levels in RCS cells and primary cultures of chondrocytes. Interestingly, and as shown in Fig. 2, d and e, Northern blot analysis confirmed that RA induced the expression of this transcription factor. Cbfa1 induction was detected at 24 h of RA treatment, thus preceding the appearance of collagenase-3 transcripts. Cbfa1 transcripts overlapping with those of collagenase-3 were also increased in RA-treated metatarsal rudiments (Fig. 3, c and d).

To further support the possibility that the effect of RA on collagenase-3 expression was mediated by Cbfa1, we performed metatarsal organ cultures from embryos with targeted deletion of the Cbfa1 gene. A comparative morphological analysis with control littermate metatarsal rudiments revealed that Cbfa1-null rudiments were shorter in both total length (3.0 ± 0.11 mm, $n = 4$ versus 3.7 ± 0.14 mm, $n = 5$, $p < 0.01$) and fraction of hypertrophic cartilage. RA treatment of rudiments from Cbfa1^{-/-} mice resulted in a

Figure 4. Collagenase-3 expression in metatarsal bone rudiments from embryos with targeted deletion of the *Cbfa1* and *MT1-MMP* genes. *Cbfa1*-null (a–d) and *MT1-MMP*-null (e–h) metatarsal rudiments were untreated (a, b, e, and f) or treated with RA (c, d, g, and h) and stained with Alcian blue and nuclear fast red (a, c, e, and g) or hybridized with a labeled antisense riboprobe for collagenase-3 (b, d, f, and h). Untreated *Cbfa1*^{-/-} rudiments showed signs of cellular disorganization, especially in the hypertrophic zone where a clear Alcian blue staining was observed (a). Treatment of *Cbfa1*^{-/-} metatarsi with RA resulted in a marked inhibition of the chondrocytic hypertrophy (c). In situ hybridization studies failed to find positive signal for collagenase-3 in either untreated (b) or RA-treated (d) rudiments. Untreated *MT1-MMP*^{-/-} rudiments showed a disorganized hypertrophic zone with an intense Alcian blue staining (e). Collagenase-3 expression was low but could be observed in cells located mainly in the perichondrium (f). Treatment of *MT1-MMP*^{-/-} metatarsi with RA induced some cytological changes (g) and resulted in an increased collagenase-3 expression (h) at the hypertrophic chondrocytes. Bars, 200 μ m.



slight decrease of longitudinal growth (2.7 ± 0.12 mm, $n = 4$ versus 3.0 ± 0.11 mm, $n = 4$, $p < 0.01$) that was proportionally lower than that observed in RA-treated *Cbfa1*^{+/+} rudiments (2.8 ± 0.14 mm, $n = 5$ versus 3.7 ± 0.14 mm, $n = 5$, $p < 0.01$). RA treatment of *Cbfa1*^{-/-} rudiments also resulted in a marked inhibition of chondrocytic hypertrophy (Fig. 4, a and c), a result similar to that observed in wild-type samples (Fig. 1). However, RA treatment did not induce any increase of perichondrial thickness in *Cbfa1*-null metatarsal rudiments, a result different from that observed in the wild-type samples. In situ hybridization studies showed that collagenase-3 expression was absent in both untreated and RA-treated *Cbfa1*^{-/-} metatarsal bone rudiments (Fig. 4, b and d), which agrees with the proposal that *Cbfa1* is required for the RA-induced increase of collagenase-3 expression. We next examined the possibility that the effect of RA on collagenase-3 expression could be influenced by the absence of *MT1-MMP*, which as mentioned above is involved in both bone formation and collagenase-3 activation. To this end, we used metatarsal cultures from *MT1-MMP*-null embryos and analyzed the effects of RA on these explants. Similar to the case of *Cbfa1*-null rudiments, *MT1-MMP*^{-/-} rudiments were shorter than wild-type littermate

