Porcine smooth muscle cell-conditioned medium stimulates LDL receptor activity in Hep G2 cells

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Summary

Paracrine factors may modulate low density lipoprotein (LDL) receptor activity in hepatocytes. To study this the effect of conditioned medium prepared from a range of cell types on the binding and internalisation of $^{125}$I-LDL in Hep G2 cells was studied. Seven of the fourteen conditioned media tested, including those from P388D1, U937, porcine smooth muscle (Pc SMC) Swiss 3T3, STO, =48 and MDCK cells, were found to increase the binding and internalisation of $^{125}$I-LDL at 37°C by Hep G2 cells ($P < 0.01$). The largest increase in LDL receptor activity was produced by conditioned medium from Pc SMC cells and was, therefore, selected for further analysis. The Pc SMC-conditioned medium increased LDL receptor number in Hep G2 cells by three-fold but had no effect on LDL receptor activity in human skin fibroblasts. DNA synthesis and cholesterol synthesis by Hep G2 cells were inhibited by Pc SMC-conditioned medium. Preliminary characterisation of the Pc SMC-derived factor(s) suggests that it is a protein(s) of low relative molecular mass.

Key words: smooth muscle cells, conditioned medium, LDL receptor, Hep G2, TGF-β.

Introduction

Despite its importance in the maintenance of the plasma cholesterol level, relatively little is known about what regulates the level of the hepatic low density lipoprotein (LDL) receptor in vivo. In vitro studies have shown that the activity of the hepatic lipoprotein can be regulated by both lipoproteins and hormones. Thus, LDL has been shown to down-regulate whilst high density lipoprotein has been shown to up-regulate LDL receptor activity in Hep G2 cells (Havekes et al., 1986). Several hormones including triiodo-L-thyronine, insulin and oestrogen, albeit at concentrations higher than those apparently present in vivo, have been shown to increase LDL receptor activity in Hep G2 cells and/or rat hepatocytes (Salter et al., 1988; Semenkovich and Ostlund, 1987) whilst dexamethasone has been shown to decrease LDL receptor activity in rat hepatocytes (Salter et al., 1988). In extrahepatic cells LDL receptor activity has also been shown to be modulated by paracrine factors. For example, LDL receptor activity is increased by platelet-derived growth factor (PDGF) in fibroblasts and smooth muscle cells (Chait et al., 1980) but decreased by transforming growth factor-beta (TGF-β) in bovine adrenocortical cells (Hotta and Baird, 1987). There have been few studies, however, on the role of cell factors in regulating LDL receptor activity in the hepatocyte. To determine whether paracrine factors may also regulate the activity of the hepatic LDL receptor we looked at the effect of conditioned medium prepared from a range of cell types on LDL receptor activity in Hep G2 cells. As serum contains several factors which can modulate LDL receptor activity, a chemically defined medium (CDM) for the culture of Hep G2 cells has been developed for these studies (Gherardi et al., 1992, accompanying paper). Cell growth and expression of LDL receptor and HMG-CoA reductase activity are comparable in Hep G2 cells cultured in CDM and in medium containing 10% fetal calf serum (FCS). Conditioned media from seven of the fourteen cell types tested increased LDL receptor activity in Hep G2 cells, but by far the biggest increase was induced by conditioned medium from porcine smooth muscle cells (Pc SMC) and was, therefore, further investigated.

Materials and methods

Origin of cells

MDCK cells (canine kidney epithelial cells) were a gift from Sir Michael Stoker (Department of Pathology, Cambridge University), =48, a mammary epithelial cell line, was a gift from Dr. P. A. W. Edwards (Department of Pathology, Cambridge University) and bovine smooth muscle cells (Bov SMC) were a gift from Dr. A. Peachey (Strangeways Research laboratory, Cambridge). Pc SMC were prepared from porcine thoracic aortas obtained from a
local abattoir by explant outgrowth (Ross, 1971). Human foreskin fibroblasts (McK fibroblasts) were obtained from Dr. J. Owen (Royal Free Hospital, London). All other cell types were obtained from the American Type Culture Collection or the European Collection of Animal Cell Cultures. All of the cell types used were free from mycoplasma as assessed using the Gen-probe mycoplasma kit.

