A study of growth inhibitory factor in rabbit mandibular condylar cartilage

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Abstract : Since the endochondral bone growth of the mandible is a most important mechanism in the field of craniofacial growth and development, an investigation which examined the acid extracts from cartilage tissues *in vitro* culture system was done in order to explore the growth regulating factors in mandibular condylar cartilage.

Cultured chondrocytes isolated from young rabbit costal cartilage were used for assay of mandibular condylar cartilage derived factors. Crude acid extract and a chromatographically separated fraction (F-IV) from the mandibular condyle had an ability to inhibit chondrocyte proliferation in cell culture. An extract from nasal septal cartilage slightly inhibited proliferation of the chondrocytes, where as those from other cartilage, capital cartilage and costal cartilage, did not inhibit the ^3H -Thymidine incorporation. However, this inhibitory activity was gradually reduced with to subculture progression.

These results suggest that this growth inhibitory factor from mandibular condylar cartilage may have a physiological role in the growth regulating mechanism of this unique cartilage.

Introduction

The mandibular condyle is a site of endochondral ossification which persists throughout life and plays an important part in the pre- and postnatal growth of the mandible. Studies on the growth potential in the mandibular condylar cartilage have been of greater interest in the characterization of its growth activity compared with those in other cartilage tissues, such as epiphyseal cartilage, nasal septal cartilage and synchondrosis¹⁻³⁾. These investigations suggested that the condyle is a site of endochondral ossification which is essential for normal bone growth. On the bases of the experiments of Kosk *et al.*^{4,5)}, they suggested that the growth potential of condylar cartilage was less than those of the epiphyseal cartilage and the synchondrosis.

Interest in the growth of the condylar cartilage has been shifted to a new question why the condylar cartilage has a uniqueness of

lower growth activity compared with other cartilage tissues. In hyperplasia of the condyle, the mandible is enlarged which may be due to the collapse of the mechanism of self control in the condylar cartilage. According to Petrovic *et al.*^{6,7)}, the presence of chondroblasts has a restraining effect on the multiplication of prechondroblasts and such a restraining effect is a cybernetic negative feedback signal, being part of the regulatory system. However, the existence of the growth regulating factor in the mandibular condylar cartilage has not yet been determined at the biochemical level. In order to achieve the degree of control seen in cartilaginous mandibular bone growth, one could speculate that a substance which inhibits the cell division might be present.

In order to know if such a cell division inhibitory substance exists, an assay system of rabbit mandibular condylar cartilage derived factor was characterized. In this article, the

effect of the extracts from rabbit mandibular condylar cartilage, nasal septum, capital cartilage and costal cartilage on the proliferation of the chondrocytes in cell culture was examined. The results in this study strongly suggest the existence of an intrinsic growth regulatory factor of the condylar cartilage.

Materials and Methods

1) Isolation and Cultivation of Chondrocytes from Costal Cartilage

Activity of a growth regulatory factor was assayed by measuring the stimulation of DNA synthesis in confluent grown monolayers of primary cultures of chondrocytes from the costal cartilage of young male Japanese white rabbits (200–400 g body weight). The preparation and culture of primary chondrocytes were performed by using a modification of the technique of Suzuki *et al.*⁸⁾. The fresh costae were removed aseptically and immediately immersed into cold Hank's balanced salt solution (Nissui Pharmaceutical Co., Tokyo). The costae's adherent muscles and perichondrium were stripped as far as possible, and the growing zone of cartilage tissue was separated.

Freshly isolated cartilage tissues were diced into small pieces and incubated with 0.2 % (v/w) Trypsin (Difco Laboratories Inc. Detroit, MC.) in Mg^{2+} and Ca^{2+} free phosphate buffered saline (PBS(-), Nissui.) at 37°C for 40 minutes. After removal of the trypsin solution, the cartilages were further incubated with 0.2 % (v/w) clostridial collagenase (Wako Pure Chemical Industries Ltd., Tokyo) in PBS (-) at 37°C for 2.5 hours. The chondrocytes were passed through a 153 μ m stainless sieve to remove debris and undigested cartilage fragments. They were then centrifugated at $1,000\times g$ for 5 minutes to remove the collagenase. The cell pellet was washed with fresh Ham's F-12 medium (Nissui) containing 10 % fetal bovine serum (FCS, Flow Laboratories, McLean, VA), 60 mg/liter kanamycin sulfate (Boehringer, Mannheim, GmbH), and then resuspended in the same medium as described above at a concentration of $1.0\text{--}1.5\times 10^5$ cells/ml. The cell viability after enzymatic digestion exceeded 90 % as judged by trypan blue exclusion. The primary chondrocytes were

plated into 25 cm² plastic tissue culture flasks (Falcon, Div. of Becton, Dickinson and Co., Oxnard, CA) and grown for 4 to 8 days at 37°C. These chondrocyte cultures were used for assessment of the extracts from various cartilage tissues at various stages of subculture period.

