Separation of cell cycle phase-specific chalones using the epidermal organ culture as an assay system

Tomohisa HIROBE* and Takeo YAMAGUCHI**

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SUMMARY

When a piece of mouse ear conch was laid on a disk of glass filter immersed in a TC 199 medium and kept at 37°C in an atmosphere of 95 % O₂+5 % CO₂ gas mixture, an abrupt increase in the number of mitoses and DNA synthesizing cells was observed between 12 and 24 h after the initiation of culture. The skin extract containing epidermal chalone was added to the culture medium at the time on the course of increase in mitoses and DNA synthesizing cells. In the case of DNA synthesizing cells ³H-thymidine was added various times later. Both G₂-chalone which inhibited the transition from G₂ to M and G₁-chalone which inhibited the transition from G₁ to S required adrenaline as cofactor.

A 80 % ethanol precipitate of crude skin extract was fractionated through Sephadex G-100 column chromatography. The activity of G₂-chalone existed in the low molecular weight fraction (10,000<MW<100,000) and that of G₁-chalone existed in the high molecular weight fraction (MW>100,000).

INTRODUCTION

We have reported that the inhibitory effect of epidermal chalone on the proliferative response of the regenerating epidermis of mouse ear surround cut. Epidermal chalones inhibited the epidermal cell proliferation in at least two different processes of the cell cycle, the transition from G₂ to M phase (G₂-chalone) and that from G₁ to S phase (G₁-chalone, Yamaguchi, Hirobe, Kinjo & Manaka, 1974; Yamaguchi & Hirobe, 1977). In order to separate G₂-chalone and G₁-chalone, our in vivo assay system using regenerating epidermis is not necessarily suitable, since it requires large quantity of chalone and obscures the hormonal and other several conditions. From these reasons, we established the organ culture system to assay the epidermal chalone activity.
The piece of mouse ear conch was laid on a disk of glass filter immersed in a TC 199 medium and kept at 37°C in an atmosphere of 95 % O₂+5 % CO₂ gas mixture. The cultured epidermis showed an active proliferation after 12- to 24- hour-culture periods.

The effects of epidermal chalone on G₁ → S transition as well as G₂ → M transition in the cell cycle and adrenaline requirement were investigated. Separation of G₂-chalone and G₁-chalone by Sephadex gel column chromatography was also carried out.

MATERIALS AND METHODS

The animals used in this study were the house mouse, *Mus musculus*, of strain C57BL/6J. They were given water, fed ad libitum on a commercial diet and maintained at 24±1°C.

A skin extract containing epidermal chalone was prepared from 4- to 6-month-old males of C57BL/6J mouse. The extraction procedure of epidermal chalone was described previously (Yamaguchi, Hirobe, Kinjo & Manaka, 1974). The scraped epidermis was homogenized with a glass homogenizer and the resulting suspension was centrifuged at 18,000 g for 1 h. To the 18,000 g supernatant of the crude skin extract, cold ethanol was added dropwise, with continuous stirring, up to a concentration of 80 % (V : V); the precipitate was collected by centrifugation at 3000 rpm for 30 min. This precipitate was applied to a Sephadex gel chromatography. A column of 1.6 cm diameter was filled in the cold room at 4°C with Sephadex G-100 up to 30 cm and equilibrated with 0.9 % NaCl. Then 80 % ethanol precipitate dissolved in 1 ml of 0.9 % NaCl was placed in the top of the Sephadex column and the elution was performed with the same 0.9 % NaCl solution at a rate of 0.045 ml/min. In order to determine the molecular weight (MW), the standard substances with known molecular weights were blue dextran (MW>200,000), hemoglobin (68,000), cytochrome C (12,000) and dinitrophenol (184). These chemicals were applied to sephadex G-100 and their elution volume was noticed. Fraction with MW more than 10,000 and that with MW less than 100,000 were separated. The samples were then concentrated by an Ulvac Diafilter G-10 T (MW=10,000) under a nitrogen pressure. The volume was reduced to one fourth or fifth.