samples (2.8 ± 0.13 mm, $n = 6$, versus 3.5 ± 0.16 mm, $n = 4$, $p < 0.01$) and showed a clear histological alteration at the hypertrophic zone where chondrocytes were smaller in size and appeared highly disorganized (Fig. 4 e). RA treatment of *MT1-MMP*^{-/-} rudiments induced a significant decrease in length (2.1 ± 0.18 mm, $n = 6$ versus 2.8 ± 0.13 mm, $n = 6$, $p < 0.01$), which was comparable to that observed in wild-type rudiments (2.9 ± 0.13 mm, $n = 4$ versus 3.5 ± 0.16 mm, $n = 4$, $p < 0.01$). The observed cytological alterations in untreated *MT1-MMP*^{-/-} samples were also enhanced in RA-treated rudiments (Fig. 4 g). In situ hybridization studies showed positive collagenase-3 expression in both untreated and RA-treated *MT1-MMP*^{-/-} metatarsi (Figs. 4, f and h). In untreated rudiments, labeling was restricted to some hypertrophic chondrocytes and cells of the perichondrium, a pattern similar to that observed in samples from control mice (Fig. 4 f). Likewise, RA-treatment of *MT1-MMP*^{-/-} rudiments resulted in an increased expression of collagenase-3. However, in these mutant bone rudiments collagenase-3 expression was found mainly at the disorganized hypertrophic chondrocytes located at the middle of the bone (Fig. 4 h). Like in *Cbfa1*^{-/-} metatarsi, RA treatment did not induce any perichondrial expansion in *MT1-*

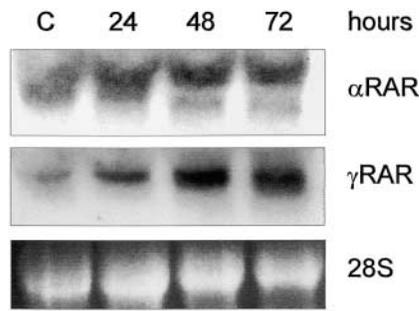


Figure 5. **Expression of RA receptors in RCS cells.** Cells were induced with 10^{-6} M RA for the times indicated, and total RNA was analyzed by Northern blot with specific probes for α RAR and γ RAR.

MMP^{-/-} rudiments. According to these results, we conclude that the RA-induced increase of collagenase-3 expression was not dependent of MT1-MMP.

Induction of collagenase-3 expression in chondrocytic cells is mediated by RAR-RXR heterodimers and involves the p38 MAPK pathway

The above observation that all-trans RA upregulated collagenase-3 expression in chondrocytic cells prompted us to examine the nature of the nuclear retinoic acid receptors presumably involved in this process. To this purpose, we first analyzed by Northern blot the expression levels of the different retinoid receptors in RCS cells (Fig. 5). This analysis revealed that RAR α and RAR γ receptors are constitutively expressed but show some variations after RA treatment, indicating that both of them could be involved in the process. We next evaluated the effect of different agonistic and antagonistic retinoids on collagenase-3 expression in these cells. As shown in Fig. 6 a, the RAR α -selective agonist Ro40-6055 induced a collagenase-3 expression similar to that observed with all-trans RA. In addition, when cells were incubated with the RAR β -selective (Ro48-2249) or the RAR γ -selective (Ro44-4753) retinoids, collagenase-3 expression was also induced, although the upregulatory effect was much lower than that observed after treatment with RAR α agonists. Furthermore, the RAR α -selective antagonist Ro41-5253 extensively blocked the RA-induced accumulation of collagenase-3, whereas the RAR β -selective antagonist LE135 only showed a slight inhibitory effect (Fig. 6 b). Finally, we examined the possibility that RXR-mediated signaling pathways could also contribute to the observed upregulation of collagenase-3 expression by retinoids. Thus,

RCS cells were incubated with different RAR agonists in the presence or absence of the RXR-selective retinoid LG100064, and collagenase-3 levels were analyzed by Northern blot. As shown in Fig. 6 a, the presence of this RXR-selective retinoid produced a significant increase in the collagenase-3 mRNA levels when compared with values obtained after incubation with RAR agonists alone. Taken together, these results indicate that RAR-RXR heterodimers, likely containing RAR α isoforms, are involved in the transduction of the retinoid signal that leads to the induction of collagenase-3 in chondrocytic cells.