2) Extraction of Growth Regulatory Factor from Mandibular Condylar Cartilage

The growth inhibitory factor from the mandibular condyle was compared with those from three different cartilage tissues; chondro-septum, capital cartilage and costal cartilage. These were removed from seventy-five Japanese white rabbits (body weight 1.5–3.0 kg). Each cartilage sample was freed from the muscles and overlying perichondrium and then homogenized in 0.1 M acetic acid utilizing a Polytron homogenizer. The extracts were clarified by centrifugation at $10,000\times g$ for 20 minutes. These samples were pooled and used in all subsequent experiments.

The supernatants were subjected to molecular sieve chromatography, using Sephadex G 200 column (3 $\times$ 100 cm). The column was eluted with 0.1 M acetic acid at a flow rate of 15.0 ml/hr and 7.0 ml fractions were then collected. The fractions were monitored for absorbance at 280 nm and the fractions containing proteins were lyophilized and weighed. The lyophilized materials were dissolved in Ham's F-12 medium as described above. Each sample was tested for its ability to stimulate DNA synthesis in confluent cultures of chondrocytes. Unless otherwise indicated, primary cultures were used.

3) Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel disc electrophoresis was used for the analysis of the extracts from mandibular condyle using 5 % sodium dodecyl sulfate. Electrophoresis was conducted for about 5 hours at room temperature at constant current of 6–7 mA/tube. For molecular weight determination, 2.5 μ g of standards of known molecular weight were used (Pharmacia Fine Chemicals, Uppsala, Sweden). The gels were stained in a 5 % coomassie blue solution for 1 hour and destained by washing in a 7 % acetic acid solution.

4) Assay for Chondrocytes Proliferation

Chondrocytes were trypsinized and resuspended at a concentration of $1.0-1.5 \times 10^5$ cells/ml. Aliquots of 200 μ l were plated into microtiter wells along with ^3H -Thymidine (10 μCi /ml, ^3H -TdR, New England Nuclear, Boston, MA.). After at least 24 hours, the medium was removed and the cells were washed in Hank's balanced salt solution. The following technique was utilized as described by Klagsbrun et al.⁹⁾ in order to measure the incorporation of ^3H -TdR. Each well was washed twice with 100 % methanol for 4 minutes and then washed with H_2O . Cold 5 % trichloroacetic acid was added twice for a period of 10 minutes and the wells were then washed 4 times with H_2O . The fixed cells were lysed with 150 μ l of 0.3 N NaOH and dissolved in 5.0 ml of scintillation cocktail. Radioactivity was measured by liquid scintillation counter.

Results

1) Characterization of Costal Cartilage Chondrocytes

The chondrocytes were plated into culture flasks at a concentration of 1.5×10^5 cells/ml and reached confluence in 4-8 days maintaining a round or polygonal shape in high density areas. The medium was changed 1 to 2 times a week. The pattern of DNA synthesis in the first six days of culture is shown in Fig. 1. The rate of ^3H -TdR incorporation increased markedly within the first four days after plating. The confluent grown chondrocytes stained with toluidine blue signified excellent ability of matrix synthesis (Fig. 2).

2) Effect of Separated Fractions on DNA Synthesis of the Chondrocytes

The acid extracts of the cartilages were chromatographed on a Sephadex G 200 column eluted with 0.1 M acetic acid. The proteins were eluted in four major components as shown in Fig. 3. Fractions corresponding to the peak were pooled as indicated. Each fraction was assayed for its effect on the proliferation of isolated chondrocytes in culture. Separated fractions were lyophilized and dissolved in Ham's F-12 medium. The protein concentration in the medium was adjusted to 1 to 10 mg/ml. Incorporation of ^3H -TdR into chondrocytes was affected by exposure to frac-

Fig. 1 "Growth curve" of the primary chondrocytes determined by ^3H -TdR incorporation.

tions which were from mandibular condylar cartilage. Although higher molecular weight substances (F-I, F-II) had ability to accelerate the DNA synthesis of cultured chondrocytes, addition of the lower molecular weight fraction (F-IV) to primary cultures of chondrocytes obviously inhibited the ^3H -TdR incorporation in comparison with control cells while no marked effect on ^3H -TdR incorporation was observed with F-III (Fig. 4, Table 1).