The mice used for the assay of chalone activity were 4- to 6-month-old males of C57BL/6J mice. Mouse ears were removed and cut to the size of ca. 3×4 mm. These pieces of ears were washed with culture medium several times. They were split along cartilage and only the one side with cartilage was put on a disk of glass filter (epidermis upside) in a glass petri dish of 4 cm in diameter. These ear pieces were cultured with TC 199 medium for 16-17 h at 37°C, pH 7.2 with humidified 95 % O₂+5 % CO₂ gas.

Chalone in a Hanks' balanced salt solution (BSS) was added to the culture medium. Colchicine in a concentration of 0.4 mg/ml Hanks' BSS and in a dose of 4 µg/ml was
added to a culture medium for 4 h. Thymidine-6-³H (³H-TdR; The Radiochemical Centre, Amersham, 26 Ci/m mole) were dissolved in Hanks’ BSS in a concentration of 0.2 mCi/ml and a dose of 2-3 µCi/ml was added to the culture medium.

Cultured ear fragments were fixed in Bouin’s solution, embedded in paraplast and sectioned at 8 µm. Autoradiographs (ARG) were made by dipping slides in Sakura NR-M2 emulsion. They were exposed with a desiccant at 4°C for 2 weeks and developed with a D-19 developer for 5 min at 20°C. They were stained with Delafield’s hematoxylin. All mitotic figures and labeled cells in the epidermis of cultured fragments were counted on each section. The figures were divided by the length of epidermis measured.

**RESULTS**

*Change in the proliferative activity of epidermal cells during organ culture*

The change in the number of mitotic figures during the culture period is shown in Fig. 1. The mitotic activity of 12-hour-culture was twice as much as that at initial time, and maintained for 24 h. The mitotic activity was declined rapidly after 36 h of culture (Fig. 1). From these results 16–17 hours of culture were selected as the time
of epidermal chalone addition when numerous cells entering M phase.

The change in the number of \(^{3}H\)-TdR labeled cells is shown in Fig. 2. \(^{3}H\)-TdR labeled cells increased in number until 24-hour-culture, then rapidly decreased in number. From the result obtained 16-17 hours of culture were selected as the time of epidermal chalone addition when numerous cells entering S phase.

Effect of skin extract on the mitotic activity in cultured epidermis

When skin extract was added to the culture medium without adrenaline, no decrease in the number of mitoses was observed. On the other hand, adrenaline (0.0025 \(\mu g/ml\)) caused an appreciable decrease. Combined treatment of cultured epidermis with skin extract and adrenaline decreased the number of mitoses significantly (P < 0.01, Table 1). Thus, epidermal G\(_2\)-chalone inhibits the transition of cells from G\(_2\) to M phase in the presence of adrenaline.

### Table 1. Effect of epidermal chalone and adrenaline on the mitotic activity in cultured epidermis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mitoses/cm (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.0 ± 2.2 (^b)</td>
</tr>
<tr>
<td>Skin extract</td>
<td>15.2 ± 2.5 (^c)</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>8.7 ± 1.1 (^d)</td>
</tr>
<tr>
<td>Skin extract + adrenaline</td>
<td>5.1 ± 0.9 (^e)</td>
</tr>
</tbody>
</table>

After pieces of mouse ears were precultured in TC 199 for 16.5 h, they were cultured with or without skin extract (2 cm\(^2\)/ml) and (or) adrenaline (0.0025 \(\mu g/ml\)) for 4 h at 37\(^\circ\)C on glass filters in the presence of colchicine (4 \(\mu g/ml\)).

\(^a\): Each value is the mean ± S. E. M. (standard error of the mean).

\(^b\)-\(^c\): not significant difference

\(^b\)-\(^d\); \(^c\)-\(^d\); \(^c\)-\(^e\); \(^d\)-\(^e\): significant differences (P<0.05)

\(^b\)-\(^d\): not significant but appreciable difference (0.05<P<0.1)

Precipitate of crude extract by 80 \% ethanol was applied to a sephadex G-100 and column. Fraction A with MW more than 100,000 and fraction B with MW less than 100,000 were pooled (Fig. 3). These two fractions were concentrated through a Diafilter (MW=10,000). Adrenaline was added to all experimental groups except otherwise specified. As Table 2 shows, treatment with the precipitate by 80 \% ethanol and fraction B decreased the number of mitoses compared with adrenaline control. Therefore, it is suggested that the G\(_2\)-chalone exists in the low molecular weight fraction. When the precipitate by 80 \% ethanol was heated at 100\(^\circ\)C for 10 min, it lost its G\(_2\)-chalone activity.
Seepation of cell cycle phase-specific chalones

