Cells and Materials, Volume 8, 1998 (Pages 57-61) 1051-6794/98$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

DISCOVERY AND CHARACTERISTIC FEATURES OF CARTILAGE-DERIVED RETINOIC ACID-SENSITIVE PROTEIN (CD-RAP)

S. Kondo[1], W.-F. Xie[2], S.H. Cha[3], and L.J. Sandell[*]

Dept. Orthopaedics, Univ. of Washington School of Medicine/VA Puget Sound Health Care Systems, Seattle, WA

(Received for publication July 21, 1997 and in revised form December 15, 1997)

Abstract

A cDNA encoding a new secreted protein has previously been isolated from two independent sources: melanoma cell lines and primary chondrocyte cultures. The protein from primary bovine chondrocytes and cartilaginous tissues is called cartilage-derived retinoic acid-sensitive protein (CD-RAP) and the protein from human melanoma cell lines is called melanoma inhibitory activity (MIA). CD-RAP/MIA is expressed normally in chondrocytes and pathologically in chondrosarcoma and melanoma. The function of CD-RAP/MIA is not known except that it suppresses cell proliferation and causes rounding of cells *in vitro*. The restricted expression of CD-RAP/MIA may provide an opportunity to monitor cartilage metabolic activity as well as the tumor activity of chondrosarcoma and melanoma.

Key Words: Cartilage-derived retinoic acid-sensitive protein (CD-RAP), melanoma inhibitory activity (MIA), chondrocytes, melanoma, chondrosarcoma, transcription factor.

[1]Present address: Dept. Orthopaedic Surgery, Nagoya University School of Medicine, 65 Tsuramai Showa-ku, Nagoya 466, Japan
[2]Present address: Department of Gastroenterology, Chang-Zeng Hospital, Shanghai 200003, P.R. China
[3]Present address: VA Medical Center, Seattle WA 98108

*Address for correspondence:
Linda J. Sandell
Washington University School of Medicine
Department of Orthopaedic Surgery
Yalem Research Building, Room 704
216 South Kingshighway
St. Louis, MO 63110
Telephone number: (314) 454-7800
FAX number: (314) 454-5900
E-mail: sandelll@msnotes.wustl.edu

Introduction

Discovery of CD-RAP

Retinoic acid (RA) is known to play a role in various aspects of skeletal development *in vivo*, including morphogenesis, growth plate maturation and apoptosis (Kochhar, 1967; Tickle *et al*., 1982; Tamarin *et al*., 1984; Iwamoto *et al*., 1993). In cell cultures, retinoic acid treatment of chondrocytes suppresses the chondrocyte phenotype characterized by production of type II collagen and aggrecan (Bernier and Goltzman, 1993; Lau and Heersche, 1993). In the growth plate, RA stimulates maturation of chondrocytes (Iwamoto *et al*., 1993). In an effort to discover molecules involved in regulation of chondrocytes or related to developmental processes such as chondrogenesis, mRNAs from bovine chondrocytes cultured with and without retinoic acid were isolated and amplified by reverse transcription-polymerase chain reaction (RT-PCR), then compared by differential display (Dietz and Sandell, 1996). After this analysis, our attention was focused on one clone that we called **C**artilage-**D**erived **R**etinoic **A**cid-sensitive **P**rotein, CD-RAP. The CD-RAP mRNA is quite small, encoding a protein of 130 amino acids in bovine, rat, and mouse and 131 amino acids in human. This sequence includes a signal peptide used to transport the nascent protein into the lumen of the endoplasmic reticulum. The remaining sequence does not contain any known (i.e., recognizable) protein motifs. In short, CD-RAP is a new molecule that is chondrocyte-specific, small and secreted, consequently, it is a good candidate for an easily detected marker for cartilage metabolism.

