Growth Factors for a Primary Chick Muscle Cell Culture from Shochu Distillery By-products

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An unidentified growth factor (UGF) was separated from shochu distillery by-products (SDBP) and its effect on the growth of a primary chick muscle cell culture was investigated. Chick muscle cells were isolated from fertile eggs (13-day-old embryos) of commercial broilers. UGF was separated on Sephadex LH-20 with a solvent system of water–methanol–ethylene dichloride (10:90:20, v/v), and the fraction eluted between 136 and 164 min was collected (fraction I). Fraction I was further purified by HPLC with an Inertsil ODS-2 column using a solvent system of methanol–butanol (80:20, v/v). Three fractions having retention times of 3.76, 4.57, and 5.12 min were collected and are referred to as fraction A, B, and C, respectively. In experiment 1, chick muscle cells were cultured in an m-199 medium containing 0.001, 0.01, or 0.1% of fraction I. In experiment 2, chick muscle cells were cultured with 0.01 or 0.005% of each fraction A, B, and C. Creatine kinase (CK) activity, protein and DNA contents were measured as indices of myotube growth, cell growth, and cell proliferation, respectively. N'-methylhistidine (N'-MH) release from the muscle cell was also measured to observe the effect on proteolysis.

In experiment 1, the protein content was significantly (p < 0.05) increased by fraction I, despite the low dose level. CK activity was significantly (p < 0.05) higher than the control when 0.001% of fraction I was added to the medium. However, increasing the level beyond 0.01% did not further increase the CK activity. The DNA content was not significantly changed. In experiment 2, the protein content, CK activity, and DNA content were significantly (p < 0.05) higher when fractions A and B were added to the medium. However, this was not the case when fraction C was added. N'-MH release was significantly (p < 0.05) higher when fraction A was added, but, was significantly (p < 0.05) lower when fraction B was added, while fraction C had no effect on N'-MH release.

The present results show that SDBP contained two growth-promoting factors for a primary chick muscle cell culture, although their modes of action may be different.

Key words: muscle cell culture; unidentified growth factor; distillery by-product

Cell culture systems have recently been widely used to determine the activities of biological substances such as hormones. For instance, it has been shown that Insulin-like growth factors (IGF)-1 and -2 stimulated growth by increasing protein synthesis and inhibiting protein degradation of the cultured skeletal muscle cell.11

In the process of producing beer, distilled liquors, alcohol, shochu (Japanese liquor), etc., various by-products remain after the fermentation. We have previously shown that shochu distillery by-products (SDBP) contained an unidentified ether-soluble growth-promoting factor (UGF) for chickens. Growth of chickens was promoted by adding an ethereal extract at the level of 0.05% to the diet.21 UGF was then separated by column chromatography with Sephadex LH-20, with a solvent system of water, methanol, and ethylene dichloride, and the UGF activity was determined in vivo by using chickens.21

The resulting UGF fraction was further separated into 3 fractions by HPLC with an Inertsil ODS-2 column, using a solvent system of methanol, butanol and 10 mM sodium acetate. However, it was difficult to get sufficient UGF for the in vivo experiment, so we examined the effect of UGF on the growth of cultured muscle cells. We observed that UGF stimulated the growth of cultured primary chick muscle cells, using CK activity and protein content as indices of the myotube and cell growth.

Materials and Methods

Separation of UGF. The UGF fraction (fraction I) was separated as reported previously.21 The shochu distillery by-products (SDBP) were obtained from Komasa Liquor Co., Ltd., (Kagoshima) and were homogenized for 3 min in a mixer. Homogenized SDBP was then freeze dried and extracted with ether. One hundred mg of an ethereal extract of SDBP was dissolved in 5 ml of solvent after removing the ether and applied to a Sephadex LH-20 column (60 x 750 mm). Elution was performed at a flow rate of 1.5 ml/min with a solvent system of water–methanol–ethylene dichloride (10:90:20) and the absorbance at 292 nm was monitored with a spectrophotometer (Shimadzu UV-150-02). The growth promoting activity of eluting fraction I was determined by feeding to broiler chicks.