To provide further insights into the mechanisms underlying the upregulating effect of RA on collagenase-3 expression in chondrocytic cells, we performed cell culture experiments in the presence of the protein synthesis inhibitor cycloheximide. As shown in Fig. 7 a, incubation of RCS cells with cycloheximide blocked the effect of RA on collagenase-3 mRNA levels. Therefore, we conclude that de novo protein synthesis is required for collagenase-3 induction by RA. We next evaluated the possibility that different signaling pathways could be involved in this process. To this purpose, RCS cells were first incubated with RA in the presence or absence of several inhibitors of these signaling pathways, and the levels of collagenase-3 were examined by Northern blot. As illustrated in Fig. 7 b, the highly specific PKC inhibitor GF109203X diminished the upregulating effect of RA on collagenase-3 expression, indicating the involvement of a PKC in this process. However, the classical but less specific PKC inhibitor staurosporine not only was unable to diminish its expression but even enhanced it, likely through an alternative mechanism (Shoshan and Linder, 1994). To determine whether a tyrosine kinase was also involved in collagenase-3 induction, RCS cells were incubated with RA in the presence or absence of genistein. As shown in Fig. 7 b, this inhibitor blocked the induction of collagenase-3 expression elicited by RA. By contrast, incubation of RCS cells with H89, a protein kinase A inhibitor, or with indomethacin, which blocks prostaglandin synthesis, did not diminish RA-mediated induction of collagenase-3 and even promoted it (Fig. 7 b). Taken together, these results indicate that the positive effect of RA on collagenase-3 expression in RCS cells is exerted through a signaling pathway involving PKC and tyrosine kinase activities.

On the other hand, it is well established that activation of tyrosine kinase-dependent signaling can activate downstream signaling cascades including MAPKs. To elucidate the putative implication of extracellular signal-regulated kinase (ERK)1,2, Jun NH₂-terminal kinase (JNK)1, and p38

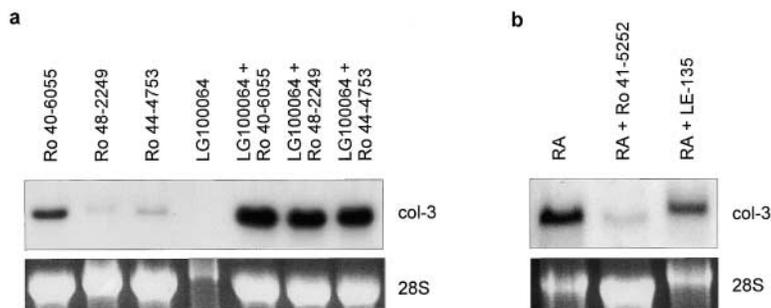


Figure 6. **Effect of different RA receptors agonists and antagonists on collagenase-3 expression.** RCS cells were cultured for 48 h in the presence of 10^{-6} M of different agonists (Ro40-6055, α RAR agonist; Ro48-2249, β RAR agonist; Ro44-4753, γ RAR agonist; RXR agonist, LG100064) (a) or with 10^{-6} M RA plus different antagonists (Ro41-5253, α RAR antagonist; LE135, β RAR antagonist) (b). Total RNA was extracted, and collagenase-3 transcripts were detected by Northern blot.

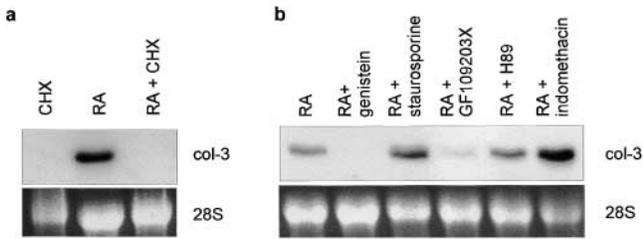


Figure 7. Analysis of the signaling pathways involved in RA-mediated induction of collagenase-3 expression. (a) Effect of cycloheximide (CHX) (0.5 $\mu\text{g/ml}$) on collagenase-3 mRNA levels induced in RCS cells treated with 10^{-6} M RA for 48 h. (b) Effect of inhibitors of different intracellular signaling pathways on RA-induced collagenase-3 mRNA levels in RCS cells. Genistein (100 $\mu\text{g/ml}$), staurosporine (2 nM), GF109203X (5 μM), H89 (500 nM), and indomethacin (5 μM) were added to the culture medium 1 h before induction with 10^{-6} M RA. Total RNA was extracted after 48 h and analyzed by Northern blot with a collagenase-3 probe.

