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Antisense c-*myc* oligonucleotide promotes chondrogenesis and enhances RA responsiveness of mouse limb mesenchymal cells in vitro

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Abstract

To examine the role of c-*myc* protein during chondrogenesis, we exposed 11 day p c mouse limb mesenchymal cells to the antisense c-*myc* oligonucleotide in micromass culture The antisense oligonucleotide inhibited the c-*myc* protein expression, and intensely promoted chondrogenesis in the exposed cells Most of the cells differentiated into cartilaginous cells, whereas they differentiated into cartilaginous and fibrous cells under the control conditions The antisense oligonucleotide increased the inhibitory efficiency of all-*trans* retinoic acid (RA) to the chondrogenesis These results suggest that the c-*myc* protein suppress the chondrogenesis and reduces RA responsiveness in the limb mesenchymal cells

Key words c-*myc*, Antisense oligonucleotide, Micromass culture, Limb mesenchymal cell, Chondrogenesis, RA responsiveness

1. Introduction

It is well known that the c-*myc* gene is highly expressed in the precartilaginous cells during chondrogenesis of mouse mesenchymal cells, while there is no c-*myc* expression in the differentiated cartilaginous tissues [1,2] Since the BrdU incorporation has indicated no differences in growth activity between the precartilaginous and surrounding cells [2], the c-*myc* expression may be related to cartilage differentiation rather than cell proliferation.

All trans-retinoic acid (RA), which has been known to decrease c-*myc* gene expression in many kinds of embryonic carcinoma cells [3,4], inhibits the chondrogenesis of mesenchymal cells both in vivo and in vitro [5,6]. The c-*myc* expression in precartilaginous cells may be related to their RA responsiveness.

To examine the role of the c-*myc* protein during chondrogenesis, we exposed 11-day p.c mouse limb mesenchymal cells to the antisense c-*myc* oligonucleotide in micromass culture Furthermore, to examine the relation between c-*myc* expression and RA responsiveness, we treated the cells with both the antisense oligonucleotide and RA We found that the antisense oligonucleotide inhibited c-*myc* protein expression, promoted chondrogenesis, and enhanced the RA responsiveness of limb mesenchymal cells

2. Materials and methods

2 1 *Synthesis and purification of oligodeoxynucleotides*

Fifteen-base unmodified oligodeoxynucleotides for both sense and antisense strands of mouse c-*myc* cDNA [7] were made on an Applied Biosystems 391 DNA synthesizer using β-cyanoethylphosphoramide chemistry We purified them using electrophoresis, ethanol precipitation, and repeated washes with 70% ethanol They were dissolved in a small amount of sterile water, and stock solutions were prepared by dilution with plain culture medium The sequence of the sense oligodeoxynucleotide was 5′-AGTCCCCTCAACGTG-3′, and that of the antisense was 5′-CACGTTGAGGGGCAT-3′ [8] They were designed to overlap the initiation codon of the c-*myc* mRNA

2 2 *Micromass culture and treatment of oligodeoxynucleotides and all-trans-retinoic acid (RA)*

The micromass culture was performed as described by Wedden [9] Distal tips of forelimb buds were dissected from 11 d p c mouse embryos (C57BL/6, 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) We plated out the cells in 10 μl drops at a final density of 2×10^7 cells/ml on tissue culture dishes (Falcon Primaria) and cultured them in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for up to 4 days We added the sense or antisense oligonucleotide to the culture medium at the final concentration of 5 0 μM every other day for 4 days, and the same volume of sterile water was added as control Ten nM RA (Sigma type XX) was added to the culture medium after one day of incubation with or without 5 0 μM antisense oligonucleotide

2 3 *Detection of* c-myc *protein by immunohistochemistry*

We fixed the cultures incubated for 2 days in 2% paraformaldehyde in PBS After permeabilization in 0 1% Triton X-100 (Sigma) in PBS, they were incubated with 500 ng/ml anti-human c-*myc* monoclonal antibody (Cambridge Research Biochemicals Co) that is known to recognize mouse c-*myc* protein (personal communication) at 4°C for 16 h, and were also incubated without the antibody as negative control For immunodetection, we used ABC kit (Vector Laboratories Inc) and HRP-DAB reaction