**Tissue culture**

All cells were cultured in 75 cm² flasks (Falcon) in DMEM (Flow laboratories) or RPMI 1640 (Flow laboratories) supplemented with 2 g/l sodium bicarbonate (BDH), 0.06 g/l penicillin G (Sigma) and 0.1 g/l streptomycin sulphate (Sigma) and further additions as below, at 37°C in equilibrium with 95% air/5% CO₂. The cells were fed every two days with fresh medium and routinely split 1:4 as soon as they had reached confluence. The fibroblast lines (McK, STO and Swiss 3T3), Pc SMC and Bov SMC and bovine aortic endothelial cells (BAEC) were cultured in DMEM supplemented with 10% FCS (Biological Industries). For passing, the cells were detached with 2 mg/ml trypsin (Flow Laboratories). ≈48 cells were cultured as above with the addition of 1 mg/ml insulin (Sigma) to the culture medium. The murine macrophage/menocyte cell line P388D1 and MDCK cells were grown in DMEM supplemented with 10% FCS. For passing, the cells were detached with a cell scraper. The non-adherent menocyte/macrophage cell lines THP-1, J774 and U937 were grown in RPMI 1640 supplemented with 20% FCS and 1.19 g/l Hepes (Sigma). Hep G2 cells, a human hepatoma cell line, were cultured in RPMI 1640 supplemented with 1.19 g/l Hepes and 10% FCS and the cells detached for passing with 2 mg/ml trypsin.

**Preparation of conditioned medium**

Cells were grown to confluence as described above, washed twice with PBS and then incubated with 15 ml of RPMI 1640 containing 2 mg/ml defatted BSA at 37°C. After 48 hours the cell-conditioned medium was collected, centrifuged at 1000 g for 10 minutes to remove cell debris and the supernatant stored at −20°C.

**Testing of conditioned medium**

The conditioned media were tested for their effect on Hep G2 cells and McK fibroblasts as follows. Hep G2 cells were plated at a density of 75,000 cells/cm² in 2 cm² multiwell dishes (Falcon) in CDM and cultured until confluent (four days). The CDM consisted of RPMI 1640 containing 2 g/l sodium bicarbonate, 1.19 g/l Hepes, 0.06 g/l penicillin G, 0.1 g/l streptomycin sulphate, 10 i.u./l prolactin (Sigma), 5 × 10⁻² M insulin (Sigma), 5 × 10⁻¹⁸ M glucagon (Sigma), 1 × 10⁻⁸ M hydrocortisone (Sigma), 1 × 10⁻⁹ M triiodo-L-thyronine (Sigma), 10 mg/ml LDL, 5 × 10⁻⁵ M insulin (Sigma), 1 × 10⁻⁷ M copper sulphate, 5 × 10⁻¹¹ M zinc sulphate, 1 × 10⁻⁷ M manganese sulphate, 5 × 10⁻¹⁵ M nickel chloride, 1 × 10⁻⁷ M iron sulphate and 3 × 10⁻⁹ M sodium selenite. McK fibroblasts were plated at a density of 50,000 cells/cm² in 2 cm² multiwell dishes in DMEM containing 10% FCS and cultured until confluent (four days). The cells were then washed two times with PBS before incubating for a further 24 hours with either 1 ml of RPMI 1640 containing 1 mg/ml defatted BSA or 1 ml of conditioned medium which had been diluted two-fold with RPMI-1640. The binding and internalisation of ¹²⁵I-LDL at 37°C or other assays were then performed as described below.

