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 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-cyanoethylphosphoramid 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 phosphate-buffered saline.

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 day p c mouse embryos (C57BL/6, Sankyo Laboratory). The mesenchymal cells removed from the ectoderm after incubation in dispase (Gibco) containing 10% fetal calf serum (JRH Biosciences) and 100 μg/ml ascorbic acid (Gibco) were plated out in 10 μl drops at a final density of 2 x 10^5 cells/ml on tissue culture dishes (Falcon Primaria) and cultured them in a humidified atmosphere of 95% air and 5% CO₂ 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 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 μM antisense oligonucleotide.

2.3 Detection of c-myc protein by immunohistochemistry
We fixed the cultures incubated for 2 days in 2% parafomaldehyde in PBS. After permeabilization in 0.1% Triton X-100 (Sigma) in PBS, we 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.
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 50 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 (Fig. 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 matrix...
Keeping the cells in an undifferentiated state during limb development 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 Rauscher 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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References