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Antisense retinoic acid receptor γ-1 oligonucleotide enhances chondrogenesis of mouse limb mesenchymal cells in vitro

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Abstract

Retinoic acid receptor (RAR) γ gene is expressed in the precartilaginous cells during chondrogenesis in mouse embryos, but the role of the gene products is still unclear To examine the role during chondrogenesis, we isolated mesenchymal cells from the limb bud of mouse embryos and exposed them to antisense RAR γ-1 oligodeoxynucleotide in micromass culture The antisense oligodeoxynucleotide inhibited RAR γ-1 protein expression and enhanced chondrogenesis in the exposed cells These results suggest that the complex of RAR γ-1 protein and its ligand RA acts as a suppressor of the chondrogenesis in the limb development

Key words Retinoic acid receptor γ-1, Antisense oligonucleotide, Micromass culture, Limb mesenchymal cell, Chondrogenesis

1. Introduction

Retinoic acid (RA), a metabolite of vitamin A, plays an important role in pattern formation during vertebrate limb development and regeneration [1–6]. Excess retinoids induce craniofacial and limb malformations involving abnormal cartilage and skeletal development [7–10], and have inhibitory effects on the chondrogenesis of craniofacial and limb mesenchymal cells in vitro [11,12] On the other hand, nuclear RA receptor (RAR) γ gene is expressed in precartilaginous mesenchymal aggregates, whereas RAR β is expressed in the surrounding cells that differentiate into fibrous tissue in mouse embryos [13,14]. The effects of RA and the spatial distribution of different RARs gene expression suggest that endogenous RA has a role in regulating the chondrogenic area mediated by RAR β and γ To examine the role of RAR γ protein during chondrogenesis, we isolated mesenchymal cells from the limb bud of 11 day p c mouse embryos and exposed them to antisense RAR γ-1 oligodeoxynucleotide in micromass culture. We found that the antisense oligodeoxynucleotides inhibited RAR γ-1 expression and enhanced the enlargement of chondrogenic area in the exposed cells.

2. Materials and methods

2 1 *Synthesis and purification of oligodeoxynucleotide*

Fifteen-base oligodeoxynucleotides for both sense and antisense strands of mouse RAR γ-1 cDNA [15,16] were made on a Milligene Cyclone Plus DNA synthesizer using beta-cyanoethylphosphoramide chemistry and were modified with a sulfurizing reagent (Beaucage reagent, Millipore) during synthesis They were purified by electrophoresis, ethanol precipitation, and repeated washes with 70% ethanol The oligodeoxynucleotides were resolved in a small volume of sterile water, and stock solutions were prepared by dilution with plain culture medium The sequence of the sense oligodeoxynucleotide (Sγ-1) was 5′-GCAGCTACCATGGCC-3′, and that of antisense (ASγ-1) was 5′-GGCCATGGTAGCTGC-3′ They were designed to overlap the initiation codon of RAR γ-1 mRNA

2 2 *Micromass culture and treatment with oligodeoxynucleotides and RA*

The micromass culture was performed as described by Wedden et al [17] Distal tips of forelimb buds were dissected from 11 d p c C57BL/6 mouse embryos (Sankyo Laboratory) The mesenchymal cells removed from the ectoderm after incubation in dispase (Godosyusei) at 4°C for 18 min were disaggregated and suspended in Ham's F-12 tissue culture medium (Gibco) containing 10% fetal calf serum (JRH Biosciences) and 200 μg/ml ascorbic acid (Gibco) They were plated out in 10 μl drops at a final density of 2×10^7 cells/ml on tissue culture dishes (Falcon Primaria) and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for up to 7 days The oligodeoxynucleotides were added to the every changed culture medium at a final concentration of 5 0 μM every other day for 7 days, and the same volume of sterile water was added as control We started the treatment with 10 nM RA (Sigma, type XX) after one day of incubation with or without the antisense oligodeoxynucleotide

2 3 *Staining and quantification of cartilage matrix*

The cultures were fixed in half strength Karnovsky's fixative [18] at 4°C for 2 h and stained with Alcian blue at pH 1 for 2 h for the detection of cartilage specific proteoglycans This staining is the promised method, because the dye specifically binds to the sulfated glycosaminoglycans that has been identified to be one of the differentiation marker in the chondrogenesis [19] Deposited Alcian blue in the cartilage matrix

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was extracted from cultures with 6 M guanidine HCl at 4°C for 16 h and the absorbance was measured at 600 nm [20] Cartilage matrix production in each culture was indicated as percentage of the absorbance against controls

2 4 Production of anti-RAR γ-1 polyclonal antibody

The polyclonal antibody was raised against fusion protein of glutathione S-transferase (GST) and human RAR γ-1 expressed in *Escherichia coli* The vector for expressing fusion protein was constructed by ligating the cDNA fragment containing the A region corresponding to amino acid residues 1–57, which is unique to RAR γ-1, into the appropriate site of pGEX-2T vector (Pharmacia), and confirmed by DNA sequencing The construct was introduced to *E coli* JM101 and expression of fusion protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Takara) The fusion protein purified by Glutathione Sepharose 4B (Pharmacia) was emulsified with an equal volume of Freund's complete adjuvant and injected into rabbits Booster injections with incomplete Freund's adjuvant were administered 3 and 6 weeks later Fifteen days after the second booster injection, serum was collected and IgG fraction was purified by using protein-G sepharose (Amersham) IgG against GST was removed by affinity chromatography using GST expressed in *E coli* The specificity of the antibody to mouse RAR γ-1 was confirmed by immunoabsorption

