Cell and Tissue Interaction in Skeletal Growth

Atsumasa UCHIDA* Toshiyuki KIKUCHI** Ikuo HARADA** and Yutaka SHIMOMURA**

ABSTRACT

For the investigation of bone, muscle and nerve tissue interaction during skeletal growth, attempts were made to discover whether cultured cells isolated from calvaria, muscle and spinal cord interact with each other during their growth in vitro. The conditioned medium from myogenic cells markedly stimulated the incorporation of \(^3\mathrm{H}\)-thymidine in calvaria cells (osteoblasts). In contrast, \(^3\mathrm{H}\)-thymidine uptake in myogenic cells was inhibited by the conditioned medium from calvaria cells. DNA synthesis in myogenic cells and calvaria cells was increased by the addition of the conditioned medium from cultured spinal cord cells. Partial purification of the myogenic cell-derived growth activity by Sephaedx G-75 gel filtration disclosed the major peak of activity at a molecular of 70,000 or more. On the other hand, the calvaria cell-derived growth inhibition activities elute in two peaks, at approximately 12,000 and 7,000. These results may suggest that bone growth in regulated by factors produced by muscle and nerve tissues, and bone tissue in turn produces a factor regulating the growth of muscle.

Kew words

calvaria cells, myogenic cells, spinal cord cells, growth, cell interaction

Introduction

It is well known that there is the interaction among different tissues and organs during the process of differentiation in the initial stages of development\(^1,2\). In the skeletal system, the formation of cartilage is thought to be linked with differentiation of skeletal muscle and fibrous connective tissue\(^3,4,5\). The inductive influence of the notochord and spinal cord on myogenesis and chondrogenesis has also been demonstrated\(^6\). The growth of post-fetal tissue, including proliferation and differentiation of various cells in the tissue, also appears to be the result of interaction between growing tissues. For example, muscles, ligaments, nerve fibers and other connective tissues grow along with the bones, to complete the balanced skeletal system. Such coordinated skeletal growth seems to be regulated mainly at the gene level, but local environmental controls should also be considered. We can postulate various environmental factors. For example, it is possible that the growth-regulating factor produced by growing tissue acts on the adjacent tissues as well as its own tissue.

* Atsumasa UCHIDA
Department of Orthopaedic Surgery, Osaka University Medical School, Fukushima 1-1-50, Fukushima-ku, Osaka 553.

** Department of Orthopaedic Surgery, National Defense Medical College
Therefore, we have undertaken a cell culture study to investigate the tissue-and-cell interaction in skeletal growth. The interaction during the growth of cultured cells isolated from calvaria, muscle and spinal cord was studied in terms of the incorporation of $^{3}\text{H}\text{-thymidine}$ in these cells.

**Materials and Methods**

Sprague-Dawley rats embryos of 19-20 days gestation were used for the following experiments.

1) Cell separation and culture

For the preparation of bone cells, the calvaria was removed from the rats described above. The calvaria was thoroughly cleaned of the adhering soft tissues, minced, immersed in a calcium-magnesium free Tyrode-balanced solution containing 0.1% EDTA and 0.2% trypsin and kept there for 1 hour at 37°C. The specimen was treated with 0.2% collagenase in Ham’s F-12 medium for 3-5 hours at 37°C to separate the cells. For the culture, isolated cells were inoculated in plastic dishes (Lux, 35 mm diameter) containing Ham’s F-12 medium supplemented with 10% fetal bovine serum (FBS, Gibco) at a concentration of $1\times10^5$ cells/ml. The culture medium was exchanged every two days.

In order to obtain myogenic cells, muscle tissue was removed from the thigh portion of the same rats, minced and treated with dispase (Godo Shusei, Tokyo) at a concentration of 500 U/ml for about 3 hours to separate individual cells. These isolated cells were cultured in the same culture medium used for the clavaria cells at the same cell density.

Spinal cord cells were isolated from the spinal cord of the same animals. The spinal cord was removed as quickly as possible after sacrifice. The specimen was minced and treated with 500 U/ml dispase for 2-3 hours, and cultured under the same conditions used for the culture of myogenic cells.

For the identification of myogenic and spinal cord cells, the enzyme-labeled antibody staining method was applied using anti-human myoglobin (DAKO) for myogenic cells and neuron specific enolase (SANBID) for spinal cord cells.

2) Exchange of conditioned medium among the cultured cells

The culture medium for each type of cell was exchanged for Dulbecco’s modified Eagles medium (DMEM) +10% FBS on the 7th day of culture, followed by culture for another 48 hours. This medium was then collected and exchanged the three types of cells after filtration and adjustment of pH. After a preincubation for 12 hours, $^{3}\text{H}\text{-thymidine}$ uptake into each kind of cell was measured. The culture medium obtained from each type of cell before the exchange served as the control.