MAPKs pathways in mediating the RA-dependent induction of collagenase-3 expression in RCS cells, we studied the activation of these different kinases after RA treatment. As illustrated in Fig. 8 a, only p38 showed constitutive levels of activation and was appreciably activated after a 90-min RA treatment. This effect continued for at least 12 h. To test the implication of p38 in RA-elicited collagenase-3 induction, we pretreated RCS cells with the p38 inhibitor SB 203580 and then analyzed the obtained RNA after incubation with RA. As shown in Fig. 8 b, treatment of these cells with SB 203580 abolished the RA-induced expression of collagenase-3. In contrast, the ERK1,2 pathway inhibitor PD 98059 strongly augmented collagenase-3 induction by RA in RCS cells. It is remarkable that PD 98059 alone, in the absence of RA, did not elicit any inductive effect on collagenase-3 expression. We also examined if the actions of these MAPK inhibitors could be extended to other RA-induced genes such as Cbfa1 and osteocalcin. As shown in Fig. 8 b, the p38 inhibitor SB 203580 abolished the RA-induced expression of Cbfa1 and osteocalcin, whereas PD 98059 increased the RA-mediated induction of both genes. These results provide evidence that the p38 MAPK signaling pathway seems essential for expression of the different genes induced by RA in chondrocytic cells, whereas ERK1,2 MAPKs play an inhibitory role on the process.

Discussion

Skeletal formation and remodeling are strictly regulated both temporally and spatially by a variety of molecules. Among these regulatory factors, retinoids have raised large interest due to their pleiotropic and profound effects in several events occurring during bone formation. Thus, RA has been implicated in limb bud development, anterior/posterior axis orientation, chondrogenesis, growth plate maturation, chondrocyte apoptosis, and matrix mineralization (Hofman and Eichele, 1994). This wide variety of RA functional roles has made difficult to identify the effector molecules involved in these different processes and to elucidate the molecular mechanisms underlying each of them. In this work, we provide evidence that collagenase-3, a potent proteolytic enzyme associ-

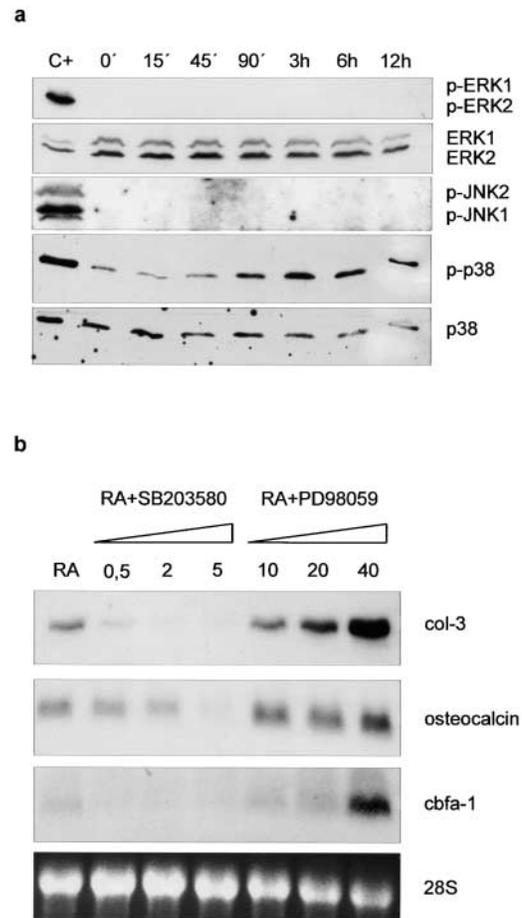


Figure 8. Implication of p38 MAPK in collagenase-3 induction by RA. (a) RCS cells were treated with 10^{-6} M RA for the times indicated. Cells were lysed, and protein extracts were analyzed by Western blot. The levels of activated ERK1/2, JNK1/2, and p38 were determined using phosphospecific antibodies for the corresponding MAPKs (p-ERK1/2, p-JNK1/2, p-p38). As controls, levels of total ERK1/2 and p38 were determined with specific antibodies. Cell lysates from HaCaT cells treated for 20 min with 10^{-7} M TPA or 20 ng/ml TNF- α were used as positive controls for activated ERK1/2 or JNK1/2 and p38, respectively. (b) Increasing amounts of p38 inhibitor SB 203580 or ERK1/2 inhibitor PD 98059 ($\mu\text{g}/\mu\text{l}$) were added to the culture medium 1 h before inducing RCS cells with 10^{-6} M RA. Total RNA was extracted, and collagenase-3 transcripts were detected by Northern blot. The same filter was subsequently hybridized with probes specific for Cbfa1 and osteocalcin.

ated with tumor and arthritic processes but whose expression in normal tissues is essentially restricted to bone formation, is induced by RA in chondrocytes during endochondral ossification. Likewise, MT1-MMP, a membrane-bound protease whose relevance in skeletal formation has been uncovered recently (Holmbeck et al., 1999; Zhou et al., 2000), is also induced by RA in chondrocytic cells. Interestingly, the production of these MMPs is concomitant with the development of an RA-induced osteogenic differentiation program mediated by Cbfa1. Finally, we demonstrate that these effects require the participation of a signaling pathway involving the activity of p38 MAPK.