CD-RAP is the Same as Melanoma Inhibitory Activity, MIA

Once the sequence of cDNA for bovine CD-RAP was available and the GenBank was screened, a similar sequence was found, that is human **M**elanoma **I**nhibitory **A**ctivity, MIA (Blesch *et al*., 1994). MIA was originally isolated from a metastatic melanoma cell line as an inhibitory activity due to its ability to inhibit DNA synthesis in some melanoma cell lines and some other neuroectodermal tumor

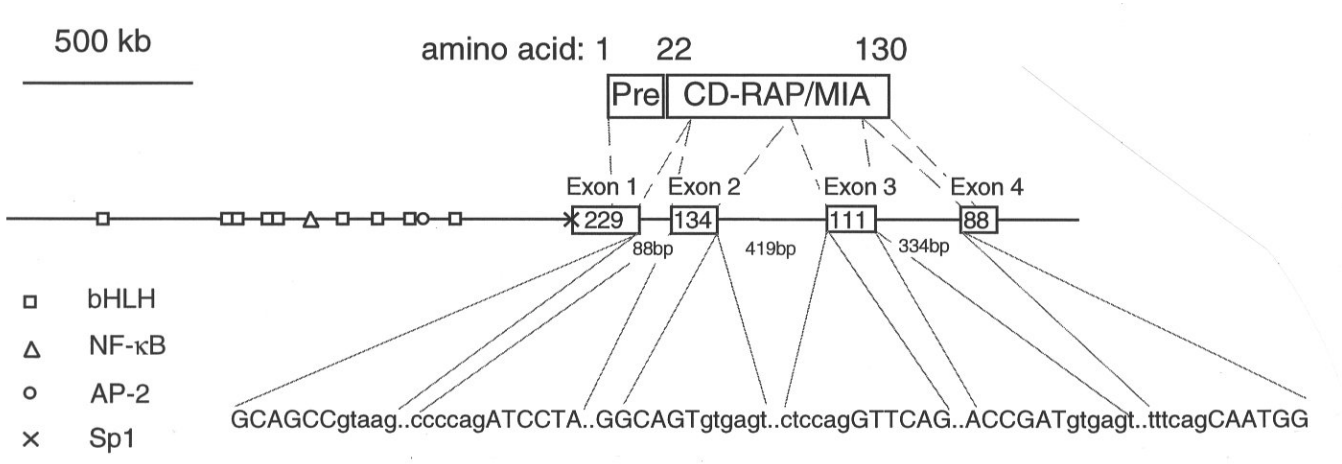

Figure 1. Structure of the CD-RAP gene. Potential *cis-acting* motifs in the promoter are indicated. Intron sequence appears in lower case, and exons in upper case.

cell lines *in vitro*, including gliomas (Bogdahn *et al*., 1989). Functional studies with MIA have shown that it inhibits DNA synthesis in melanoma cell lines and causes rounding of cells. Initially, MIA was thought to be exclusively expressed by melanoma tumors and cell lines.

Recently, CD-RAP/MIA expression has been detected during the development of mammary tumors (Lu *et al*., 1997). Similar to melanoma and chondrosarcoma, mouse mammary tumors induced by methyl-1-nitrosourea demonstrate overexpression of CD-RAP/ MIA containing no mutations. While CD-RAP/MIA expression is not observed in normal mammary tissue, we have detected endogenous expression in the pre-mammary tissue of transgenic mice in which LacZ expression is driven by the CD-RAP promoter (Xie and Sandell, unpublished observations).

Structure of the CD-RAP/MIA Gene

A diagram of the mouse CD-RAP/MIA gene is shown in Figure 1. The structure is fundamentally the same as the human gene, with slight differences in the size of the introns and exons (Bosserhoff *et al*., 1996, 1997a). A comparison of the genomic and cDNA sequences revealed that the CD-RAP/MIA gene consists of four small exons interrupted by three introns. The intron-exon boundaries are in accordance with consensus splice sites. The entire locus encoding the CD-RAP/ MIA protein is encompassed within a small region of approximately 2.5 kilo-base pairs (kb). Both the human and mouse genes have neither a CAAT box nor a TATA box close to the transcription start site. The Sp1 site is conserved in both genes as is the case for many extracellular matrix genes. An NF-kB site is conserved although at a different location. Removal or mutation of the NF-kB site from the human gene reduces activity of the gene construct in a melanoma cell line. However, this site is not responsible for the stimulatory effect of the tumor promoter, PMA (Bosserhoff *et al*., 1996). A characteristic feature of the CD-RAP/MIA promoter is the abundance of the bHLH motif, whose binding proteins can modulate determination of expression of differentiated cellular functions (Murre and Baltimore, 1992). Recent functional studies from our laboratory indicate that AP-2 (-469 base pairs from the tranlational start codon) is involved in the biphasic regulation of CD-RAP gene in chondrocytes (Xie *et al*., in press), as well as, *SOX9* at -401 (Xie and Sandell, unpublished data).

