Growth control of primary culture hepatocytes by nonparenchymal liver cells
Role of interferon produced by liver sinusoidal cells

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Summary

Using the primary culture of liver cells, we showed that interferon produced by nonparenchymal liver cells inhibits the proliferation of cultured parenchymal liver cells. DNA synthesis of parenchymal liver cells was suppressed not only by their coculture with nonparenchymal liver cells but also by the addition of the culture supernatant of nonparenchymal liver cells. The suppressive activity of the supernatant correlated closely with the interferon (\(\alpha + \beta\)) level in the supernatant and was reduced by anti interferon (\(\alpha + \beta\)) serum. Furthermore, purified interferon (\(\alpha + \beta\)) also suppressed parenchymal liver cell proliferation in a dose-dependent manner and the suppression was released by anti interferon (\(\alpha + \beta\)) serum.

The interferon level of the supernatant necessary for suppressing parenchymal liver cell proliferation, however, was extraordinarily low compared with purified interferon. The possibility exists that IFN in the culture supernatant of nonparenchymal liver cells works synergistically with other factors in the supernatant to suppress the cell proliferation.

Key words: parenchymal liver cells (hepatocytes), nonparenchymal liver cells, interferon, growth inhibition

Introduction

In general, the cells in a multicellular organism proliferate and differentiate under the rigid control of other cells. One of the control mechanisms is carried through hormones or nerve fibers from distant cells. The other one is mediated through local chemical mediators from neighboring cells or through their direct contact with target cells.

There have been numerous reports concerning various active factors in the blood\(^\(1\)\) which regulate liver regeneration. Little is known, however, concerning the influence of the microenvironment surrounding parenchymal liver cells on their regeneration. It is possible that the proliferation of parenchymal liver cells is controlled by their intercellular matrix and nonparenchymal liver cells. It has been reported that various active factors, interleukin-1 (IL-1)\(^2\), tumor necrosis factor (TNF)\(^3\), prostaglandin E\(_2\) (PGE\(_2\))\(^4\), interferon (IFN)\(^5\), heparin-binding growth
factor type 1 (HBGF-1), transforming growth factor β (TGF β), interleukin 6 (IL-6) and colony stimulating factor (CSF), which may have great influence on the proliferation of neighboring cells, are secreted by nonparenchymal liver cells (NP-LC). Since liver regeneration is suppressed by the injection of polyinosinic acid: polycytidylic acid (poly I:C), poly I:C treated syngeneic spleen cells, or IFN itself at an appropriate time after hepatectomy, interferon may play an especially important role in the regulation of liver regeneration. However, it is not clear from these reports where the IFN suppressing the liver regeneration is produced.

This report reveals that IFN produced in NP-LC inhibits the proliferation of cultured parenchymal liver cells (P-LC). The IFN level in the culture supernatant of NP-LC necessary to inhibit P-LC proliferation is low compared with purified IFN added to P-LC culture instead of the supernatant. The difference between the suppressive activity of the purified IFN and the supernatant IFN is discussed.

Materials and Methods

(1) Mice

6-8 week-old female C3H/Heslc mice were purchased from Sankyo Laboservice Co., (Tokyo, Japan).

(2) Culture media

Cell cultures were performed employing RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with fresh L-glutamine (0.3 mg/ml, Gibco), penicillin (25 u/ml, Meiji Pharmaceutical Co., Tokyo, Japan), 2-mercaptoethanol (50 µm, Wako Pure Chemical Industries, Osaka, Japan), HEPES (15 mM, Sigma Chemical Company, St. Louis, Mo.), sodium pyruvate (1 mM, Sigma) and 5% fetal calf serum (FCS, Hyclone Laboratories Inc., Logan, Ut.).

(3) Preparation of P-LC and NP-LC suspensions

P-LC suspensions were prepared from normal livers. Isolation of liver cells was performed according to the Berry and Friend collagenase method. Under sodium pentobarbital anesthesia (50 mg/kg, ip., Abbot Laboratories, North Chicago, IL) the portal vein was canulated with a 26-gauge needle. The liver was perfused with Mg2+- and Ca2+-free HBSS, containing 25 u/ml penicillin, 25 µg/ml streptomycin and 0.08% collagenase (Type 1, 300 u/mg, Sigma) at 37°C. The liver was perfused until blood-free with 10 to 15 ml of buffer. The perfused liver was minced with scissors and reacted with the collagenase solution at 37°C for 2 min. The livers were then passed through a 50-mesh stainless steel sieve and the resulting suspension was washed five times by centrifugation at 35 ×g for 3 min.