Fraction I was further fractionated by HPLC with an Inertsil ODS-2 column (4.6 x 250 mm). Dried fraction I (225 µg) was dissolved in 10 ml of methanol, and a 100-µl aliquot was applied to HPLC. Separation was

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performed at a constant flow rate (1 ml/min) with a solvent system of methanol-butanol-10 mm sodium acetate (800:200:1) and monitored at 282 nm with a UV detector (Tosoh 8010). Three fractions were obtained as shown in the Fig. These fractions (A, B, and C) were then collected, and the solvents were removed with a rotary evaporator under reduced pressure before their activities were determined by a cell culture system. Each fraction was again examined by HPLC to confirm its separation.

Isolation and cultivation of chick cells. Fertile commercial broiler eggs were presented by Kumi Hina Center (Kagoshima, Japan). The muscle tissue obtained from four embryos was digested with PBS (+) containing dispase (1000 IU/ml). After 10 min of incubation at 37°C, the cell suspension was passed through gauze and centrifuged. The supernatant was aspirated, and the cell pellet was dispersed in 3 ml of a growth medium (m-199 containing 15% calf serum and 2.5% embryo extract). The cell suspension was transferred to a 50-mm uncoated culture dish to allow fibroblast attachment. After 40 min, the unattached cells were recovered and transferred to the next dish. This procedure was repeated 3 times, cell suspensions containing a minimum of 96% myogenic cells being provided by this protocol. The recovered muscle cells were seeded on 2 gelatin-coated 6-well tissue culture plates at a density 2.5 x 10⁶/well. The cells were grown in a basal medium consisting of 82.5% m-199, 15% calf serum, 2.5% chick embryo extract, streptomycin (100 mg/l), and penicillin (10 U/l) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After 24 h, the medium was replaced with a medium containing the UGF fraction separated from SDBP. The medium was changed every other day for a 6-day incubation period. In experiment 1, fraction I was added to the medium at a level of 0.001, 0.01, or 0.1%, while in experiment 2, fractions A, B, and C were each added at a level of 0.005 or 0.01%. On day 6, after collecting the medium, the cell monolayer was washed three times with ice-cold PBS, and the cells were detached by scraping and suspended in a Tris-HCl buffer (pH 6.8). The collected medium and cells were stored at -80°C until needed for analysis. The cells were homogenized with a Polytron homogenizer and centrifuged at 2500 rpm for 10 min at 4°C, and the CK activity and protein and DNA contents were measured in the resulting supernatant. The protein content was determined according to the Lowry method, using bovine serum albumin (BSA) as a standard.

Measurements of creatine kinase activity and protein and DNA contents. Creatine kinase (CK) activity was determined according to the modified method of Rosalki. A 2.9 ml substrate mixture contained adenosine diphosphate (ADP), creatine phosphate (CP), glucose, magnesium chloride, hexokinase, glucose-6-phosphate (G6P), and nicotinamide adenine dinucleotide phosphate (NADP) at concentrations chosen to provide the optimal reaction conditions. Adenosine monophosphate (AMP) and cysteine hydrochloride were added to inhibit myokinase activity. All reagents were dissolved in a Tris buffer at pH 6.8 and 25°C. To 2.9 ml of this solution, 0.1 ml of a sample was added, and the mixture incubated at 30 (±1)°C for 6 min. CK activity was determined by measuring the increase in optical density per minute at 340 nm.

DNA content was determined according to the modified method described by Erwin et al. The suspended cells were centrifuged at 3000 rpm and 4°C for 15 min, and the precipitate was resuspended by vortexing in 500 μl of 5% TCA. The suspension was then hydrolyzed at 90°C for 30 min, and centrifuged at 3000 rpm and 4°C for 15 min after the samples were cooled to 4°C. A 500-μl portion of the supernatant was subjected to 200 μl of dinitrophenylhydrazine (DNPH) and kept at 60°C for 1 h. 2 ml of 1 N HCl then being added to terminate the reaction. DNA was quantified by a fluorescence spectrophotometer (F-200, Hitachi Electric, Japan), using an excitation wavelength of 314 nm and emission wavelength of 520 nm.