2 5 Western blotting analysis

We cultured the cells in the presence of 5 0 μM sense, antisense RAR γ-1 oligodeoxynucleotide or control for 2 days, washed them with PBS and removed by scraping with a rubber policeman and centrifuged The pellets were homogenized in 10 volumes of buffer (10 mM Tris-HCl, pH 6 8, 1% SDS, 0 15 M NaCl, 1 mM EDTA) at 0°C, and then mixed in a vortex mixer at 0°C for 10 min After centrifugation at 10,000 × *g* at 4°C for 1 h, we measured protein concentration of the supernatants by the modified Lowry's method (Pierce) Ten μg of protein were fractionated by electrophoresis on a sodium dodecyl sulfate/10% polyacrylamide gel, and electroblotted onto nitrocellulose sheets The blots were blocked with PBS containing 5% dried milk and incubated with anti-RAR γ-1 IgG fraction (1 μg/ml) diluted in PBS containing 3% bovine serum albumin at 20°C for 16 h After washing with PBS, they were incubated with horseradish peroxidase conjugated anti-rabbit-IgG (Amersham) at 20°C for 1 h, and washed five times with PBS We incubated them in the detection solution (Amersham) to allow chemiluminescence to occur for 1 min and then exposed them to autoradiography film (Kodak)

3. Results

In Western blotting analysis, the polyclonal antibody against fusion protein of GST and RAR γ-1 A region detected a band with a relative molecular weight of 50 kDa in the proteins isolated from 2 day's cultures (Fig 1, lane A) and the band disappeared by the addition of excess antigen (Fig 1, lane B). The antibody specifically recognized endogenous RAR γ-1 protein because molecular weight of mouse RAR γ-1 has been already determined as 50 kDa [15,16]. The treatment with 5 0 μM ASγ-1 for 2 days reduced the intensity of RAR γ-1 protein expression (Fig 1, lane D), while the same concentration of Sγ-1 had no effect (Fig 1, lane C) Thus, AS-γ-1 specifically inhibited the RAR γ-1 protein expression in the exposed cells

The cells isolated from limb buds developed numerous discrete cartilaginous aggregates intensely stained with Alcian blue for 7 days under the control condition (Fig. 2A) Five μM ASγ-1 increased the extent of cartilage aggregates in exposed cells (Fig. 2B) The cartilaginous aggregates occupied more than 50% of the total area of the cultures (Fig 2B), while about 30% was occupied in the control and the treatment with 5 0 μM Sγ-1 (Fig 2A,C) By measuring the amount of accumulated Alcian blue-positive cartilage matrix, we confirmed that ASγ-1 increased the accumulation to almost 160% compared to that of controls, while Sγ-1 had no effect (Fig 3)

Under the treatment with 5 0 μM ASγ-1, 10 nM RA inhibited the cartilage matrix accumulation at 40% compared to the cultures treated with ASγ-1 alone (Fig 2E, Fig 3) Only 10 nM RA also inhibited the accumulation at 40% compared to the control (Fig. 2D, Fig 3). Thus, there was no difference in RA responsiveness between the cultures treated with and without ASγ-1

4. Discussion

We demonstrated that the inhibition of RAR γ-1 protein expression by ASγ-1 increased the extent of precartilaginous aggregates, indicating that RAR γ-1 is related to the inhibition of the enlargement of precartilaginous aggregates (Fig. 2A,B), and suggesting that endogenous RA inhibits chondrogenesis mediated by RAR γ-1 even under the control condition. What is the source of providing endogenous RA in the limb development? RAR β gene has been known to express in surrounding cartilaginous aggregates in mouse embryo [13,14] and to be

A B C D

Fig 1 Immunodetection of RAR γ-1 in whole cell extracts of mouse limb mesenchymal cells cultured for 2 days After electrophoresis and electrotransfer to nitrocellulose sheets, the blots were incubated with anti GST-RAR γ-1 fusion protein Ig fraction, without (lanes A, C and D) or with (lane B) previous immunoabsorption with GST-RAR γ-1 fusion protein RAR γ-1 protein is detected in the cells under control condition (lane A) (lane B) Because this reactivity is disappeared by immunoabsorption, the band detected in lane A indicates endogenous RAR γ-1 protein (lane C) Cells cultured in the presence of sense oligodeoxynucleotide possess similar reactivity (lane D) This reactivity is reduced by treatment with antisense RAR γ-1 oligodeoxynucleotide Position of the 50 kDa band (arrow) is noted Molecular weight markers from Bio-Rad were used for size determination