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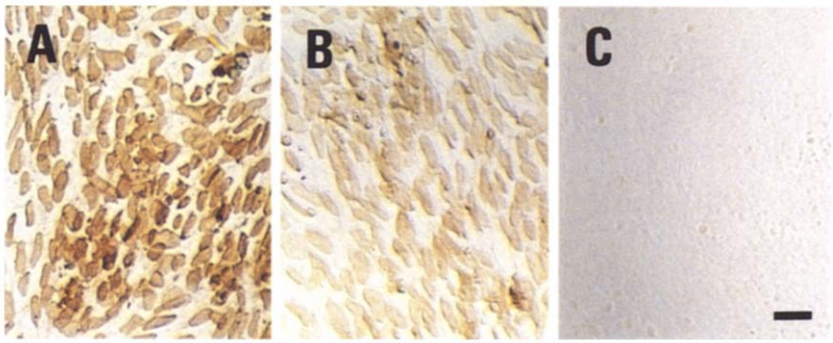

Fig 1 Immunodetection of c-*myc* protein in the cells cultured in the presence of sense (A) and antisense (B) c-*myc* oligonucleotide for 2 days (A) Cells cultured in the presence of sense oligonucleotide possess c-*myc* reactivity in nuclei (B) The antisense oligonucleotide reduced the reactivity (C) There was no signal in negative control Scale bar = 10 μm

2 4 Staining and quantification of cartilage matrix

The cultures were fixed in half strength Karnovsky's fixative [10] at 4°C for 2 h and stained with Alcian blue at pH 1 for 2 h [11] This staining is a promising method because the dye specifically binds to the sulfated glycosaminoglycans that have been identified to be one of the differentiation markers in the chondrogenesis [11] We extracted accumulated Alcian blue from each culture with 6 M guanidine HCl at 4°C for 16 h and measured the absorbance at 600 nm [12] Cartilage matrix production in each culture was indicated as percentage of the absorbance against controls

3. Results

The c-*myc* protein expressed ubiquitously in the nucleus of the cells cultured for 2 days (Fig. 1A). The treatment with 5 0 μM antisense c-*myc* oligonucleotide for 1 day reduced the expression (Fig 1B) while the same concentration of sense oligonucleotide had no effect (data not shown) Thus, the antisense oligonucleotide specifically inhibited the c-*myc* protein expression in the exposed cells. There was no c-*myc* expression in negative controls (Fic 1C)

The cells isolated from limb buds developed numerous discrete cartilage aggregates intensely stained with Alcian blue for 4 days under the control conditions (Fig 2A) The antisense oligonucleotide drastically increased the number and the extent of cartilage aggregates and many of them fused each other (Fig 2B) Most of the cells were stained with Alcian blue in the exposed cultures Accompanied by the expansion of cartilaginous area, the accumulation of Alcian blue-positive cartilage