**Measurement of ¹²⁵I-LDL binding at 4°C, ¹²⁵I-LDL binding and internalisation at 37°C and ¹²⁵I-LDL degradation**

¹²⁵I-LDL binding at 4°C, ¹²⁵I-LDL binding and internalisation at 37°C and ¹²⁵I-LDL degradation were measured essentially as described by Brown and Goldstein (1974). For binding at 4°C the cells were preincubated for 30 minutes on ice before adding ¹²⁵I-LDL to 15 µg/ml with a specific activity of 100 cts/min per ng and incubating for a further 2 hours on ice with shaking. Measurement of the degradation and binding and internalisation of ¹²⁵I-LDL was carried out using a concentration of 5 µg/ml ¹²⁵I-LDL with a specific activity of 100 cts/min per ng over a 4 hour time period at 37°C. The rate of degradation of ¹²⁵I-LDL was measured in terms of the accumulation of TCA (trichloroacetic acid)-soluble material. In each case incubations were carried out in the absence of unlabelled LDL to measure total binding and internalisation of LDL or in the presence of 0.25 mg/ml unlabelled LDL to measure low affinity binding of LDL. The high-affinity or receptor-mediated binding was then calculated by subtracting the low-affinity binding from the total binding. Results were calculated in terms of the protein per dish (Markwell et al., 1978).

**Measurement of cholesterol synthesis**

The rate of cholesterol synthesis by Hep G2 cells was determined by measuring the incorporation of [¹³C]acetate into non-saponifiable cholesterol. Briefly, the cells were incubated with 0.5 ml of RPMI 1640 containing 1 mM [¹³C]acetate with a specific activity of 2 mCi/m mole for 2 hours at 37°C. The total lipids were extracted and saponified. The cholesterol was then isolated by thin-layer chromatography and the radioactivity associated with it was determined by scintillation counting. Results were standardised in terms of mg of cell protein.

**Measurement of DNA synthesis**

The rate of DNA synthesis by the cells was determined by measuring the incorporation of [³H]thymidine into trichloroacetic acid-precipitable material as follows. The preincubation medium was removed and the cells were incubated with 0.5 ml of RPMI 1640 containing 5 µM [³H]thymidine (Amersham) with a specific activity of 2 mCi/m mole at 37°C for 2 hours. The cells were then washed two times with PBS before incubating with 50 g/l trichloroacetic acid for 30 minutes. The cells were washed two times with water and then solubilised by incubating with 0.4 ml of 0.2 M NaOH for 30 minutes at 37°C. The cells were harvested into scintillation vials and the solution neutralised with 0.1 ml of 0.8 M HCl. Radioactivity was measured after the addition of 5 ml of Hisafe II scintillation fluid in a Nuclear Chicago MK2 scintillation counter.

**Measurement of cell cholesterol**

For measurement of cell cholesterol, total lipids were extracted from the cells and then separated by thin-layer chromatography. Lipids were visualised with iodine vapour and those spots containing cholesterol and cholesteryl ester as judged by reference to standards were eluted from the plate with 1 ml of chloroform (Bowyer and King, 1977). The cholesterol was quantified in a fluorometric assay by reference to standards in the range 0.1 to 10 µg cholesterol in chloroform (Bondjers and Bjökerud, 1971). A stock solution of zinc chloride was made by heating 4 g of anhydrous zinc chloride in 15 ml of glacial acetic acid at 80°C for 2 hours. Immediately before use 0.5 ml of the stock solution was added to 10 ml of acetyl chloride. A 1.5 ml sample of the working solution was added to 0.5 ml of sample in chloroform and then heated at 55°C for 30 minutes before cooling rapidly to halt the reaction. Fluorescence was measured at an excitation wavelength and a fluorescent wavelength of 565 nm.

**Concentration of Pc SMC-conditioned medium by dialysis against polyethylene glycol (PEG) M., 20 × 10³**

Pc SMC-conditioned medium was placed in 6 to 8 (×10³) Mₗ cut-off dialysis membrane (Spectrum) and dialysed against a 35% w/v solution of PEG (Mₗ 20 × 10³) (Sigma) in RPMI 1640 at room
temperature until it had reduced in volume approximately 15 times. The concentrate was recovered and dialysed against RPMI 1640 for 14 hours with two changes of buffer. The dialysate was then filtered through a 0.22 μm membrane and stored at −20°C.

**Freeze/thawing of Pc SMC-conditioned medium**
A sample of Pc SMC-conditioned medium was subjected to four cycles of freezing at −20°C and thawing at room temperature over a period of 7 days.

**Heat treatment of Pc SMC-conditioned medium**
Samples of Pc SMC-conditioned medium were heated at 100°C for 5 minutes or 1 hour.