Expression of CD-RAP/MIA During Development

In situ hybridization of mouse embryos showed that during development, CD-RAP/MIA expression is initiated at day 10. Stronger expression occurs at day 11.5, particularly in cartilage of developing bones (Bosserhoff *et al*., 1997a). Expression is observed in the nasal process, basioccipital bone of the cranium, vertebral bodies, and pelvis by day 12.5. Expression of CD-RAP/MIA correlates with the beginning of overt chondrogenesis, localized to differentiating chondroblasts and chondrocytes. However, it is not observed in other non-cartilaginous tissues such as somites, notochord, neuroepithelium, periosteum, perichondrium, and osteogenic cells of the mandible (Fig. 2). Immunohistochemistry demonstrated that CD-RAP/MIA is detectable in the cytoplasm of chondrocytes (Bosserhoff *et al*., 1997a). Very little CD-RAP/MIA is detected in the interterritorial matrix between lacunae.

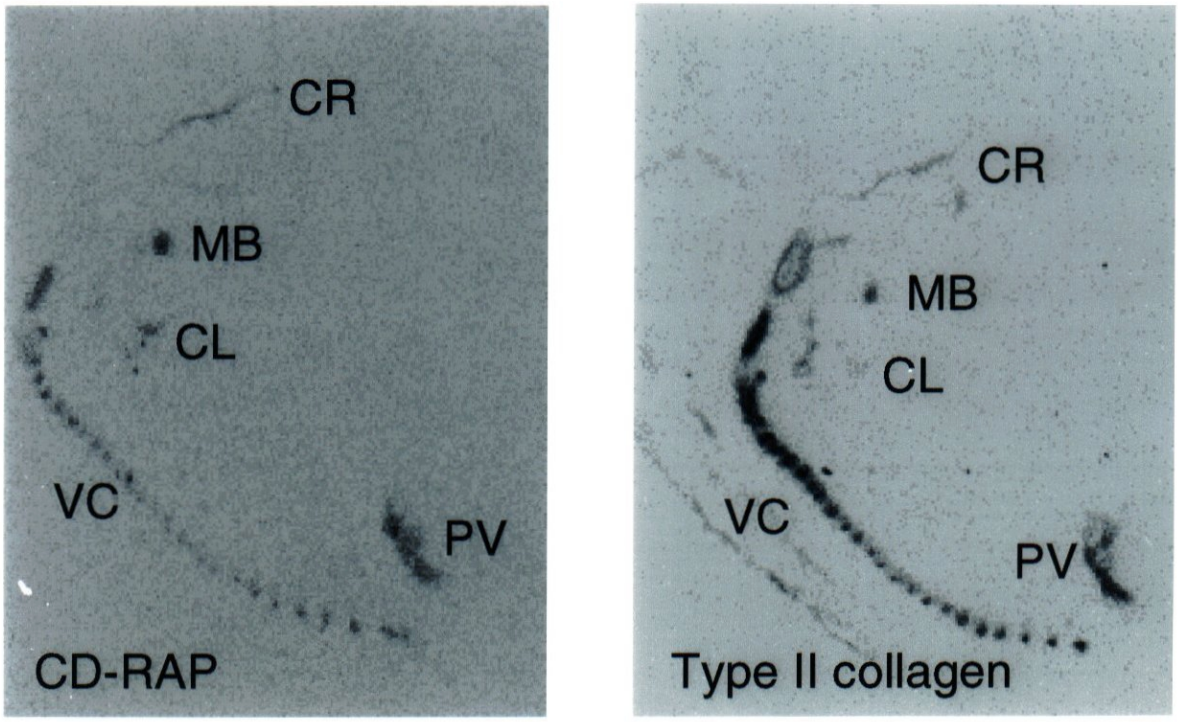

Figure 2. Autoradiographs of *in situ* hybridization with CD-RAP and type II collagen. Tissues sections were from a 13.5 day mouse embryo. Tissues labeled are the primordia of the cranium (CR), mandible (MB), clavicle (CL), vertebral column (VC), and pelvis (PV).

