A novel recombinant molecule, termed IL-6c and consisting of a chimera of interleukin 6 (IL-6) and its soluble receptor is extremely potent in stimulating proliferation of hematopoietic progenitors. We investigated the effect of the IL-6c on the proliferation and differentiation of E14 fetal hepatocytes. IL-6c, in a dose-dependent manner, stimulated proliferation of E14 fetal rat hepatocytes. Adult hepatocyte mitogens together with IL-6c showed no further effect on proliferation. Hematopoietic stem cells mitogens SCF and flt3 ligand (FL) were also mitogenic for fetal hepatocytes, but did not further enhance the effect of IL-6c on cell proliferation. IL-6c decreased expression of fetal markers α-fetoprotein (AFP) and γ-glutamyltranspeptidase, and induced expression of adult enzyme glucose-6-phosphatase (Gluc-6-P) in E14 hepatocytes. On the other hand, IL-6c strongly reduced, in a dose-dependent manner, expression of albumin and tyrosine aminotransferase (TAT). However, when the cells were grown for 3 days with IL-6c, and IL-6c was removed for the next 5 days, expression of albumin and TAT returned to levels found in control cultures. In conclusion, IL-6c stimulated proliferation and affected gene expression in fetal hepatocytes in culture.
has been designed and expressed in CHO cells (Chebath et al., 1997; Katz et al., 1998). A previous similar construct was shown to be 100–1,000 fold more potent than the unlinked IL-6 and IL-6r in stimulating proliferation of hematopoietic progenitors (Fischer et al., 1997). IL-6c was more potent than IL-6 in increasing survival of IL-6 deficient mice after CCl4-induced liver damage (Katz et al., 1998). An IL-6c molecule was also shown to increase liver regeneration of murine liver (Peters et al., 2000) and to increase liver regeneration in two different models of hepatic liver failure and to reduce hepatocyte apoptosis and hepatotoxicity (Galun et al., 2000).

Biliary epithelial cells, as well as “oval cells,” transformed liver progenitor cells, express the hematopoietic stem cell receptors c-kit andflt3 (Omori et al., 1997). The presence of these receptors may indicate that stem cell factor (SCF) (the c-kit ligand) and flt3 ligand (FL) could be mitogens for both hematopoietic as well as for hepatic stem cells.

The purpose of the present studies was to determine the role of the IL-6 chimera, by itself or in combination with fetal and adult hepatic mitogens, on the proliferation and maturation of fetal hepatocytes. Identification of selective fetal hepatic mitogens may be extremely helpful for eventual use of fetal hepatocytes during cell therapy for liver disease.

EXPERIMENTAL PROCEDURES

Primary cultures of fetal hepatocytes

Fischer 344 rats with known durations of pregnancy were obtained from the Tel Aviv Sourasky Medical Center Animal Facility. By convention, the first day of gestation is defined as day 0. Use of animals was approved by the Animal Care and Use Committee of the Tel Aviv Sourasky Medical Center.

Cell suspensions enriched for liver parenchymal cells were prepared as described (Brill et al., 1999). Livers from E14 embryos were removed and placed in Hank’s Balanced Salt Solution (HBSS) with 0.8 mM MgCl2 and 20 mM HEPES (Sigma Chemical Company, Israel), pH 7.3 (modHBSS). EGTA was added to 1 mM final concentration. The livers were trituriated, centrifuged at 400g for 5 min at 4°C. The pellet was resuspended in 50 ml of 0.6% Collagenase Type I (333 U/mg, Worthington Biochemical Corp., NJ) in HBSS containing 1 mM CaCl2 and stirred at 37°C for 20 min in an Erlenmeyer flask. The dispersed cells were resuspended in modHBSS. Cell number and viability were determined using a hemocytometer and trypan blue exclusion.

Proliferation assays

Fetal liver cells were plated in 24-well plates, on collagen I coated plates and in the hormonally defined medium (HDM) described by Block et al. (1996), with or without 300 ng/ml IL-6 or various doses of IL-6c (10 ng/ml, 100 ng/ml, and 1 µg/ml). The growth factors tested were: 50 ng/ml human SCF, 50 ng/ml human FL (Pepro Tech, Rocky Hill, NJ), 10 ng/ml heregulin α (the EGF binding domain), 10 ng/ml HGF, 10 ng/ml human heparin-like binding EGF (HB-EGF) (Sigma Chemical Company). The cells were trysinized at various time points and counted in duplicates using a hemocytometer. Each experiment was repeated three-times.