0.10
0.08
0.06
0.04
0.02
0.00

BD HB CYT DNP

4

Fig. 3. Elution pattern of the 80% ethanol precipitate of skin extract through a Sephadex G-100 gel chromatography. Arrows indicate molecular weight standard (BD: blue dextran, MW > 200,000; HB: hemoglobin, MW 68,000; CYT: cytochrome C, MW = 12,000; DNP: dinitrophenol, MW = 184). Fractions A (MW more than 100,000) and B (MW between 10,000 and 100,000) were used in Tables 2 and 4.

Table 2. Effect of fractionated epidermal chalone on the mitotic activity in cultured epidermis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mitoses/cm a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.6 ± 1.4 b</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>6.5 ± 1.5 c</td>
</tr>
<tr>
<td>80% EtOH ppt + adrenaline</td>
<td>3.9 ± 0.8 d</td>
</tr>
<tr>
<td>Fraction A + adrenaline</td>
<td>6.3 ± 0.5 e</td>
</tr>
<tr>
<td>Fraction B + adrenaline</td>
<td>3.5 ± 0.9 f</td>
</tr>
<tr>
<td>EtOH ppt, 100°C, 10 min + adrenaline</td>
<td>5.8 ± 0.3 g</td>
</tr>
</tbody>
</table>

After pieces of mouse ears were precultured in TC 199 for 17 h, they were cultured with or without 80% ethanol precipitate of the crude skin extract (3 cm²/ml) and gel filtrated fractions in the presence of adrenaline (0.0025 μg/ml) and colchicine (4 μg/ml) for 4 h at 37°C.

a: Each value is the mean ± S. E. M. (standard error of the mean)  
b-c: not significant but appreciable differences (0.05 < P < 0.1)  
b-d: significant differences (0.01 < P < 0.02; P < 0.001; P < 0.001)  
c-e: not significant but appreciable differences (0.05 < P < 0.1)  
c-f: not significant differences  
e-f: significant differences (0.02 < P < 0.05)
Effect of skin extract on DNA synthesizing cells in cultured epidermis

After the ear pieces were precultured for 16-17 h, they were cultured further with or without skin extract and (or) adrenaline for 4 h. During the last 1 h, \(^{3}\text{H}-\text{TdR}\) was added to the culture medium and then ear pieces were fixed with Bouin's solution. ARGs were exposed for 2 weeks and the number of labeled epidermal cells was counted.

As Table 3 shows, no decrease in the number of labeled cells was observed in the epidermis cultured with medium containing skin extract alone or adrenaline alone. On the other hand, a significant (P<0.05) decrease was observed in the epidermis cultured with medium containing skin extract and adrenaline. Therefore, epidermal chalone is considered to inhibit the transition from G1 to S phase, being cooperated with adrenaline similar to G2 chalone.

Table 3. Effect of epidermal chalone and adrenaline on DNA synthesizing cells in cultured epidermis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of labeled cells/cm (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0±10.9 (^b)</td>
</tr>
<tr>
<td>Skin extract</td>
<td>91.9±12.8 (^c)</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>90.2±13.1 (^d)</td>
</tr>
<tr>
<td>Skin extract + adrenaline</td>
<td>57.2±9.5 (^e)</td>
</tr>
</tbody>
</table>

\(^{a}\): Each value is the mean ± S. E. M. (standard error of the mean)  
\(^b\)-\(^c\); \(^b\)-\(^d\); \(^c\)-\(^d\): not significant differences  
\(^b\)-\(^e\); \(^c\)-\(^e\); \(^d\)-\(^e\): significant differences (P<0.05)

After pieces of mouse ears were precultured in TC 199 on glass filter at 37°C for 16 h, they were cultured with or without skin extract (2 cm\(^2\)/ml) and (or) adrenaline (0.0025 µg/ml) for 5 h. During the last 1 h, \(^{3}\text{H}-\text{TdR}\) (2 µCi/ml) was added, and they were fixed with Bouin's solution. Exposure for ARG was 2 weeks. Data are expressed as percent of the cultured control.