**Acid/alkali treatment of Pc SMC-conditioned medium**
Samples of Pc SMC-conditioned medium were placed in 6 to 8 (×10³) M C₄ cut-off dialysis tubing and dialysed for 16 hours at 4°C against a 50-fold volume excess of buffers of pH 3 (0.05 M glycine/HCl) and pH 11 (0.2 M sodium phosphate/NaOH) with two changes of buffer. They were then dialysed back into RPMI 1640 pH 8.2 over 24 hours at 4°C with three changes of buffer and the dialysates were filtered through a 0.22 μm filter.

**Reductive alkylation of Pc SMC-conditioned medium**
Two samples of Pc SMC-conditioned medium were buffer-exchanged into 0.05 M Tris-HCl, pH 8.0, by dialysis in 6 to 8 (×10³) M C₄ cut-off dialysis membrane for 16 hours at 4°C. To one sample dithiothreitol (DTT) was added to a final concentration of 0.05 M (from a 0.5 M stock solution in PBS) and to the other sample an equal volume of buffer and the tubes were incubated at 37°C for 1 hour. The tubes were then placed on ice for 30 minutes, before adding iodoacetic acid (IAA), to 0.005 M, to the tube containing the DTT (from a 0.5 M stock solution in PBS) and an equal volume of buffer to the other tube, and then incubated on ice for 1 hour. They were then dialysed back into RPMI 1640 pH 8.2 over 40 hours with 6 changes of buffer and the dialysates filtered through a 0.22 μm filter.

**Tryptic digestion of Pc SMC-conditioned medium**
Trypsin (Sigma) was added to a final concentration of 0.5 mg/ml to Pc SMC-conditioned medium and the tube incubated at 37°C for 4 hours. Soybean trypsin inhibitor (Sigma) was then added to a final concentration of 1 mg/ml to halt the digestion. As a control Pc SMC-conditioned medium was incubated with trypsin in the presence of trypsin inhibitor from the start of the 4 hour incubation at 37°C.

**Fractionation of Pc SMC-conditioned medium by gel filtration on a Superose 12 column**
Pc SMC-conditioned medium was concentrated approximately 20 times as described above by dialysis against a 35% (w/v) solution of PEG 20,000 in RPMI 1640. A 0.5 ml sample of the concentrate was applied to a 30 ml Superose 12 column equilibrated in phenol red-free RPMI 1640, pH 8.2, containing 1 mg/ml defatted BSA. The sample was eluted at a flow rate of 0.5 ml per minute and the eluent was collected in 1 ml fractions. In order to provide sufficient material the fractions from three consecutive runs were pooled. The Mr of fractions was estimated by reference to the elution volumes of a 0.5 ml sample of proteins of known Mr run under identical conditions.

**Effect of TGF-β1 and TGF-β antisera on LDL receptor activity**
TGF-β1, prepared from human platelets, (British Biotechnology Ltd.) was reconstituted in 4 mM HCl. A 50 ng/ml solution was prepared in RPMI 1640 containing 1 mg/ml defatted BSA.

To determine whether the Pc SMC LDL receptor stimulator was TGF-β the Pc SMC-conditioned medium was preincubated for 30 minutes with 50 μg/ml of anti-porcine TGF-β antisera (British Biotechnology Ltd.) before determining its effect on the binding and internalisation of 125I-LDL by Hep G2 cells. As a control to test for the effect of the antisera per se on LDL receptor activity parallel incubations were set up of 50 μg/ml anti-porcine TGF-β in RPMI 1640 alone. The efficiency of the anti-porcine TGF-β antisera was tested by its ability to neutralise the LDL receptor stimulatory activity of TGF-β1 from human platelets in Hep G2 cells.