According to *in situ* hybridization, Northern blot, and Western blot analysis, RA is a potent inducer of colla-

nase-3 expression in cartilage rudiment cultures, primary chondrocyte cultures, and chondrosarcoma cells. This effect is time and dose dependent, requires protein synthesis, and is mediated by RAR-RXR heterodimers, likely involving RAR α isoforms. The finding of an upregulatory effect of RA on the production of proteolytic enzymes like MT1-MMP and collagenase-3 confirms and extends previous observations indicating that different MMPs can be induced by retinoids in human, murine, and avian cells (Ballock et al., 1994; Connolly et al., 1994; Varghese et al., 1994; Guérin et al., 1997; Nie et al., 1998). However, these findings are in clear contrast with many studies demonstrating that retinoids are repressors of MMP expression (Lafyatis et al., 1990; Nicholson et al., 1990; Schüle et al., 1991; Schroen and Brinckerhoff, 1996; Benbow et al., 1999). These RA-induced inhibitory mechanisms have been proposed to be mediated mainly through interaction of RAR-RXR heterodimers with AP-1 transcription factors, which bind to the AP-1 site present in the promoter region of most MMP genes. Therefore, it is paradoxical that the collagenase-3 gene, which contains a functional AP-1 site (Pendás et al., 1997), is not repressed by RA. Some studies have reported that AP-1 sites may also mediate RA stimulatory effects (Desai and Niles, 1997); however, we have been unable to find any indication of the involvement of the AP-1 site in the RA-induced expression of the collagenase-3 gene as assessed by lack of JNK activation, induction of *fos* or *jun* genes, augmented translocation of these factors to the nucleus, or enhanced binding to the AP-1 element (unpublished data). These results indicate that sequences other than AP-1 influence the responsiveness of the collagenase-3 gene to RA. Therefore, we can conclude that regulation of MMP gene expression by retinoids is complex and markedly dependent on cell type or on available factors mediating RA effects in different normal or pathological conditions.

Previous works have reported conflicting results regarding the role of retinoids in the process of chondrocyte maturation. Thus, several studies have proposed a central role for RA in skeletal development, promoting chondrocyte terminal maturation and matrix mineralization (Iwamoto et al., 1994; Cancedda et al., 1995; Koyama et al., 1999). By contrast, other groups have reported that RA negatively regulates bone growth by inhibiting chondrocyte maturation and bone matrix synthesis (Ballock et al., 1994; De Luca et al., 2000). Our results derived from morphological studies and gene expression analysis using murine cartilage rudiments and chondrocytic cells support the possibility that RA is a signaling molecule that in cell cultures promotes an osteogenic differentiation program. This effect is attenuated in metatarsal rudiments in which RA induces the invasion of perichondrial osteogenic cells from the perichondrium into the cartilage rudiment. Consistent with previous studies (Ballock et al., 1994), RA treatment of murine chondrocytic cells induced morphological changes, resulting in more elongated and flattened cells with stellate-like shape resembling osteoblasts. Furthermore, the coordinated expression of markers such as *Cbfa1*, osteocalcin, MT1-MMP, and collagenase-3 may reflect defined stages of this differentiation process. Interestingly, RA-treatment of metatarsi from MT1-MMP^{-/-} mice induced collagenase-3 expression in

hypertrophic-like chondrocytes but did not lead to formation of collagenase-3-positive cells with osteoblastic characteristics, suggesting that the presence of both proteases is needed for the development of this osteogenic differentiation process. Finally, the observation that RA induces the formation of a mineralized bone matrix also suggests that chondrocytes have acquired some functional capabilities necessary to act as osteoblast-like cells. Nevertheless, we must consider the possibility that the addition of relatively high concentrations of RA may lead to a series of phenotypic changes that do not reflect a true osteogenic differentiation of hypertrophic chondrocytes. However, it is remarkable that concentrations as low as 10⁻⁸ M RA, which fall within physiological levels (Gentili et al., 1993), still have an upregulatory effect on MMP expression in metatarsal rudiments. Therefore, it is tempting to speculate that endogenous retinoids may trigger the regulatory cascade described in this work, leading to induction of MT1-MMP and collagenase-3 that in turn may play a direct role in replacement of cartilage by bone during development.