Expression of CD-RAP/MIA in Melanoma Cell Lines

Bosserhoff and colleagues showed that melanoma cell lines express very high levels of CD-RAP/MIA mRNA, and this expression parallels closely the malignancy of pigmented skin tumors (Bosserhoff *et al*., 1996). Cultures of non-neoplastic skin cells including fibroblasts, keratinocytes, or melanocytes do not express CD-RAP/MIA mRNA. By RT-PCR and differential display, von Groningen and colleagues independently identified CD-RAP/MIA mRNA in melanoma cell lines with different metastastic capacity (von Groningen *et al*., 1995). They reported that both non-metastasizing melanoma cell lines and metastasic melanoma lesions express CD-RAP/MIA mRNA, while the expression is absent in highly metastasizing cell lines and pretumor stages.

Treatment of melanoma cell line with purified CD-RAP/MIA protein *in vitro* results in growth inhibition paralleled by significant change in cell morphology (Bogdahn *et al*., 1989). Melanoma cells appear rounded within 2 hours after the addition of CD-RAP/MIA protein to the medium. It is therefore possible that secretion of CD-RAP/MIA *in vivo* leads to decreased melanoma cell adhesion, thus promoting melanoma invasion and progression.

Effects of CD-RAP/MIA on Chondrocytes

To examine the effects of CD-RAP/MIA on chondrocytes, we produced recombinant CD-RAP/MIA using a glutathione sulfate transferase (GST) fusion protein system (Kondo *et al*., 1997). Bovine articular chondrocytes were incubated with CD-RAP or GST alone and [^{3}H]-thymidine was added to the medium for the final 6 hour. CD-RAP/MIA inhibited incorporation of [^{3}H]-thymidine into DNA in cultured primary chondrocytes in a dose dependent manner by approximately 50 % (Fig. 3). Though CD-RAP/MIA inhibits DNA synthesis, it has no significant effect on proteoglycan synthesis as detected on the basis of ^{35}S-Sulfate incorporation (data not shown).

Serum Level of CD-RAP/MIA in Patients with Melanoma

Bosserhoff and colleagues measured serum level of CD-RAP/MIA in patients with melanoma using a quantitative, non-radioactive enzyme-linked immunosorbent assay (ELISA) (Bosserhoff *et al*., 1997b). They detected higher CD-RAP/MIA serum levels in 18% and 22% of patients with stage I and stage II disease, respectively, and in 100 % with stage III and IV disease. Compared to S-100 and

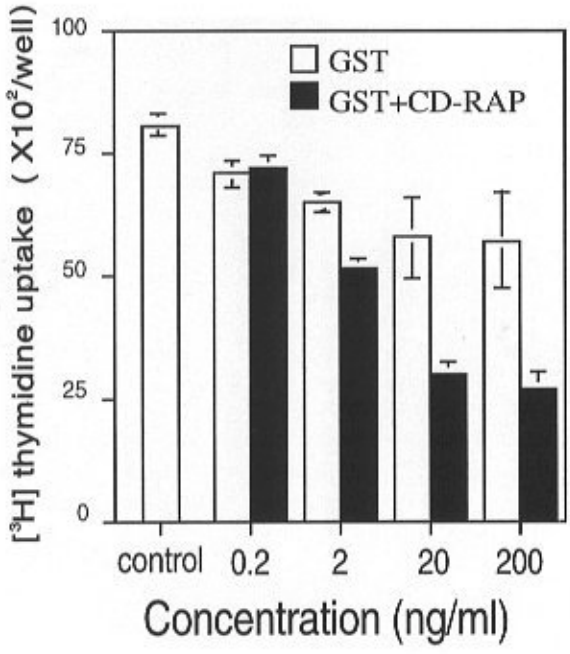

Figure 3. Effect of CD-RAP on DNA synthesis. Primary chondrocytes were cultured with GST + CD-RAP or GST alone for 48 hours [^{3}H]-thymidine was added to the medium 6 hours before harvesting.