For the preparation of P-LC suspension, the cells were passed through a 200-mesh nylon and were resuspended in a culture medium. The suspension contained more than 99% P-LC.

To prepare NP-LC suspensions from normal mice, the isolation of liver cells was performed according to the method described above. The isolated liver cells were suspended in Eagle’s minimum essential medium (E-MEM) (Nissui Seiyaku Co., Tokyo, Japan) and then centrifuged at 35 ×g for 3 min to remove P-LC. After the centrifugation, the supernatant was recentrifuged at 125 ×g for 3 min at 4°C. The pellet was resuspended in E-MEM and washed twice by centrifugation at 125 ×g for 8 min. After two washes, cells resuspended in the culture medium were centrifuged at 35 ×g for 3 min to completely remove P-LC. Phagocytic NP-LC were measured
by the method of Friedrich et al\textsuperscript{14}. In this fraction, P-LC contamination was lower than 1%.

(4) Preparation of nonparenchymal adherent liver cells (NPA-LC) and detached NPA-LC

Mitomycin C (MMC, Kyowa Hakko Kogyo Co., Tokyo Japan) treated NP-LC suspended in 0.2 ml of culture medium at various concentrations were plated into each well of a 96-well flat-bottomed tissue culture plate and then cultured. Two hours later, each well was washed twice by gently pipetting 2 washes of warm medium over the surface to remove nonadherent cells. The viability of NPA-LC was greater than 95%. FcR and CR positive cells in NPA-LC were measured by the EA- and EAC-rosette methods\textsuperscript{15,16}. 91.4% of NPA-LC were FcR positive and 54.8% were CR positive. To prepare detached NPA-LC, 1-2 $\times 10^7$ of NP-LC suspended in 3 ml of E-MEM were plated onto 55-mm glass Petri dishes. Following a 2 hour incubation period, the nonadherent cells were removed by gentle pipetting and the Petri dishes were washed twice with warm serum-free E-MEM. After further incubation for 30 min at 4ºC in cold Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-free phosphate-buffered saline [PBS(−)], NPA-LC were collected with a rubber policeman.

(5) Culture of P-LC

0.2 ml of culture medium containing 2 $\times 10^4$ P-LC was added to each well of a 96-well flat-bottomed tissue culture plate. Twenty-four hours later, nonadherent cells were removed by pipetting two washes of warm medium over the surface. To investigate the influence of culture supernatants, purified IFN, anti IFN serum and NPA-LC on P-LC proliferation, the adherent P-LC were cultured in 0.2 ml culture medium with these substances or MMC treated detached NPA-LC at various concentrations for 3 days in a humidified atmosphere of 5% CO\textsubscript{2}. The cells were pulsed with 1 $\mu$Ci $^3$H-TdR (New England Nuclear, Boston, Mass, specific activity 6.7 Ci/n moles) during the last 18 h of the incubation period. The labeled cells were detached by a rubber policeman and then harvested on an automatic cell harvester (Flow Laboratories, Rockville, MD). The amount of incorporated radioactivity was measured with a Packard Tri-Carb Liquid Scintillation Counter.

(6) Preparation of NPA-LC and P-LC culture supernatant (NPA-LC.sup and P-LC.sup)

To prepare the culture supernatants, 3 $\times 10^5$ NP-LC or 2 $\times 10^4$ P-LC were placed into each well of the plate. The procedures followed were almost the same as the methods described in “culture of P-LC” and “preparation of NP-LC”, except that nonadherent cells were removed from P-LC by washing 3 hr after the culture. The cells were cultured in 0.2 ml of culture medium and the supernatant was harvested at different culture periods (2 or 3-24 h, 24-48 h and 48-72 h). Endotoxin concentrations in all culture supernatants were checked by the Limulus amoebocyte lysate synthetic substrate methods\textsuperscript{17} and they were shown to contain <0.2 ng/ml endotoxin.

(7) IFN assay

Interferon was assayed by the inhibition of the cytopathic effect (CPE) of vesicular stomatitis virus (VSV) on mouse L cells\textsuperscript{18}. Briefly, each well of a 96 well flat-bottomed tissue culture plate was seeded with 5 $\times 10^4$ mouse L cells in 0.1 ml of tissue culture medium. An equal volume of serial dilution of the material to be tested was added to quadruplicate L cell cultures and the plates were incubated at 37ºC. After 16 to 24 h of incubation, 80 to 120 tissue culture infective doses of VSV were added to each culture well, and the plates were incubated an additional 48 h to allow development of the CPE.