The induction of MMPs by RA is a transient event. The lag between the initiation of treatment and the expression of MMPs together with the observation that the effect of RA on collagenase-3 production is dependent of de novo synthesis of proteins indicate that the induction of an intermediate factor may be involved in this process. An interesting possibility is that *Cbfa1*, a key regulator of osteoblast differentiation and function (Karsenty, 1999), may act as this intermediate factor. Several experimental findings provide support to this proposal. Thus, we have shown that *Cbfa1* expression in RCS cells is also strongly upregulated by RA in a time-dependent manner. We have also found that *Cbfa1* is necessary for collagenase-3 expression as assessed by the lack of expression of this gene in both untreated and RA-treated metatarsal rudiments obtained from *Cbfa1*-null mice. Furthermore, recent *in vivo* and *in vitro* studies have demonstrated that *Cbfa1* is essential for controlling transcriptional activation of genes encoding osteoblastic proteins through binding to *Cbfa* functional elements present in these genes (Ducy et al., 1997; Jiménez et al., 1999). Finally, we have also observed the presence of putative *Cbfa* elements in the MT1-MMP promoter, which is also induced by RA in these cells (Fig. 2; unpublished data). Assuming that this differentiation process triggered by RA is mediated by *Cbfa1* and leads to the induction of MMPs, we can speculate about the potential role of these proteolytic enzymes during bone development. Thus, the wide substrate specificity of collagenase-3 could facilitate the degradation of different matrix components of the bone anlagen in order to initiate the formation of mature bone. In addition, collagenase-3 in collaboration with other MMPs (Engsig et al., 2000) may regulate the availability of bone growth factors sequestered as inactive molecules in the matrix or blocked by interaction with their binding proteins. Finally, collagenase-3 could facilitate the matrix invasive processes occurring after cartilage calcification in a manner similar to that proposed for MMPs during tumor invasion (MacDougall and Matriasian, 1995). On the other hand, the importance of MT1-MMP for bone formation seems clear if we consider that

mice deficient in this protease exhibit severe skeletal abnormalities that lead to the early death of these mutant animals (Holmbeck et al., 1999; Zhou et al., 2000). It has been proposed that inadequate collagen turnover and defects in angiogenesis underlie the observed phenotype, although the possibility that impaired collagenase-3 activation could be responsible at least in part of the observed phenotype cannot be ruled out. Nevertheless, and contrary to the case of *Cbfa1*, MT1-MMP is not necessary for the observed RA-induced expression of collagenase-3 during bone formation as assessed by the presence of collagenase-3 transcripts in chondrocytes from metatarsal rudiments obtained from MT1-MMP-null mice.

In this work, we have also analyzed the signaling pathways mediating the effect of RA on MMP expression in chondrocytic cells. By using a series of specific inhibitors for different signaling pathways, we have found that the RA action on collagenase-3 is mediated by PKC and tyrosine kinase signal transduction pathways. The finding that a specific PKC inhibitor is effective in blocking the RA stimulatory process on collagenase-3 provides a functional link between PKC and retinoid pathways, which are generally considered to have antagonistic activities on different processes. On the other hand, the observation that induction of collagenase-3 is also dependent on tyrosine kinase activity agrees with previous findings, indicating that these kinases play an essential role in signaling pathways regulating the proteolytic activity of diverse cells (Ravanti et al., 1999b; Westermarck and Kähäri, 1999). Interestingly, we have found that two different MAPK pathways mediate opposite effects on collagenase-3 expression. In fact, inhibition of the ERK1,2 pathway results in collagenase-3 induction, indicating that these MAPKs generate an inhibitory rather than an activating signal on the production of this protease. By contrast, inhibition of the p38 MAPK pathway extensively abolishes the RA stimulatory effect on collagenase-3 expression. Similar results were obtained in the analysis of the effect of both MAPK inhibitors on *Cbfa1* and osteocalcin expression. Therefore, it is likely that the balance between p38 and ERK1,2 MAPK pathways will finally determine if these genes are expressed or not. Identification of p38 MAPK as an essential signaling pathway in the induction of *Cbfa1*, osteocalcin, and collagenase-3 suggests that this pathway might be important during osteogenic differentiation processes. It is also of interest that p38 MAPK has been proposed to play a crucial role in the invasive phenotype of transformed keratinocytes (Johansson et al., 2000). The finding that collagenase-3 expression is suppressed by p38 MAPK inhibitors in bone-forming cells supports the proposal that this protease may facilitate some of the invasive processes that take place during bone formation and remodeling.