sICAM-1 serum levels, CD-RAP/MIA is the most sensitive marker for melanoma. Response to chemotherapy in stage IV disease correlated with changes in CD-RAP/MIA serum levels. Repeated measurement of sera of 239 patients with a history of stage I or II melanoma during follow-up revealed that 16 patients developed positive CD-RAP/MIA serum level. At the time of analysis, 9 of the 16 patients had developed metastases, one had numerous melanocytic nevi, and 6 had no clinical signs of metastasis. None of the patients with normal MIA serum levels developed metastases during follow-up period of 6 months. In addition, after surgical removal of the tumor, CD-RAP/MIA serum levels of patients with stage III dropped significantly.

Expression of CD-RAP in Skeletal Tumors

We investigated the production of CD-RAP/MIA in a series of skeletal tumors (Chansky *et al*., 1997). In a preliminary study, 29 skeletal tumors were analyzed for the presence of the CD-RAP/MIA protein: 14 chondrosarcomas, 5 osteosarcomas, 1 Ewing's sarcoma and 9 miscellaneous non-malignant tumors. One melanoma was tested as a positive control. Proteins in the tissues were extracted from 3 mm^3 specimen by three repeated freeze/thaw cycles in 0.15 ml of phosphate buffered saline. The extracts were spun at 5000 x g for 10 minutes and the supernatants were tested by ELISA using the human CD-RAP/MIA monoclonal antibody. Every chondrosarcoma expressed high levels of CD-RAP/MIA, and all but one were higher than the melanoma. In contrast, all of the osteosarcomas were negative. Interestingly, three tumors originally classified as non-malignant enchondromas at the time of surgery showed very high CD-RAP/MIA levels. These three tumors subsequently recurred and were diagnosed as chondrosarcoma by the pathologist. There appears to be a trend toward more CD-RAP/MIA production as the grade of the chondrogenic tumor increases.

Future of CD-RAP/MIA

Cartilage damage is one of the most intractable problems in orthopaedics. Many experiments and clinical trials have been focused on the repair of cartilage damage and so far, the results have not been satisfactory. There are several possible roles for CD-RAP/MIA in clinical applications. First, CD-RAP/MIA itself could be used as a cytokine applied directly to joint spaces. The preliminary results indicate that CD-RAP/MIA has inhibitory effect on cell proliferation and is the cause of cell rounding. However, the full effect of CD-RAP/ MIA on chondrocytes or, possibly more importantly, chondroprogenitor cells, is still unknown. To further understand the function of CD-RAP/MIA *in vivo*, we are currently developing a gene knockout mouse.

Secondly, CD-RAP/MIA may be used as a disease marker. As we reviewed in this paper, serum levels of CD-RAP/MIA in patients with melanoma are significantly higher than those in healthy controls and correlate with later stages of the disease. In orthopaedics, CD-RAP/MIA might be very useful in diagnosing chondrogenic tumors or monitoring changes in cartilage metabolism as seen in osteoarthritis. Based on our expression studies of CD-RAP/MIA, we would anticipate that a mutation or a malfunction of the CD-RAP/MIA gene would affect the development and/or maintenance of cartilage. To date, we have not uncovered any diseases that are associated with defective CD-RAP/MIA.

The third potential clinical application is to use the chondrocyte specificity of the CD-RAP/MIA promoter in gene therapy. We have already developed transgenic mice harboring the CD-RAP/MIA promoter and confirmed that it contains elements that specifically drive expression in chondrocytes.

Acknowledgment

We are grateful to Dr. Pamela Enayati for critical review of the manuscript.

References

Bernier SM, Goltzman D (1993) Regulation of expression of the chondrocytic phenotype in a skeletal cell line (CKF2) *in vitro*. J Bone Min Res **8**: 475-483.

Blesch A, Bosserhoff AK, Apfel R, Behl C, Hessdorfer B, Schmitt A, Jachimczak P, Lottspeich F, Buettner R, Bogdahn U (1994) Cloning of a novel malignant melanoma-derived growth-regulatory protein, MIA. Cancer Res **54**: 5695-56701.