3) $^{3}\text{H}\text{-thymidine}$ incorporation

After preincubation, 1$\mu$Ci/ml $^{3}\text{H}\text{-thymidine}$ (26 Ci/mmol, Amersham International plc., UK) was added to each type of cell for pulse-labeling over a period of 3 hours, followed by removal of the culture medium. After washing the cultured cells 5 times with ice-cold phosphate buffered saline, 5% trichloroacetic acid (TCA) was added and the fraction insoluble in TCA was collected, dissolved in NaOH and neutralized in 1N HCl. Its radioactivity was then measured in a liquid scintillation counter (Aloka).

4) Partial purification by Sephadex G-75 gel filtration

Each sample of serum-free conditioned medium (100 ml) was concentrated 10-fold using an Amicon ultrafiltration membrane, dialyzed against deionized distilled water and lyophilized. The lyophilized material was then reconstituted to 0.5 ml with phosphate buffered saline (pH 7.2) and subjected to gel
filtration over a Sephadex G-75 column (0.930 cm). The flow rate was 4 ml/hr and fractions of 0.4-0.5 ml were collected. Elution was monitored by measurement of absorption at 280 nm. Aliquots of each fraction were assayed for the ability to stimulate incorporation of \(^{3}H\)-thymidine into the DNA.

**Results**

1) Cell culture

Cells isolated from the calvaria mostly consisting of osteoblasts grew favorably in culture, were polygonal in shape and almost reached confluence on the 7th day. Intense staining for alkaline phosphatase showed their function as osteoblasts (Fig. 1).

Some of the myogenic cells fused together in culture to become multinucleated cells, distinctly stained by enzyme-labeled antibody against anti-human myoglobin (Fig. 2). The

![Fig. 1](image1)

**Fig. 1** Phase contrast micrograph and alkaline phosphatase staining of calvaria cells. Cultured cells are polygonal (A) and have an intense activity of alkaline phosphatase (B) after 7 days cultivation. (×100)

![Fig. 2](image2)

**Fig. 2** Phase contrast micrograph (A) and enzyme-labeled antibody staining against anti-human myoglobin (B) of cultured myogenic cells. For enzyme-labeled antibody staining, myogenic cells after 3 days cultivation were used. (×100)

![Fig. 3](image3)

**Fig. 3** Phase contrast micrograph of spinal cord cells after 7 days cultivation (A). Enzyme-labeled antibody staining against neuron specific enolase was done on the third day of culture (B). (×100)

<table>
<thead>
<tr>
<th>Table 1 Effect of conditioned medium from myoblasts on DNA synthesis in calvaria cells and skin fibroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calvaria cells</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Conditioned medium from myoblasts</td>
</tr>
<tr>
<td><strong>Skin fibroblasts</strong></td>
</tr>
</tbody>
</table>

Calvaria cells and skin fibroblasts after 7 days cultivation were used for this experiment. Values are average ± SD.
rate of growth for the myogenic cells was the same as that for the calvaria cells.

Compared to these two types of cells, the growth rate was lower for spinal cord cells. At first, dissociated spinal cord cells appeared round. The cytoplasmic processes of some cells became extremely elongated, while other cells became fibroblastic. Both types of cells were stained with enzyme-labeled antibody against neuron specific enolase (Fig. 3).

2) Exchange of culture mediums

As shown in Table 1, the conditioned medium obtained from myogenic cells markedly stimulated the \(^3\)H-thymidine uptake in calvaria cells. In contrast, incorporation of \(^3\)H-thymidine in myogenic cells was inhibited by about 50% in response to contact with the conditioned medium from calvaria cells. This inhibitory effect almost completely disappeared on replacement of the conditioned medium 15 hours later with fresh DMEM followed by further culture for 24 hours (Table 2).

Neither of these two types of conditioned mediums showed any effect rat skin fibroblasts.

When the conditioned medium obtained from spinal cord cells was added to the calvaria cells and myogenic cells in culture, the \(^3\)H-thymidine uptake in these cells was moderately stimulated (Table 3).