Fluorescence activated cell sorter (FACS) analysis

The E14 freshly isolated liver cells were cultured for 1 hr on ice with anti rat albumin (1:400, Sigma, St. Louis, MI) diluted in DMEM +10% fetal calf serum (FCS) + 0.05% sodium azide and 0.15% saponin. The cells were washed and then incubated for 1 hr on ice with an anti-rabbit F Ab fragment conjugated with fluorescein isothiocyanate (FITC). At the end of the incubation, the cells were washed and fixed in 0.5% paraformaldehyde in phosphate buffered saline (PBS).

Plated E14 fetal liver cells were cultured with IL-6c (1 µg/ml), SCF (10 ng/ml) and FL (10 ng/ml) for various time periods, then the cells were trypsinized and stained for albumin as described above.

Albumin-positive cells were analyzed using a Becton-Dickson FACS and the results were analyzed using the WinMDI software.

Histochemistry and immunohistochemistry

Histochemical assays for hepatic enzymes were performed on fetal hepatocytes. The quantitation score used is: –, negative staining; +/−, weak staining in few cells; +, positive staining; ++, strong positive staining in most cells; and ++++, strong positive staining in all cells.

GGT (gamma-glutamyltranspeptidase) activity was detected as described in Rutenberg et al. (1969). The cells were incubated for 30 min at 37°C with substrate solution, γ-glutamyl-4-methoxy-2-naphthylamide, glycylglycine, and fast blue BB salt. After saline wash, the plates were incubated with 0.1 M Cu₂SO₄ for 2 min at room temperature and GGT activity was detected as red staining of the cells.

DPP IV (dipeptidylpeptidase IV) activity was detected by incubating cells with the substrate solution: Gly-Pro H₂O-8-sulphonic acid, glycine, and fast blue BB salt. After 20 min incubation, the wells were washed and incubated with 0.1 M Cu₂SO₄ for 2 min. DPP IV activity was viewed as red staining.

ATPase (adenosyl triphosphatase) activity was detected by incubating the cells at 37°C for 3 hr in substrate solution (ATP as a sodium salt dissolved in 0.1 M MgSO₄ and 2% lead nitrate). Dark brown color appears in areas of ATPase activity after the addition of 0.22% ammonium sulfide.

Gluc-6-P activity was determined by histochemistry after incubation with the substrate solution composed of sucrose, Gluc-6-P, and lead nitrate for 10 min at room temperature. Brown staining is visualized after addition of ammonium sulfide (0.27%) for 30 sec.

Western blots

Total protein from cultured fetal hepatocytes was extracted by incubating the pelleted cells for 30 min on ice in lysis buffer (250 mM sucrose, 5 mM MgCl₂, 10 mM Tris pH 8.0, 0.5% Triton X-100 and 1 mM phenylmethysulfonylfluoride). The extracts normalized to total protein content, were separated by SDS–PAGE and blotted onto Hybond C extra (Amersham, Arlington Heights, IL). The blots were blocked in Tris-saline buffer pH 7.5, 0.05% Tween-20 and 5% milk. Blots were incubated with antibodies to albumin and α-fetoprotein...
(AFP) (Sigma; and ICN, Irvine, CA), washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson Laboratories, Bar Harbour, ME). The blots were subjected to chemiluminescent detection and fluorography using X-ray film (NEN Life Science Products, Boston, MA). Equal protein loading was determined by staining the blots with Ponceau S solution.