N₂-MH analysis. N₂-MH was analyzed according to the modified method described by Hayashi et al. One ml of the culture medium was hydrolyzed in 6 M hydrochloric acid at 110°C for 20 h. The hydrochloric acid was removed by evaporating under reduced pressure, and the residue was dissolved in 5 ml of 0.2 M pyridine. A 3 ml portion of this solution was applied to an anion-exchange column (7 x 60 mm, Dowex 50×8, 200-400 mesh, pyridine form). After washing out most of the acid and neutral amino acids with 20 ml of 0.2 M pyridine, N₂-MH was eluted with 20 ml of 1 M pyridine and collected. The solvent was then evaporated, and the residue was dissolved in 2 ml of the mobile phase (15 mm sodium octane sulfonate in 20 mm KH₂PO₄). A 100-μl aliquot was injected into Shimadzu LC-6A HPLC apparatus equipped with an ion-pairing Shim-pack ODS column (6.0 x 150 mm). A Shimadzu RF-535 fluorometer set at an excitation wavelength of 350 nm and an emission wavelength of 460 nm was used to monitor the fluorescence derived from the reaction with orthophthalaldehyde (OPA).

Statistical analyses. Data were analyzed by analysis of variance, and means were further tested by Duncan's multiple-range test. A p value <0.05 is considered to be statistically significant. Each result is the mean ± standard deviation of the values obtained from two plates (twelve determinations).

Results

In experiment 1, we observed the effects of fraction I, which had been separated by a Sephadex LH-20 column, on the CK activity, protein and DNA content of cultured muscle cells (Table I). The protein content and CK activity were increased when fraction I was added to the medium, although no clear dose response could be obtained. The DNA content was not influenced by fraction I.

In experiment 2, we evaluated the effects of fractions A, B, and C which had been separated in an Inertisil ODS-2 column by HPLC, on the protein content, CK activity, DNA content, and N₂-MH release in cultured muscle cells (Table II). Fractions A and B significantly (p<0.05) increased the protein content by 12% and 22%, respectively, but fraction C had no effect on protein content. CK activity was higher in all the treated groups. DNA content was increased by fractions A and B by 34% and 39%, respectively, but fraction C had no effect. N₂-MH release was increased by fraction A, while fraction B decreased it, and fraction C had no effect. This latter result shows that
Table I. Effect of UGF Separated by Sephadex LH-20 from Shochu Distillery By-products (Fraction I) on the Creatine Kinase (CK) Activity, and Protein and DNA Contents in a Primary Chick Muscle Cell Culture (Experiment 1)

<table>
<thead>
<tr>
<th>Parameter*</th>
<th>C</th>
<th>0.001</th>
<th>0.01</th>
<th>0.1</th>
<th>Analysis of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein content (mg/well)</td>
<td>1.11 ± 0.5a</td>
<td>3.01 ± 0.5b</td>
<td>2.34 ± 0.3b</td>
<td>2.73 ± 0.1b</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>CK activity (IU/well)</td>
<td>25.8 ± 1.5a</td>
<td>29.7 ± 0.9b</td>
<td>27.5 ± 0.6b</td>
<td>34.7 ± 0.7b</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>DNA content (μg/well)</td>
<td>17.8 ± 4.8b</td>
<td>16.7 ± 3.6b</td>
<td>14.6 ± 2.9b</td>
<td>15.9 ± 3.2b</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Each value is the mean ± S.D. Means not sharing the same superscript letters are significantly different (p < 0.05).