Treatment with the 80 % ethanol precipitate and fraction A decreased the number of labeled cells significantly (P<0.05) compared with the control with or without adrenaline. On the other hand, in the epidermis treated with fraction B, no decrease in the number of labeled cells was observed (Table 4). The decrease in the number of DNA synthesizing cells by the 80 % ethanol precipitate or fraction A was ca. 33 %. Thus, the G1-chalone activity is considered to exist in high molecular weight fraction. When the precipitate by 80 % ethanol was heated at 100°C for 10 min, it retained its G1-chalone activity.
Table 4. Effect of fractionated epidermal chalone on DNA synthesizing cells in cultured epidermis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of labeled cells/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0±16.5 b</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>102.9± 6.3 c</td>
</tr>
<tr>
<td>EtOH ppt + adrenaline</td>
<td>65.1± 7.4 d</td>
</tr>
<tr>
<td>Fraction A + adrenaline</td>
<td>68.5± 8.0 e</td>
</tr>
<tr>
<td>Fraction B + adrenaline</td>
<td>99.2±10.5 f</td>
</tr>
<tr>
<td>EtOH ppt, 100°C, 10 min + adrenaline</td>
<td>68.7± 6.7 g</td>
</tr>
</tbody>
</table>

After pieces of mouse ears were precultured for 17 h, they were cultured with or without 80% ethanol precipitates of the crude skin extract (3 cm²/ml) and gel filtrated fractions in the presence of adrenaline (0.0025 µg/ml) for 5 h. During the last 1 h, 3H-TdR (3 µCi/ml) was added and they were fixed with Bouin's solution. Exposure for ARG was 2 weeks. Data are expressed as percent of the cultured control.

Each value is the mean ± S. E. M. (standard error of the mean)

b-c; b-f; c-f; d-e; d-g; e-g: not significant differences
b-d; b-e; b-g; c-d; c-e; c-g; f-d; f-e; f-g: significant differences (P<0.05)

DISCUSSION

Effect of epidermal G₂-chalone on the mitotic activity

Adrenaline plays an important role in the regulation of cell division. Low mitotic rate by stress of starvation or insomnia, high mitotic activity by adrenalectomy and participation in the regulation of mitotic diurnal rhythm were documented (Bullough & Laurence, 1961a, b; Pilgrim, Erb & Maurer, 1963). The present study confirms the inhibitory effect of adrenaline on the mitotic activity in the cultured epidermis. The present study well agree with the report by Bullough & Laurence (1964) in that epidermal G₂-chalone required adrenaline as cofactor for the inhibition of mitotic activity in mouse ear epidermis.

The fraction B (10,000 < MW < 100,000) obtained by Sephadex gel chromatography had the G₂-chalone activity. This indicates that G₂-chalone has a low molecular weight. When the precipitate by 80% ethanol was heated at 100°C for 10 min, it lost its G₂-chalone activity, indicating that G₂-chalone is heat labile. Our results confirm the findings of Maars & Voorhees (1971).

Effect of epidermal G₁-chalone on DNA synthesizing cells

The fraction A (MW>100,000) obtained by Sephadex gel chromatography had G₁-chalone activity, indicating that G₁-chalone had a rather high molecular weight. When
the precipitate by 80 % ethanol was heated at 100°C for 10 min, it retained its G₁-chalone activity. Thus, epidermal G₁-chalone is suggested to be heat stable.

The present study shows that G₁-chalone requires adrenaline as cofactor. Adrenaline alone did not inhibit the transition from G₁ to S of epidermal cells, in spite of the fact that adrenaline alone inhibited the transition of cells from G₂ to M. This suggests that the mechanisms of action of G₁ chalone are different from G₂ chalone.

Marks (1971, 1973) reported that skin contained an epidermis-specific and adrenaline-nondependent G₁-chalone which was different from adrenaline-dependent G₂-chalone. G₁-chalone was a glycoprotein with an apparent molecular weight of 100,000—300,000. Although the discrepancy in the adrenaline dependence between his results and our findings can not be fully explained at present, it might be attributed to the difference in the assay system. He investigated the epidermal chalone effect by counting the radioactivity of homogenized skin after chalone treatments.

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REFERENCES