(8) Mouse IFN (α + β) and rabbit anti-mouse IFN (α + β) serum

Two $\times 10^5$ IU IFN (α + β) (lot number 85001, spec. act. 4.2 $\times 10^6$ IRU/mg), rabbit anti-mouse
IFN (α + β) serum (containing 10,000 neutralization IFN reference units) and control preimmune rabbit serum were purchased from Lee Biomolecular Research Laboratories (San Diego, Calif. USA).

**Results**

1. **Suppression of P-LC proliferation by NPA-LC**

   Since Kupffer cells and endothelial cells in NP-LC are located close to parenchymal liver cells, they seem responsible for the control of P-LC proliferation after a partial hepatectomy. So NPA-LC, consisting mainly of Kupffer cells and endothelial cells\(^{19}\), were separated from NP-LC to measure their suppressive activity. These NPA-LC were detached using a rubber policeman before the coculture with P-LC. The detached NPA-LC suppressed P-LC proliferation depending on the dose of added NPA-LC (**Table 1**).

2. **Suppression of P-LC proliferation by NPA-LC.sup**

   Since P-LC proliferation was suppressed by the co-cultured NPA-LC (**Table 1**), it is conceivable that NPA-LC.sup may suppress the proliferation, so we studied the suppressor activities of the supernatant harvested at different culture periods. When P-LC were cultured in a medium containing culture supernatant from 2 to 24 h or 24 to 48 h culture of NPA-LC, the suppressor activity was always higher in the 2 to 24 h culture supernatant than in the 24 to 48 h culture supernatant (**Fig. 1**). Whatever the culture period, however, the P-LC.sup did not significantly suppress the P-LC proliferation.

![Graph showing the suppression of P-LC by NPA-LC.sup](image)

**Fig. 1** The suppression of P-LC by NPA-LC.sup is always greater in 2-24 h sup than that in 24-48 h sup. The P-LC monolayer was cultured for 3 days in a culture medium containing various kinds of NPA-LC.sup (2-24 h and 24-48 h sup in lot 810 and lot 818) at the concentrations indicated in **Fig. 1** and \(^3\text{H}-\text{TdR}\) uptake was measured. In some experiments, P-LC.sup was added to P-LC culture instead of NPA-LC.sup. Typical results from 3 separate experiments are shown.
Table 1  NPA-LC suppress the DNA synthesis of P-LC

<table>
<thead>
<tr>
<th>No. of NPA-LC added to P-LC culture</th>
<th>(^{3})H-TdR uptake of P-LC (cpm)</th>
<th>%inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20,981±288</td>
<td>—</td>
</tr>
<tr>
<td>3×10(^{4})</td>
<td>14,740±3,398</td>
<td>29.7</td>
</tr>
<tr>
<td>9×10(^{4})</td>
<td>11,952±162</td>
<td>43.0</td>
</tr>
<tr>
<td>15×10(^{4})</td>
<td>9,691±122</td>
<td>53.8</td>
</tr>
</tbody>
</table>

a) For preparation of P-LC monolayer, 2×10\(^{4}\) P-LC per well were cultured for 24 h in a 96-well flat-bottomed tissue culture plate and then washed 3 times with E-MEM to remove nonadherent P-LC. The P-LC monolayer was cocultured for 3 days with various numbers of MMC treated NPA-LC. The incorporation of \(^{3}\)H-TdR during the last 18 h of the culture period was measured. Typical results from 3 separate experiments are shown and each value represents the mean±SD of triplicate cultures.

Table 2  The IFN level of liver cell culture supernatants

<table>
<thead>
<tr>
<th>culture period</th>
<th>NPA-LC sup</th>
<th>P-LC sup</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>lot 810</td>
<td>lot 818</td>
</tr>
<tr>
<td>2−24 h</td>
<td>84.8 IU/ml</td>
<td>59.8 IU/ml</td>
</tr>
<tr>
<td>24−48 h</td>
<td>10.6</td>
<td>5.3</td>
</tr>
<tr>
<td>48−72 h</td>
<td>&lt;2.7</td>
<td>&lt;2.7</td>
</tr>
</tbody>
</table>

Anti viral activity was measured in each supernatant which was harvested at various liver cell culture periods. Typical results from 6 separate experiments are shown.

3. Relationship between culture period of liver cells and IFN level in their supernatant

IFN is well known to possess suppressor activity on cell proliferation\(^{20}\), and the IFN levels were measured in the culture supernatant of liver cells harvested at various culture periods. The IFN level of NPA-LC.sup varied according to the lot of the supernatant. For example, it was higher in all supernatants of lot 810 harvested at various culture periods than in the corresponding supernatants of lot 818. However, IFN levels of NPA-LC.sup were always highest in 2 to 24 h culture supernatant, second highest in 24 to 48 h culture supernatant and unmeasurable in 48 to 72 h culture supernatant. On the other hand, P-LC.sup harvested at any culture period contained a very low or zero IFN level (Table 2).