Table II. Effect of UGF Separated by HPLC from Shochu Distillery By-products (Fractions A, B, and C) on the Protein Concentration, Creatine Kinase (CK) Activity, DNA Content, and N’-Methylhistidine (N’-MH) Release in a Primary Chick Muscle Cell Culture (Experiment 2)

<table>
<thead>
<tr>
<th>Parameter*</th>
<th>C</th>
<th>Fraction A (%)</th>
<th>Fraction B (%)</th>
<th>Fraction C (%)</th>
<th>Analysis of variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein content (mg/well)</td>
<td>0.96 ± 0.04a</td>
<td>1.27 ± 0.22a</td>
<td>1.23 ± 0.54a</td>
<td>1.34 ± 0.52a</td>
<td>1.42 ± 0.41a</td>
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<tr>
<td>CK activity (IU/well)</td>
<td>25.9 ± 3.8a</td>
<td>37.5 ± 2.3b</td>
<td>43.2 ± 3.3b</td>
<td>38.8 ± 4.2b</td>
<td>41.4 ± 3.7b</td>
</tr>
<tr>
<td>DNA content (μg/well)</td>
<td>18.0 ± 2.4a</td>
<td>19.8 ± 2.3b</td>
<td>24.3 ± 2.7b</td>
<td>23.2 ± 3.1b</td>
<td>25.2 ± 2.9b</td>
</tr>
<tr>
<td>N’-MH release (nmol/mg of protein)</td>
<td>4.16 ± 0.5a</td>
<td>5.28 ± 0.6b</td>
<td>7.04 ± 0.8b</td>
<td>1.94 ± 0.3a</td>
<td>1.96 ± 0.4b</td>
</tr>
</tbody>
</table>

* Each value is the mean ± S.D. Means not sharing the same superscript letters are significantly different (p < 0.05).

fraction A accelerated muscle proteolysis, while fraction B reduced it.

Discussion

The protein content of cultured cells is an index of growth of muscle cells,1–9 and CK activity is a parameter of myotube formation.9–11 The protein content and CK activity of the cultured muscle cells were increased when fraction I was added to the medium. On the other hand, DNA content has been measured as an index for the proliferation of cultured muscle cells.11–13 DNA content was not significantly influenced by fraction I. These results indicate that cell proliferation was not accelerated by fraction I, although muscle cell growth was stimulated.

When fractions A and B were included in the medium, the protein content was significantly increased, but this was not the case with fraction C, indicating that UGF existed in both fractions A and B. Increases in CK activity by fractions A, B, and C show that all these fractions promoted myotube formation in the cultured chick muscle cells. Fraction C accelerated myogenesis, although it did not affect the protein and DNA contents. The DNA content was increased by supplementation with fractions A and B, but not with fraction C. This indicates that fractions A and B both stimulated the proliferation of muscle cells, although this is not consistent with the results of experiment 1. Consequently, a growth inhibiting factor might have been contained in fraction I (experiment 1). Myotube formation (creatine kinase activity) was stimulated by the present UGF to the same extent as that by the thyroid hormone, being increased by the thyroid hormone to 1.5 times that of the control (Nakashima et al., unpublished data). N’-MH release from cells to the medium was measured as an index of proteolysis in experiment 2, being increased by fraction A and decreased by fraction B, while not being affected by fraction C. These results show that fraction A accelerated muscle proteolysis while fraction B reduced it. Fraction B may have been a protease inhibitor originating from a microorganism used for fermentation as lactacystin (a specific inhibitor of proteasome activity) is synthesized by Streptomyces.15

Protein accumulation (growth) is represented as the difference between protein synthesis and protein degradation, growth being stimulated when the rate of synthesis exceeds that of degradation. Thus, the mechanism for the growth-promoting effects of fractions A and B may have been different. A study on the chemical nature, protease inhibition activity and structure of fractions A and B is in progress.

In conclusion, the results of the present study show that shochu distillery by-products contain two growth-promoting factors for culturing chick muscle cells whose modes of action may be different.

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References