**Northern blot analysis**

Fetal hepatocytes were washed with cold PBS and total RNA isolated by the method of Chomczynsky and Sacchi (1987). RNA samples were resolved by electrophoresis through 1% agarose, denaturing formaldehyde gels in MOPS buffer and transferred to Hybond N paper. The blots were hybridized with cDNA probes used were hepatic nuclear factor 4 (HNF-4), TAT, and albumin. The expression of liver-specific genes was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

**RESULTS**

**Effect of IL-6c in combination with fetal and adult hepatic mitogens on the proliferation of E14 hepatocytes**

The effect of different doses of IL-6c on the proliferation of E14 hepatocytes was investigated for a period of up to 5 days, and the peak of proliferation is after 3 days in culture. IL-6c affects E14 hepatocytes proliferation in a dose-responsive manner: 10 ng/ml of IL-6c is growth stimulatory, but 100 ng/ml and 1 μg/ml are even better mitogens for fetal hepatocyte proliferation. (Fig. 1A). IL-6 (300 ng/ml) was not mitogenic for E14 fetal hepatocytes (Fig. 1A).

Bile duct epithelial cells express the hemopoietic stem cell receptors c-kit and flt3 (14). The same receptors are also present in fetal liver. We, therefore, investigated the effect of the ligands for these receptors, SCF and FL, on the proliferation of E14 hepatocytes, by themselves and in combination with IL6-c. Both SCF (in a statistically significant manner) and FL were mitogenic for fetal hepatocytes (Fig. 1A), but less so than 1 μg/ml IL-6c. There was no additive effect of either SCF or FL to the mitogenic effect of IL-6c (Fig. 1A).

We next investigated whether IL-6c is mitogenic for fetal hepatocytes from more advanced gestational ages. Fetal hepatocytes from E18 embryos showed strong proliferation when grown in the presence of 1 μg/ml IL-6c, while IL-6 had a very weak mitogenic effect (data not shown).

Since the liver cell cultures contain mixed populations of cells, we tested whether IL-6c, SCF, and FL are mitogenic for hepatoblasts, by determining the percentage of albumin-positive cells in the original population and in cells cultured for 3 days. As shown in Table 1, between 57 and 86% of the cells cultured with IL-6c, SCF, and FL were positive for albumin, though the intensity of albumin expression was lower than in the freshly isolated population. These results indicate that IL-6c, SCF, and FL induce proliferation of fetal hepatocytes in mixed cell cultures.

**TABLE 1. FACs analysis for albumin staining in freshly isolated E14 cells and in cells cultured for 3 days with IL-6c, SCF, and FL**

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Gated albumin positive cells</th>
<th>Mean intensity staining for albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly isolated liver cells</td>
<td>94±/−1.4</td>
<td>104±/−2.5</td>
</tr>
<tr>
<td>Control</td>
<td>76±/−15</td>
<td>58±/−2.8</td>
</tr>
<tr>
<td>IL-6c</td>
<td>80±/−6.3</td>
<td>37±/−4.9</td>
</tr>
<tr>
<td>SCF</td>
<td>75±/−9.9</td>
<td>39±/−12</td>
</tr>
<tr>
<td>FL</td>
<td>73±/−12</td>
<td>44±/−21</td>
</tr>
<tr>
<td>FL + SCF</td>
<td>78±/−2.1</td>
<td>51±/−7</td>
</tr>
<tr>
<td>IL-6c + SCF</td>
<td>80±/−8.4</td>
<td>44±/−10.6</td>
</tr>
<tr>
<td>IL-6c + FL</td>
<td>69±/−17</td>
<td>40±/−1.4</td>
</tr>
</tbody>
</table>

We investigated the possibility that IL-6c synergizes with adult hepatic mitogens to stimulate fetal hepatocyte proliferation. No mitogenic effect of HGF, HB-EGF, and heregulin for fetal hepatocytes was observed (Fig. 1B). Moreover, these adult hepatic mitogens did not further enhance the stimulatory effect of IL-6c (Fig. 1B).
Effect of IL-6c on the expression of liver specific genes and enzymes in cultured E14 hepatocytes

Expression of various liver specific proteins in E14 hepatocytes was determined after 2, 5, and 8 days of culture in the presence of 1 μg/ml IL-6c. Expression of α-fetoprotein (AFP) was strongly reduced (Fig. 2A). Rat liver expresses multiple AFP mRNAs, that are translated into different size variants. The major AFP fetal form is a 2.1 kb mRNA which is translated into 68,000 and 70,000 molecular weight proteins, while the major adult liver AFP mRNA is translated into smaller proteins of 58,000, 54,000, and 44,000 (Lemire and Fausto, 1991). We observed that after 2 days E14 hepatoblasts produce the larger AFP molecular weight form (Fig. 2A). With time in culture, after 5 and 8 days, the cells switched to smaller, more mature AFP forms, and AFP expression is decreased in the presence of IL-6c (Fig. 2A). Albumin expression, that increased during time in culture under the control conditions, was slightly reduced after 8 days in cells grown in the presence of IL-6c (Fig. 2B).