3) Partial purification of growth regulating activities

When the concentrated serum-free conditioned medium obtained from myogenic cells was gel filtrated on Sephadex G-75, only one major peak of activity was observed. The growth promoting activity for calvaria cells

---

### Table 2: Effect of conditioned medium from calvaria cells on DNA synthesis in myoblasts and skin fibroblasts

<table>
<thead>
<tr>
<th></th>
<th>(^3)H-thymidine uptake (×10^4 dpm/mg protein)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myoblasts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.89±0.19</td>
<td></td>
</tr>
<tr>
<td>Conditioned medium from calvaria cells</td>
<td>1.02±0.11</td>
<td>54.0</td>
</tr>
<tr>
<td>Control</td>
<td>1.69±0.74</td>
<td></td>
</tr>
<tr>
<td>Removal of conditioned medium*</td>
<td>1.73±0.61</td>
<td>102.4</td>
</tr>
<tr>
<td><strong>Skin fibroblasts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.64±0.79</td>
<td></td>
</tr>
<tr>
<td>Conditioned medium from calvaria cells</td>
<td>7.88±1.02</td>
<td>103.1</td>
</tr>
</tbody>
</table>

Both cells were cultured for 7 days. Values are average±SD.

### Table 3: Effect of conditioned medium from spinal nerve cells (CMS) on the incorporation of \(^3\)H-thymidine

<table>
<thead>
<tr>
<th></th>
<th>(^3)H-thymidine uptake (×10^4 dpm/mg protein)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calvaria cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.21±0.57</td>
<td></td>
</tr>
<tr>
<td>CMS</td>
<td>7.80±1.45</td>
<td>149.7</td>
</tr>
<tr>
<td><strong>Myoblasts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.89±0.19</td>
<td></td>
</tr>
<tr>
<td>CMS</td>
<td>2.86±0.32</td>
<td>151.3</td>
</tr>
</tbody>
</table>

Calvaria cells and myogenic cells were cultured for 7 days, and then their media were exchange with CMS. Values are average±SD.
was found in the fraction with a molecular weight of 70,000 or more (Fig. 4).

The growth inhibition effect of the calvaria cell-conditioned medium on the myogenic cells showed two major peaks. These peaks eluted at molecular weights of about 12,000 and 7,000 (Fig. 5).

Discussion

Prior to a discussion of the interaction in skeletal growth, the relationship between the differentiation and the growth capacity of tissues should be described. In general, the expression of differentiation (functional behavior) and that of proliferation in tissues or cells are thought to be complementary. In proliferating cells, functional behavior is assumed to be either lacking or scarcely present. In contrast, the cells in the functional phase have lost their capacity to divide. For example, neurons, well-differentiated and well functioning cells, never divide while less differentiated cells (less functioning cells), such as the basal layer cells of the epidermis, divide actively.

However, the growth process in differentiated tissues is very complex, because of the coexistence of proliferation and differentiation of cells. In this paper, the effect of cultured cells isolated from skeletal tissues on each other’s DNA synthesis has been demonstrated. It is very interesting to note the opposite effects of calvaria cells (osteoblasts) and myogenic cells; stimulation of proliferation on the one hand and inhibition on the other. The regulation of stimulation and inhibition in the proliferation and differentiation of cells are thought to be basic control mechanisms in the tissue growth to complete a balanced individual organism.

The myogenic cell-derived growth promoting effect on calvaria cells occurred at a relatively high molecular weight. The relationship between this effect and that of other myoblast products of biological interest, including troponin and actin, which have similar molecular weight should be explored. Moreover, comparative studies of other mitogenic factors, including bone derived growth factor, cartilage derived factor, macro-
phage derived growth factor\(^\text{10}\), should be done through further experiments.

Calvaria cells, on the other hand, released factors inhibiting the proliferation of myogenic cells, and this activity revealed two major peaks by gel filtration study. Although the significance of these peaks is unknown, the higher molecular weight peak may represent an aggregate of smaller one with biological activity. Tissue-specific inhibitors of cell mitosis are now well recognized under the term ‘chalone’ and have recently been extensively reviewed\(^\text{11,12,13}\). In tissue repair, for example, the stimulation and inhibition of cellular proliferation proceeds in perfect harmony until the completion of the repair processes. Chalones are inhibitory factors released from the cells on such occasions, and may play an important role in the negative feed-back control mechanism necessary for normal remodelling of tissue. While this is a control mechanism within a tissue, a similar controlling and harmonizing mechanism might be operating within a unit such as the skeletal system.

It is well known that nerve cells produce many physiologically active substances. Almost all of these substances are thought to act as chemical neurotransmitters. The present results have demonstrate that spinal cord cells produce a mitogenic factor which affects the cells isolated from skeletal tissues. The relationship between this factor and neurotransmitters, although well-known, is still unclear. However, clinical cases are sometimes encountered in which the growth of bone and muscle is under neurological control. These experimental results are, therefore, useful for the interpretation of the clinical evidence, and suggest that there may be neurological control mechanisms in skeletal growth.

Based on these results, further experiments will be directed towards whether the controlling effects among these tissues actually exist in the tissue growth in a living organism.

**References**