The inhibitory effect of low molecular weight fractions from mandibular condylar cartilage was compared with those from other cartilage tissues, such as capital cartilage, chondro-septal cartilage and costal cartilage (Fig. 5). The relative inhibitory activity of the mandibular condylar cartilage extract was greater than those of the others. It was found that some quantity of inhibitory activities was present in the chondroseptum extract at the same concentration. The low molecular weight fractions of acid extract from capital cartilage and costal cartilage did not inhibit DNA synthesis in secondary cultured chondrocytes. The fraction from the mandibular condylar cartilage demonstrated the inhibitory effect in a concentration dependent manner as shown in Fig. 6. When the inhibitory substance was added to subcultured chondrocytes at different stages of cultivation, this inhibitory activity was gradually reduced according to

Fig. 2 a) Phase contrast photomicrograph of chondrocytes in seven days cultivation. These cells were plated at a concentration of 1.0×10^5 cells/ml in 10 % FCS containing Ham's F-12 medium. They maintained a round or polygonal shape ($\times 100$). b) Matrix synthesis ability of the monolayered chondrocytes. Staining by toluidine blue ($\times 100$).

subcultured progression. It is of interest that after about the fifth subcultured period, 80 % of the inhibitory activities disappeared (Fig. 7).

3) SDS-PAGE

Growth inhibitory substance from the mandibular condyle was electrophoresed in order

to examine its heterogeneity. An SDS-PAGE analysis indicated the presence of two major polypeptides. These apparent molecular weights were then estimated to be approximately 10,000 and 8,000 (Fig. 8).

Fig. 3 Gel filtration of various different cartilage tissues extracts.
 a) Mandibular condylar cartilage b) Chondroseptum c) Costal cartilage
 These sample were chromatographed on Sephadex G 200 (3×100cm).
 Fractions were collected and monitored for absorbance at 280 nm. They
 were separated into fractions indicated by bars.

Fig. 4 Diversity of DNA synthesis in rabbit primary chondrocytes by separated extracts of mandibular condylar cartilage on ^3H -TdR incorporation.

Discussion

The growth of condylar cartilage of the mandible is affected by many factors, such as growth hormones, steroid hormones and some local environmental factors¹³⁾. The influence on condylar growth exerted by the orthopaedic or orthodontic appliance is a very important problem not only in orthodontics but also other dental practices. One of the key point in mandibular growth regulation is at the level of cartilage cell proliferation. Petrovic *et al.*⁷⁾ stated that when the condylar cartilage was

Fig. 5 The effect of low molecular weight fractions from mandibular condylar cartilage, chondroseptum and costal cartilage on ^3H -TdR incorporation.

plated as an organ culture, the proliferation of prechondroblasts showed a lower growth rate. The results of a number of transplantation experiments seemingly showed a fundamental difference between epiphysial and synchondrotic cartilage.

The acid extract collected from rabbit mandibular condylar cartilage contained at least four fractions in the Sephadex G 200 chromatogram. Proliferation of chondrocytes was increased 2 times by exposure to the higher molecular weight fractions F-I and F-II (Fig. 4), while F-III had no effect. High molecular weight components like F-I and F-II, which are probably matrix components of cartilage tissue, can provide an excellent

Table 1

	^3H -TdR incorporation ($\times 10^3$ cpm/well)			
	24 hours culture	T/C	48 hours culture	T/C
Control	0.81 $\pm$ 0.12		1.15 $\pm$ 0.19	
F I	1.50 $\pm$ 0.08*	1.85	2.49 $\pm$ 0.53*	3.17
F II	1.39 $\pm$ 0.11*	1.71	2.26 $\pm$ 0.13*	1.97
F III	0.56 $\pm$ 0.21	0.69	1.01 $\pm$ 0.34	0.87
F IV	0.12 $\pm$ 0.05*	0.15	0.19 $\pm$ 0.04*	0.17

* Significantly different from Control, $p < 0.001$

Fig. 6 Concentration dependent effect of the low molecular weight fraction from mandibular condylar cartilage on ³H-TdR incorporation.

Fig. 7 The changing of the effect of inhibitory substance according to the sub-cultured periods.