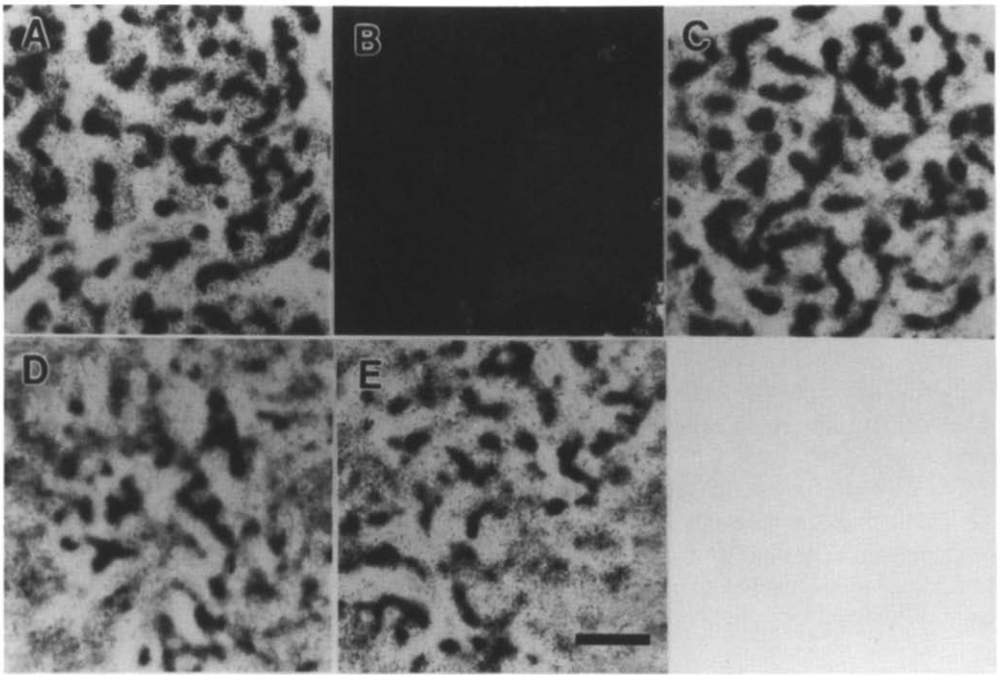

Fig 2 Accumulation of Alcian blue-positive cartilage matrix cultured under the control condition (A), in the presence of antisense (B), and sense (C) c-*myc* oligonucleotides for 7 days (B) 5 0 μM antisense oligonucleotide promotes chondrogenesis Most of the cells form cartilage aggregates and fuse to each other (C) The sense oligonucleotide has no effect (D) 10 nM RA inhibits the chondrogenesis (E) RA responsiveness under treatment with the antisense oligonucleotide is higher than that under the control conditions Scale bar = 500 μm

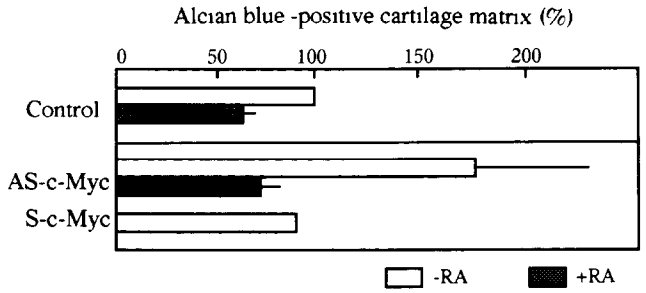

Fig 3 Quantitative comparison of the effects of the treatment with antisense or sence c-*myc* oligonucleotide and RA on the accumulation of cartilage matrix stained with Alcian blue Bar represents mean ± S E of determinations from five experiments Two to five replicate cultures were carried out in each experiment The antisense oligonucleotide promotes Alcian blue accumulation to about 1 8-fold compared to the control The sense oligonucleotide has no effect With treatment of the antisense oligonucleotide, RA suppress 63% of cartilage accumulation, while it inhibits 40% of control under the control condition

matrix also increased to about 1 8-fold of control (Fig. 3), while sense c-*myc* oligonucleotide had no such effect (Figs 2C and 3). These results indicated that inhibition of c-*myc* protein expression promoted chondrogenesis of the limb mesenchymal cells

Under the treatment with 5.0 μM antisense oligonucleotide, 10 nM RA reduced the cartilage matrix accumulation at 37% compared to the cultures treated with the antisense oligonucleotide alone (Figs. 2E and 3) Only RA reduced the matrix accumulation at 60% compared to control (Figs. 2D and 3) Thus, under a low amount of c-*myc* protein, the RA responsiveness in the mesenchymal cells was much higher than that of the controls

4. Discussion

We demonstrated that most of the limb mesenchymal cells differentiated into cartilage by the inhibition of c-*myc* protein expression, indicating that c-*myc* protein is related to the inhibition of the cartilage differentiation both in precartilaginous cells and surrounding mesenchymal cells that differentiate into fibrous cells during limb development. On the other hand, the inhibition of c-*myc* protein expression enhanced the RA responsiveness. Thus, c-*myc* protein may be involved not only in the inhibition of the cartilage differentiation, but also in the suppression of the response to RA. If the inhibition of chondrogenesis caused by RA is another kind of differentiation of the mesenchymal cells, it may be reasonable to interpret that c-*myc* proteins have a role in keeping the cells in an undifferentiated state during limb chondrogenesis

The results do not appear contradictory to the interpretation for the c-*myc* gene expressed in precartilaginous aggregates in vivo [1,2]. When the precartilaginous cells form mesenchymal condensations as a core of chondrogenesis, cartilage matrix is not produced vigorously (unpublished data) During the condensation, c-*myc* protein may inhibit differentiation into cartilage not only in the surrounding mesenchymal cells but also in the precartilaginous cells in vivo.

Staurosporine, a potent inhibitor of protein kinase C (PKC), is known to promote chondrogenesis of chick limb mesenchymal cells in micromass culture [13], suggesting that PKC is related to the negative regulation of determination to differentiate into cartilaginous cells In addition, it has been known that the PKC-dependent signal transduction pathway regulates c-*myc* gene expression in the Rausher virus-transformed Epo-responsive cells [14]. These data suggest that both c-*myc* and PKC are the members of negative regulation during chondrogenesis To prove this hypothesis, we have to elucidate the relationship between c-*myc* expression and PKC activation during chondrogenesis in developing limb bud

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