Northern blot analysis showed that HNF-4 mRNA levels were not changed by IL-6c, but TAT mRNA was decreased in fetal hepatocytes grown for 5 and 8 days with 1 μg/ml IL-6c (Fig. 3).

As shown in Table 2 and Figure 4, IL-6c induced expression of various differentiation markers in fetal hepatocytes: both Gluc-6-P and ATPase activity were increased after 5 days in culture. Strong staining for glycogen granules was also observed (Table 2). The expression of GGT, a fetal marker, was reduced after 5 days in the presence of IL-6c (Fig. 2B).

Since enzymatically the cells appeared more differentiated in the presence of IL-6c, we hypothesized that the down-regulation of albumin and TAT were merely transient effects on these genes, such as those induced by IL-6 in the negative acute-phase response. We therefore determined whether the effect of IL-6c on the expression of albumin and TAT genes was reversible. We grew E14 hepatocytes with IL-6c for 3 days, then IL-6c was removed and the cells were cultured in control medium for 5 additional days. As shown before, E14 grown for 8 days with IL-6c had very low expression of TAT, however, removal of IL-6 after 3 days and further culturing without IL-6c for 5 days resulted in recovery of the TAT mRNA expression (Fig. 5A). The same results were obtained when we tested for albumin expression (Fig. 5B). IL-6c reduced albumin expression in fetal hepatocytes in a dose-dependent manner after 8 days in culture (Fig. 5B). Similarly to its effect on TAT expression, the effect of IL-6c on albumin expression was also reversible. When IL-6c was added for 3 days, then removed for 5 days, albumin levels returned to those of the control cultures (Fig. 5B).

We also analyzed albumin expression by FACS analysis in cultures grown for 8 days with IL-6c, and for 3 days with IL-6c and for 5 more days without IL-6c (Fig. 5C and Table 3). In the control cultures after 8 days, 77% of the cells are positive for albumin.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>IL-6c</th>
<th>IL-6c&lt;sup&gt;a&lt;/sup&gt; 1d/4d w/o</th>
<th>IL-6c&lt;sup&gt;b&lt;/sup&gt; 2d/3d w/o</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gluc-6-P</td>
<td>--/-</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>ATPase</td>
<td>-+</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>GGT</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Gly</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>DPPIV</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done; Gluc-6-P, glucose-6-phosphatase; GGT, gamma glutamyl transpeptidase; Gly, glycogen accumulation by PAS staining; DPPIV, dipeptidyl peptidase IV.

The cells were cultured in the presence of IL-6c for 2 days, then the medium was removed and the cells cultured without IL-6c for an additional 4 days.
Cultures grown for 8 days in the presence of IL-6c have a smaller percentage of albumin positive cells and the mean intensity of albumin staining is lower than in the control cells (Table 3), suggesting that the cultures contain a preponderance of hepatocytes, but that the overall albumin expression per cell is lower that in cells grown without IL-6c. When IL-6c was added for 3 days only, and the cells were cultured for 5 more days without IL-6c, there was the highest percentage of albumin positive cells (Fig. 5C and Table 3).

It has been reported that fetal murine liver cultures differentiate when grown with oncostatin M or IL-6c (Kamiya et al., 1999). When cultured in medium containing 10% FCS, fetal murine hepatocytes did not express Gluc-6-P or TAT. Addition of oncostatin M or IL-6c induced expression of these genes (Kamiya et al.,...
We decided to test why our results were different, and whether FCS prevented expression of TAT, since in our studies, fetal rat hepatocytes grown in a serum-free medium showed high expression of this enzyme. Indeed, as shown in Figure 6, FCS prevented expression of TAT. Under these culture conditions, addition of IL-6c actually induced expression of TAT (Fig. 6).