A final consideration makes reference to the potential relevance of the present results in the context of the therapeutic use of retinoids in several pathological conditions (Lippman et al., 1995). Retinoids have been proven to be effective in treating hyperproliferative and inflammatory diseases, skin pathologies, and cancer. Because it is generally accepted that retinoids inhibit MMPs, it was anticipated that these enzymes, which play important roles in these pathologies,

could be efficiently targeted by retinoid-based treatments. However, the finding that these compounds have a paradoxical effect on other MMPs like collagenase-3 and MT1-MMP may represent a limitation for the therapeutic use of retinoids in those pathological conditions, including malignant tumors, with ability to produce these proteases. The design and synthesis of novel dissociating retinoids, which are devoid of transactivation properties (Chen et al., 1995), could allow an efficient treatment of these diseases without the undesired properties derived from the induction of factors such as MMPs.

Materials and methods

Materials

All media and supplements for cell culture were obtained from GIBCO BRL. All-trans RA was from Sigma-Aldrich. Ro40-6055, Ro40-6973, Ro41-5253, Ro46-5471, Ro48-2249, Ro44-4753, and Ro40-8757 were from F. Hoffmann-La Roche Ltd. RXR-retinoid LG100064 was a gift from Dr. U. Reichert (Galderma, Valbonne, France). TNF- α , TPA, cycloheximide, staurosporine, genistein, and indomethacin were from Sigma-Aldrich. H-89 was from Calbiochem.

DNA probes

The collagenase-3 probe was a 2.1-kbp fragment generated by RT-PCR from mouse embryo RNA and corresponding to cDNA positions 334–2,473 (sequence data available from GenBank/EMBL/DDBJ under accession no. X66473). The MT1-MMP probe was a 640-bp PCR fragment from positions 1,060–1,700 in the human cDNA sequence (sequence data available from GenBank/EMBL/DDBJ under accession no. D26512). The gelatinase A probe was a 2.8-kbp EcoRI fragment containing the full-length cDNA for mouse gelatinase A (sequence data available from GenBank/EMBL/DDBJ under accession no. M84324). Type II collagen probe was a 550-bp PstI fragment cloned in PGEM 3Zf vector kindly provided by Dr. Y. Yamada (National Institute of Dental and Craniofacial Research, Bethesda, MD). Type X collagen probe was a 650-bp HindIII fragment of the mouse type X collagen gene (Apte et al., 1992). *Cbfa1* probe was a 1.7-kbp EcoRI fragment from plasmid pCMV-Osf2/*Cbfa1* (Ducy et al., 1997) kindly provided by Dr. G. Karsenty (M.D. Anderson Cancer Center, Houston, TX). Osteocalcin probe was a 209-bp fragment generated by RT-PCR from mouse embryo RNA and corresponding to cDNA positions 59–267 (sequence data available from GenBank/EMBL/DDBJ under accession no. X04142).