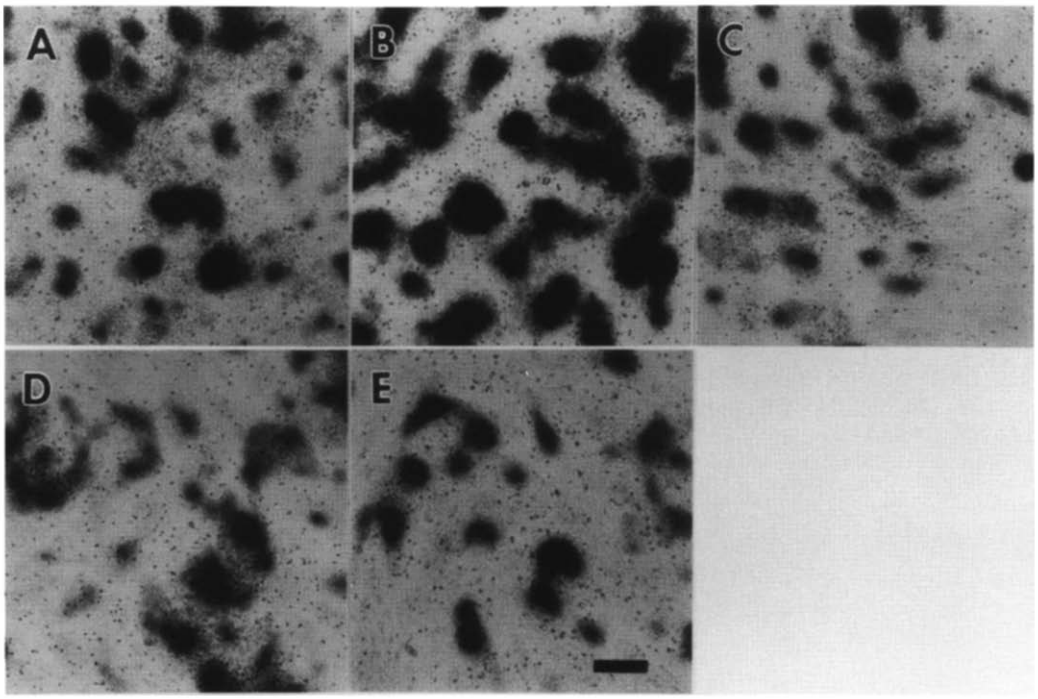

Fig 2 Accumulation of Alcian blue-positive cartilage matrix in the culture under a control condition (A), in the presence of antisense (B), and sense (C) RAR γ-1 oligodeoxynucleotide for 7 days (A) Cartilaginous aggregates stained with Alcian blue are formed (B) 5 0 μM antisense RAR γ-1 oligodeoxynucleotide increases the extent of Alcian blue-positive cartilage aggregates (C) 5 0 μM sense oligodeoxynucleotide has no effect (D) 10 nM RA inhibited chondrogenesis (E) The inhibitory effects of RA are also observed in the culture treated with the antisense oligodeoxynucleotide Scale bar, 100 μm

up-regulated by the complex of the receptor itself and its ligand RA mediated by RA responsive element [21–23]. The spatial distribution and the RA dependent expressions suggest that endogenous RA concentration in the surrounding aggregates is higher than that in other area. Thus, RA provided from the surrounding cells may inhibit the enlargement of cartilaginous aggregates The RA may be captured by RAR γ-1 in the cartilaginous cells, and the complex may be crucial in transducing RA signals at the transcriptional level, resulting in restriction of the chondrogenic area.

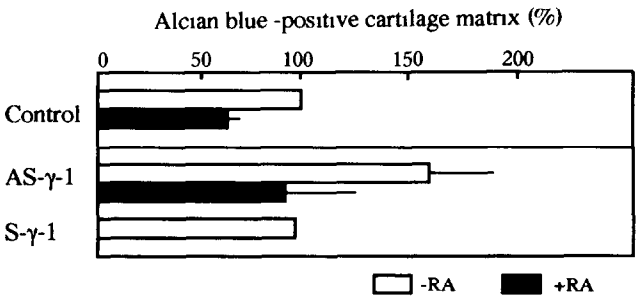

Fig 3 Quantitative comparison of the effects of the antisense or sense RAR γ-1 oligodeoxynucleotide and RA on the accumulation of Alcian blue-positive cartilage matrix Bars represent mean ± S E of determinations from six experiments Five replicate cultures were carried out in each experiment The antisense oligodeoxynucleotide increased the quantity of the accumulation of cartilage matrix Sense RAR γ-1 oligodeoxynucleotide has no effect Ten nM RA inhibits 40% of the accumulation compared to that of controls It inhibits cartilage accumulation at a similar ratio

ASγ-1 did not abolish the exogenous RA effects on the chondrogenesis (Fig. 2E). The remaining RAR γ-1 proteins in the cells even after ASγ-1 treatment may be enough to mediate the exogenous RA effects (Fig 1, lane D), or RAR γ-1 may not be involved in the pathway that mediates the effects thus inhibiting chondrogenesis

Further studies are required to determine whether the endogenous RA concentration in the surrounding of the cartilaginous aggregates is really higher than that in the precartilaginous cells, and whether endogenous RA has a role during chondrogenesis in mouse limb development

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