**DISCUSSION**

We have examined the effect of IL-6c on the proliferation and differentiation of fetal rat hepatocytes. Our results indicate that IL-6c is a strong mitogen for fetal hepatocytes, inducing maximal proliferation of after 3 days in culture. There was no additional mitogenic effect when the cells were grown with known mitogens for adult hepatocytes or with hematopoietic stem cells mitogens. However, the combination of SCF and FL was also mitogenic for fetal hepatocytes, up to 5 days in culture. Recently, IL-6c was shown to stimulate proliferation of the early hemopoietic stem cells progenitors CD34+/CD38low, without affecting their differentiation (Kollet et al., 1999). In these cells, as with fetal hepatocytes, the effect of IL-6c on cell proliferation was better than that of a combination of SCF and FL (Kollet et al., 1999).

IL-6c causes proliferation of fetal liver parenchymal cells, with partial differentiation. It allows cells to acquire expression of certain differentiated markers for hepatocytes, such as Gluc-6-P and glycogen granules storage. We found that expression of albumin and TAT remained low when fetal hepatocytes were cultured in

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% Gated albumin positive cells</th>
<th>Mean intensity staining for albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 8 days</td>
<td>77+/−9.1</td>
<td>36+/−20</td>
</tr>
<tr>
<td>IL-6c-8 days</td>
<td>72+/−12</td>
<td>26+/−7.7</td>
</tr>
<tr>
<td>IL-6c (3 days with, 5 days without)</td>
<td>79+/−17</td>
<td>31+/−11</td>
</tr>
</tbody>
</table>

Fig. 5. (Continued)

Fig. 6. Northern blot showing expression of TAT in E14 fetal hepatocytes cultured for 8 days with 10% FCS, with or without 1 μg/ml IL-6c. The numbers below the blot denote value of TAT normalized to GAPDH expression.
the presence of IL-6c. However, inhibition of albumin and TAT gene expression was reversible, since removal of IL-6c after 3 days and further culture of the cells for 5 more days resulted in albumin and TAT levels similar to those of control cultures.

IL-6 induces an acute-phase response in the liver, which includes turning on the expression of acute-phase proteins, such as haptoglobin, α1-acid glycoprotein, fibrinogen, α1-antichymotrypsin, and serum amyloid A (Wang et al., 1999). Albumin is a negative acute-phase protein, so its down-regulation by IL-6c is expected.

The role of IL-6 in liver regeneration is well documented. During liver injury, TNF-α induces expression of IL-6 by Kupffer, endothelial, and hepatic stellate cells. IL-6 activates STAT3 via the gp80/gp130 complex, and this is crucial for the differentiation of murine fetal hepatocytes. IL-6 activates STAT3 via the gp80/gp130 complex, and this is crucial for the differentiation of murine fetal hepatocytes. IL-6 activates STAT3 via the gp80/gp130 complex, and this is crucial for the differentiation of murine fetal hepatocytes.

In our hormonally conditioned medium, in the absence of IL-6c, expression of Gluc-6-P was detectable in murine fetal hepatocytes cultured in the presence of 10% FCS and dexamethasone (Kamiya et al., 1999). Moreover, IL-6c had the same effect. Knockout mice for gp130 had deficient glycogen granules accumulation in the liver and reduced expression of TAT (Kamiya et al., 1999), suggesting that for complete development of genes of the glycolysis pathway, signaling by both dexamethasone and oncostatin M are necessary (Kamiya et al., 1999).

In our hormonally conditioned medium, in the absence of serum and in the presence of dexamethasone, fetal rat hepatocytes strongly expressed TAT mRNA. In the presence of serum, cells turned down expression of TAT, and under these conditions, addition of IL-6c induced expression of the gene. Moreover, when cultured in hormonally defined medium, E14 hepatocytes showed strong staining for glycogen after 2 days in culture.

In conclusion, we have shown that IL-6c is a strong mitogen for fetal rat hepatocytes, and induces expression of various differentiation markers. IL-6c may be a mitogen for transplanted fetal hepatocytes in cell therapy for liver disease. Further studies of IL-6c effect on the proliferation of transplanted fetal hepatocytes are warranted.

LITERATURE CITED