4. Role of IFN in suppression of P-LC proliferation

The results in Table 2 and Fig. 1 show the close correlation between the suppressor activity and IFN level of NPA-LC.sup. We performed the following experiments to confirm the role of IFN in the suppression of P-LC proliferation.

5. Effect of anti IFN (α + β) serum on P-LC suppression induced by NPA-LC.sup

NPA-LC.sup is supposed to contain suppressor factors other than IFN. Furthermore, the suppressor activity of IFN in the supernatant may increase because of its synergistic cooperation with other active factors in the supernatant. In order to clarify the extent of suppression by IFN of NPA-LC.sup, we tried to negate the suppression by using anti-IFN serum. The suppression of P-LC proliferation by the supernatant was totally negated by the addition of anti IFN (α + β) serum at a final concentration of 100 neutralizing units/ml, but not by the preimmune serum at a concentration equivalent to that of antiserum (Table 3). The DNA synthesis of P-LC was not changed significantly in control culture groups containing anti IFN (α + β) serum alone (data not shown).
6. Suppression of P-LC proliferation by purified IFN

The suppression of P-LC proliferation by NPA-LC.sup was reduced by the addition of anti IFN (α+β) serum, suggesting that IFN plays an important role in the suppression (Table 3). Therefore, purified IFN (α+β) was added to P-LC culture in order to confirm the suppressive role of IFN in NPA-LC.sup. The DNA synthesis of P-LC was decreased depending on the dose of purified IFN added to the P-LC culture. However, approximately 100 IU/ml of IFN was necessary to reduce P-LC proliferation to 50% that of a control group cultured without IFN (Fig. 2). Compared with the IFN doses in NPA-LC.sup required to suppress the P-LC proliferation (Fig. 1 and Table 2), high doses of purified IFN were necessary to suppress P-LC proliferation to the same extent.
Table 4  The suppression of P-LC proliferation using purified IFN is released by anti IFN (α+β) serum

<table>
<thead>
<tr>
<th>culture conditions</th>
<th>3H-Tdr uptake of P-LC (cmp) cultured with</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>medium</td>
</tr>
<tr>
<td>P-LC+ medium</td>
<td>17,410±27</td>
</tr>
<tr>
<td>P-LC+IFN (α/β)</td>
<td>4,249±1,032</td>
</tr>
<tr>
<td>P-LC+anti-IFN(α+β)</td>
<td>17,955±1,169</td>
</tr>
<tr>
<td>P-LC+preimmune</td>
<td>18,370±1,067</td>
</tr>
<tr>
<td></td>
<td>serum</td>
</tr>
<tr>
<td>P-LC+ medium</td>
<td>12,928±1,406</td>
</tr>
<tr>
<td>P-LC+IFN (α/β)</td>
<td>5,016±852</td>
</tr>
<tr>
<td>P-LC+anti-IFN(α+β)</td>
<td>100 neutralizing u/ml</td>
</tr>
<tr>
<td>P-LC+preimmune</td>
<td>100 neutralizing u/ml</td>
</tr>
</tbody>
</table>

The P-LC monolayer was cultured with or without purified IFN (α+β) and anti IFN (α+β) serum. The preimmune rabbit serum was added to the control groups at a final concentration equivalent to that of antiserum. The concentration of purified IFN (α+β) was 1,000 IU/ml and that of anti IFN (α+β) serum was 100 neutralizing u/ml. Typical results from 3 separate experiments are shown and each value represents the mean±SD of triplicate cultures.

7. Effect of anti IFN (α+β) serum on P-LC suppression induced by purified IFN (α+β)

Anti IFN (α+β) serum removed the suppression of P-LC proliferation by NPA-LC.sup (Table 3). In order to investigate whether or not the reduction of the suppression by the antiserum depended on the inactivation of IFN in NPA-LC.sup, 100 neutralizing units/ml of anti IFN (α+β) serum were added to P-LC culture containing purified IFN (α+β) at a final concentration of 1,000 IU/ml. The suppression of P-LC using purified IFN (α+β) was not completely but mostly overcome by the addition of anti IFN (α+β) serum (Table 4). These results would seem to indicate that the reduction of P-LC suppression using anti IFN (α+β) serum depends on the specific activity of the anti serum.

Discussion

As is well known, the microenvironment plays an important role in the control of cell proliferation. Since NAP-LC, mainly consisting of Kupffer cells and endothelial cells, are found near P-LC and produce many kinds of active factors, they would appear to have a great influence on the microenvironment surrounding parenchymal liver cells.