Embryonic metatarsal rudiment organ cultures

The three central metatarsal rudiments were isolated from 15.5-d-post-coitum wild-type, *Cbfa1*^{-/-}, and MT1-MMP^{-/-} mouse embryos. The generation of *Cbfa1* and MT1-MMP-deficient mice has been described previously (Komori et al., 1997; Holmbeck et al., 1999). Isolated rudiments were cultured in 1 ml of medium containing MEM (GIBCO BRL) supplemented with 0.05 mg/ml ascorbic acid, 0.3 mg/ml L-glutamine, 0.05 mg/ml gentamicin, 1 mM glycerophosphate, and 0.2% BSA. Explants were grown at 37°C in a humidified 5% CO₂ incubator. Retinoids were added to cultures 12–14 h after dissection. Medium was changed on the third day of culture. Metatarsal length was calculated at 24 h, 3 d, and 7 d of treatment by using a Nikon microscope equipped with a micrometric eyepiece. Data are shown as the mean \pm SD. For statistical analysis, data were compared among the different groups using a one-way analysis of variance followed by Student-Newman *t* test. Metatarsal rudiments were fixed overnight at 4°C in fresh 4% paraformaldehyde and then processed according to two different protocols. Half of them were dehydrated with a graded series of acetone and embedded in Durkupan-ACM (Sigma-Aldrich). 1- μ m-thick sections were obtained on a Reicher Ultracut E ultramicrotome and stained with toluidine blue for structural studies and with von Kossa staining for mineralization. The remaining metatarsal rudiments were decalcified in 1 mM Tris, pH 7.5, and 10% EDTA at 4°C for 2 h, dehydrated in ethanol, and embedded in paraffin. 5- μ m-thick sections were used for cytochemical detection of proteoglycans and for *in situ* hybridization.

Cell culture

Primary chondrocytes were isolated from ribs of newborn rats. Rib cages were dissected, and the cartilage parts were cut and placed in DME sup-

plemented with 10% FCS. They were incubated with 0.05 mg proteinase K for 30 min at 37°C, washed, and treated with 1.5 mg collagenase (Type IA; Sigma-Aldrich) for 3 h at 37°C. Chondrocytes were recovered by centrifugation, filtered through a 150 µm mesh, and plated onto Petri dishes. Cells were maintained for 3–7 d in DME supplemented with insulin-transferrin-selenium (Sigma-Aldrich) and thereafter treated with RA for 1–7 d in the same medium also containing 0.05 mg/ml ascorbic acid, 0.3 mg/ml L-glutamine, 0.05 mg/ml gentamicin, and 1 mM glycerophosphate. RCS cells were provided by Dr. J.H. Kimura (Henry Ford Hospital, Detroit, MI). Cells were maintained routinely in DME supplemented with 10% FCS and 100 µg/ml gentamicin. To test the effect of retinoids, cells were plated and allowed to adhere for 24 h in DME containing 10% FCS. Afterwards, the serum concentration was reduced to 2%, and retinoids were added at different concentrations. Media were changed every 2 d.

Cytochemistry

Mineralization was detected by von Kossa staining. After fixation in 4% paraformaldehyde in PBS for 30 min, cells were treated with 1% AgNO₃ for 60 min at room temperature and fixed with 5% sodium hyposulfite. Proteoglycan deposition in cell cultures was analyzed by Alcian blue staining.

In situ hybridization

Experiments were basically performed as reported previously (Alvarez et al., 2000). Hybridization with collagenase-3, Cbfa1, and osteocalcin probes was performed at 58°C for 16 h in a humid chamber with 400 ng/ml of DIG-labeled probe diluted in the same solution used for prehybridization. Parallel sections were hybridized with a labeled sense riboprobe and used as negative controls.

Northern blot analysis

Cells were treated with retinoids or different agonists at the concentrations and for the times indicated. Before RNA extraction, cells were pretreated with 1 mg/ml hyaluronidase and 0.15 mg/ml trypsin-EDTA for 2 min at room temperature. Total RNA isolated from the cells was separated by electrophoresis in 1% agarose gels, blotted onto nylon membranes, and hybridized as described previously (Jiménez et al., 1999).

Western blot analysis

Conditioned media were obtained after incubation of cells with the corresponding agents. Proteins from conditioned media were precipitated in 5% trichloroacetic acid, separated by SDS-PAGE, and transferred to nitrocellulose membranes. For detection of collagenase-3, membranes were incubated with a 1:5,000 dilution of monoclonal antibody 141-15A12 (Fuji Chemicals), washed, and then incubated with a goat anti-mouse IgG anti-sera conjugated to HRP. Finally, the membranes were washed and developed with an HRP chemiluminescence detection reagent (ECL system; Amersham Pharmacia Biotech).

Determination of MAPK activation

Cells treated with the corresponding agonists were lysed in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue), heated at 95°C for 10 min, fractionated in 10% SDS-PAGE, and transferred to nylon membranes. Immunodetection of JNK/stress-activated protein kinase, ERK 1,2, and p38 MAPK was performed as described by the antibody supplier (New England Biolabs, Inc.) and developed with the ECL system.

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