Fig. 8 SDS-PAGE gel electrophoresis of the acid extract from mandibular condylar cartilage.

environment for cell proliferation. On the other hand, as can be seen in Fig. 4, addition of F-IV to the cells markedly inhibited the thymidine incorporation after 24 hours (17 % of control). These results demonstrate that there is an inhibitory substance as well as stimulatory factors in the growth regulation mechanism of mandibular condylar cartilage. The best known inhibitors of cell proliferation are chalcones, originally defined as substance which maintained tissue homeostasis by inhibition of cellular mitosis¹³⁾. They were stated to be non-cytotoxic, tissue specific and species non-specific. In order to determine whether inhibitory factors like chalcones are present in various cartilages (chondroseptum, capital cartilage and costal cartilage), extracts from the cartilages were compared with the mandibular cartilage. Only mandibular condylar cartilage had a growth inhibition factor which was not

found in other epiphyseal cartilages. Thus, the fundamental difference of growth activity in this tissue may depend upon the existence of this growth inhibiting substance.

The reduction of growth inhibitory activity of the condylar cartilage extract (F-IV), according to the subculture sequence (Fig. 7) suggest that the nature of the target cells was gradually altered during their subculture due to a loss of the receptor on the cell membrane by dedifferentiation process. This speculation, however, is contradictory to the principle of cell biology. In endochondral bone growth, chondrocytes cannot proliferates without undergoing dedifferentiation. Only undifferentiated mesenchymal cells can proliferate by cell division and develop into prechondrocytes. For identification of proliferation of chondrocytes, the following conditions should be taken into account. 1) What was the number of

undifferentiated mesenchymal cells present in the cartilage culture when the growth accelerated agent was added to it? 2) Whether the number of undifferentiated mesenchymal cells were increased or decreased at a certain period of time after the agent was added to the culture? If one can find the mesenchymal cells were decreased in number under the latter condition, no further cell proliferation would occur. On the contrary in case of increase in the number of mesenchymal cells further cell proliferation should take place, the proliferated cells eventually could develop into prechondrocytes.

Recently many growth factors, such as fibroblast growth factor (FGF), epidermal growth factor (EGF), and cartilage derived factor (CDF), have been isolated and well characterized¹⁰⁻¹²⁾. These growth factors range in molecular weight from approximately 5,000 to 10,000. The growth inhibitory factor derived from condylar cartilage appears to be a protein of relatively low molecular weight, which is similar to the molecular weight of the growth factors, but opposite in action. The identification of the growth inhibitory factor in the mandibular condyle shows a presence of unique growth control mechanism in this tissue. Whether this unique growth inhibitory factor has a functional role not only in cellular proliferation but also in cellular metabolism needs investigation to understand mechanism of the growth control.

Conclusions

An inhibitory factor was found to be uniquely present in the mandibular condylar cartilage, and although a slight inhibitory activity was found in chondroseptum, no effect of inhibition on ³H-TdR incorporation of cultured chondrocytes in other cartilages. It is still unknown whether the growth inhibitory factor found in mandibular condylar cartilage has any physiological role *in vitro*. Since the inhibitory factor is only in the mandibular condylar cartilage, it is possible that this factor constitutes part of the local growth regulating-mechanism.

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抄録：下顎骨の成長発育は主として下顎頭部の軟骨性化骨により進行することは良く知られているが、その様相は極めて複雑である。そこでそれを物質レベルで解明する目的で以下の実験を行った。

日本白色家兎75羽より下顎頭軟骨を単離し0.1M酢酸でhomogenize後、抽出物質に対してSephadex G200によるGel chromatographyを行った。得られた4つのピーク物質を、通法にしたがい初代培養された家兎肋軟骨培養系に添加し³H-Thymidineの取り込み能に与える影響を検索した。

その結果、抽出物中の低分子分画物質、すなわち分子量約10,000の蛋白に、培養軟骨細胞のDNA合成能を著しく抑制する効果が認められた。一方、他の軟骨組織、すなわち大腿骨関節頭軟骨、肋軟骨、鼻中隔軟骨から同様の方法で抽出された低分子分画物質には、鼻中隔軟骨由来の物質に若干の抑制効果を認めたのみで、他の軟骨由来の物質には有意な効果は認められなかった。したがってこの物質は組織特異的に存在していることが確認された。またこの抑制効果はbioassayに供する軟骨細胞の性質により変化した。すなわち長期にわたり継代を繰り返すほど効果が低減する傾向が認められた。その理由として細胞の脱分化によるreceptorの継時的変化、あるいは培養系にcell selectionが生じた可能性が示唆された。

以上のことから下顎頭軟骨には軟骨細胞の分裂を抑制する物質が存在し、これが関節軟骨の成長を調節している可能性が強く示唆された。

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