The proliferation of P-LC is suppressed by NPA-LC and NPA-LC.sup. The suppressor activity of NPA-LC.sup corresponds to the IFN level in the supernatant (Fig. 1 and Table 2) and this suppression is reduced by anti IFN (α+β) serum (Table 3). Purified IFN (α+β) added to P-LC culture instead of the supernatant suppresses P-LC proliferation and the suppression is also reduced by anti IFN (α+β) serum (Fig. 2 and Table 4). These results suggest that the IFN in the supernatant plays an important role in the suppression of P-LC proliferation. The suppression mechanisms of cell proliferation by IFN are not clear. It has been reported that in some cells increases in ornithine decarboxylase (ODC) are associated with the proliferation responses of cells in culture. The inhibition by IFN of DNA synthesis in mouse regenerating liver was accompanied by the inhibition or increases in the activities of adenylate cyclase and ODC after partial hepatectomy. Since the inhibition of ODC was prevented by the administration of dibutyryl cAMP, the suppression of DNA synthesis by IFN might be caused by the inhibition of adenylate cyclase activity. There is, however, evidence that concomitant inhibition by IFN of DNA synthesis and of activation of the enzyme is observed in Swiss 3T3 cells only when polypeptide hormones are used as a stimulant; for instance, cholera toxin stimulated DNA synthesis was
inhibited, while toxin-stimulated ODC activation was not\[^{22}\]. These results indicate that it depends on the cells and stimulants used in experiments whether concomitant inhibition is induced by IFN or not.

The IFN (\(\alpha + \beta\)) activity in NPA-LC.sup from the regenerating liver is at the same level as that in the supernatant of NPA-LC which have not been stimulated in vivo (data not shown). The NPA-LC may be stimulated in the course of cell separation and start to produce IFN. The spontaneous release of IFN from cultured liver cells has been reported by Arnheiter et al\[^{21}\]. In their experiments, the cells were a mixture of parenchymal and nonparenchymal liver cells. We separated each cell fraction, NPA-LC and P-LC, from whole liver cell suspensions and demonstrated that the IFN level is high in NPA-LC.sup but low or zero in P-LC.sup (Table 2). The NPA-LC.sup inhibits P-LC proliferation (Fig. 1 and Table 3) but the P-LC.sup does not (Fig. 1). These results seem to be caused by the differences of the IFN levels in these two culture supernatants. The IFN level of NPA-LC.sup necessary to suppress P-LC proliferation to 50% that of the control is extraordinarily low as compared with purified IFN (Fig. 1, Fig. 2, Table 3 and Table 4). The reason for this is not clear, but the following are presented as suggestions: 1) The ratio between IFN\(\alpha\) and IFN\(\beta\) is different in the supernatant and the purified IFN (\(\alpha + \beta\)). 2) The suppression activity of the supernatant may be due to the synergistic interaction of IFN and other active factors in NPA-LC.sup. Many reports state that in addition to IFN\(\alpha\), nonparenchymal liver cells produce many kinds of active factors, IL-1\[^{26}\], arginase\[^{25}\], CSF\[^{9}\], PGE\(^4\), HBGF-1\[^{10}\], TGF \(\beta^7\), IL-6\[^{8}\] and TNF\[^{3}\] which modulate directly/indirectly cell proliferation. In fact, we observed GM-CSF and IL-1 activity in the NPA-LC.sup\[^{28}\]. The reports showing that IFN works synergistically with IL-1\[^{26}\] or arginase\[^{27}\] to suppress cell proliferation suggest that the combination of these factors in the supernatant results in an increase in the cytostatic effect. In addition to the difference of IFN levels necessary for the suppression of P-LC proliferation between P-LC.sup and purified IFN (\(\alpha + \beta\)), the suppression by 1000 IU/ml of purified IFN (\(\alpha + \beta\)) was mostly released by 100 neutralizing units/ml of anti IFN (\(\alpha + \beta\)) serum (Table 4). The suppression takes a rapid sigmoidal curve in 500 to 5000 IU/ml of purified IFN (Fig. 2). Therefore, a small difference of purified IFN levels would have a great influence on P-LC proliferation. Since, however, the anti IFN (\(\alpha + \beta\)) serum used in these experiments is not a monoclonal antibody, we can not rule out the possibility that the antibodies in the serum react not only with IFN (\(\alpha + \beta\)) but also with different molecules to augment the P-LC proliferation.

To work synergistically with other active factors, IFN should be produced in their production periods. Now we are investigating the production periods of active factors which would appear to have a great influence on liver regeneration.

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References


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