Bogdahn U, Apfel R, Hahn M, Gerlach M, Behl C, Hoppe J, Martin R (1989) Autocrine tumor cell growth-inhibiting activities from human malignant melanoma. Cancer Res **49**: 5358-5363.

Bosserhoff AK, Hein R, Bogdahn U, Buettner R (1996) Structure and promoter analysis of the gene encoding the human melanoma-inhibiting protein MIA. J Biol Chem **271**: 490-495.

Bosserhoff AK, Kondo S, Moser M, Dietz UH, Copel NG, Gilbert DG, Jenkins NA, Buettner R, Sandell LJ (1997a) The mouse MIA/CD-RAP gene: Structure, chromosomal localization and expression in cartilage and chondrosarcoma. Dev Dynamics **208**: 516-525.

Bosserhoff AK, Kaufmann M, Kaluza B, Bartke I, Zirngibl H, Hein R, Stoltz W, Buettner R (1997b) MIA, a noble serum marker for progression of malignant melanoma. Cancer Res **57**: 3149-3153.

Chansky H, Robbins JR, Cha SH, Raskind WH, Conrad EU (1997) Expression of cartilage extracellular matrix and potential regulatory genes in a new chondrosarcoma cell line (Ch-1). J Orthop Res **16**: 521-530.

Dietz UH, Sandell LJ (1996) Cloning of a retinoic acid-sensitive mRNA expressed in cartilage and during chondrogenesis. J Biol Chem **271**: 3311-3316.

Iwamoto M, Shapiro IM, Yagami K, Boskey AL, Leboy PS, Adams SL, Pacifici M (1993) Retinoic acid induces rapid mineralization and expression of mineralization-related genes in chondrocytes. Exp Cell Res **207**: 413-420.

Kochhar D (1967) Teratogenic activity of retinoic acid. Acta Pathol Microbiol Scand **70**: 398-404.

Kondo S, Cha SH, Zhu Y, Xie WF, Sandell LJ (1997) Regulation and function of CD-RAP, a new cartilage specific protein. Trans Ortho Res Soc. **22** (sec. 1): 338 (abstract).

Lau WF, Heersche JNM (1993) Effects of retinoic acid on cartilage differentiation in a chondrogenic cell line. Teratology **47**: 555-563.

Lu J, Pei H, Kaeck M, Thompson HJ (1997) Gene expression changes associated with chemically-induced rat mammary carcinogenesis. Mol Carcinogen **20**: 204-215.

Murre C, Baltimore D (1992) Transcriptional Regulation. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 861-879.

Tamarin A, Crawley A, Lee J, Tickle C (1984) Analysis of upper beak defects in chicken embryos following with retinoic acid. J Embryol Exp Morphol **84**: 105-123.

Tickle C, Alberts B, Wolpert L, Lee J (1982) Local application of retinoic acid to the limb bud mimics the action of the polarizing region. Nature **296**: 564-566.

von Groningen JJM, Bloemers HPJ, Swart GWM (1995) Identification of melanoma inhibitory activity and other differentially expressed messenger RNAs in human melanoma cell lines with different metastatic capacity by messenger RNA differential display. Cancer Res **55**: 6237-6243.

Xie W-F, Kondo S, Sandell LJ (1998) Regulation of the mouse cartilage-derived retinoic acid-sensitive protein (CD-RAP) gene by the transcription factor AP-2. J Biol Chem **273**: 5026-5032.

Discussion with Reviewers

R.S. Tuan: The authors have made an interesting suggestion of the possible clinical applicationof CD-RAP in cartilage repair. However, since CD-RAP appears to inhibit chondrocyte proliferation, whereas cartilage repair will require chondroinductive or chondro-proliferative activities, how do the authors envision the applicability of CD-RAP in this process?

Authors: CD-RaP inhibits chondrocyte proliferation, but its effect on chondroprogenitor cells and chondroblasts may not be the same. To further understand the function of CD-RAP *in vivo*, a gene knockout mouse is being developed.