**Results**

**Effect of conditioned medium from different cell types on LDL receptor activity in Hep G2 cells**
A significant increase (P < 0.01, Student-Newman Keuls test) in the binding and internalisation of 125I-LDL at 37°C by Hep G2 cells was observed after a 24 hour incubation with cell-conditioned media from seven of the fourteen different cell types tested (P388D1, U937, Pc SMC, swiss 3T3, STO, =48 and MDCK) compared to cells incubated with RPMI 1640 containing 1 mg/ml defatted BSA (Fig. 1). This increase was small (approximately 25%-40% higher than the value for control cells for all of these conditioned media except that from Pc SMC, which caused a larger increase of 112% of the control. The conditioned medium from the remaining seven of the cell types tested (J774, THP-1, Bov SMC, BAEC, McK, HeLa and Hep G2) had no significant effect on the binding and internalisation of 125I-LDL at 37°C by Hep G2 cells. There was no clear relationship between the embryological origin of the cell and the effect of its conditioned medium on LDL receptor activity in Hep G2 cells. As conditioned medium from Pc SMC produced by far the largest increase in LDL receptor activity of the conditioned media tested it was selected for further analysis.

**Effect of Pc SMC-conditioned medium on LDL receptor activity in McK fibroblasts**
In McK fibroblasts Pc SMC-conditioned medium had no significant effect on LDL receptor activity after a 24 hour incubation (Fig. 2).

**Effect of Pc SMC-conditioned medium on LDL receptor activity in Hep G2 cells**
125I-LDL binding at 4°C, 125I-LDL binding and internalisation at 37°C and 125I-LDL degradation at 37°C by Hep G2 cells were increased to a similar extent after a 24 hour incubation with Pc SMC-conditioned medium (Table 1). Scatchard analysis of the binding at 4°C (Scatchard, 1949) showed that whilst the apparent KG was unaltered after incubation of the cells in Pc SMC-conditioned medium the Bmax was increased from 16 ng LDL apolipoprotein/mg cell protein to 46 ng LDL apolipoprotein/mg cell protein, indicating that there was an increase in the number of LDL receptors on the cell surface (Fig. 3(a),(b)).

A well-behaved dose-response curve for the effect of Pc SMC-conditioned medium on LDL receptor activity was...
observed (Fig. 4). At the highest concentration tested Pc SMC-conditioned medium increased LDL receptor activity by 265 ± 6% relative to control cells.

When the time-course of the stimulation of LDL receptor activity by Pc SMC-conditioned medium was studied it showed that 125I-LDL binding and internalisation at 37°C to Hep G2 cells was maximally stimulated between 6 to 18 hours after the addition of Pc SMC-conditioned medium to the cells (Fig. 5(a)). The effect of Pc SMC-conditioned medium on LDL receptor activity was, however, transient and with 48 hours incubation the level of LDL receptor activity was lower in cells incubated in Pc SMC-conditioned medium than in RPMI 1640 containing 1 mg/ml defatted BSA.

Other effects of Pc SMC-conditioned medium on Hep G2 cells
Changes in LDL receptor activity are frequently a result of alterations in the rate of cholesterol synthesis, changes in the cell cholesterol content or alterations in cell growth. These parameters were, therefore, measured in Hep G2 cells after incubation with Pc SMC-conditioned medium to try and elucidate the mechanism by which Pc SMC-conditioned medium stimulates LDL receptor activity in Hep G2 cells (Table 1; Fig. 5).

Cholesterol synthesis by Hep G2 cells as measured by the incorporation of 14C-acetate into cholesterol was inhibited by Pc SMC-conditioned medium (Fig. 5(b)). The time-
Regulation of LDL receptor in Hep G2 cells

The course of the effect was, however, different compared with the stimulation of LDL receptor activity. Thus, cholesterol synthesis was not significantly inhibited until after 18 hours of incubation and was not maximally inhibited until after 24 hours incubation. The level of cholesterol synthesis then remained the same with up to 48 hours of incubation of the cells with Pc SMC-conditioned medium.

The cholesterol and cholesterol ester content of Hep G2 cells was not significantly different in cells incubated for 24 hours with Pc SMC-conditioned medium or in RPMI 1640 containing 1 mg/ml defatted BSA (Table 1). The number of cells per dish was not significantly different for cells incubated for 24 hours with Pc SMC-conditioned medium compared with control cells (Table 1). The rate of DNA synthesis was decreased by approximately 18% \( (P < 0.05) \) as measured by the rate of \( \text{[3H]} \) thymidine incorporation into TCA-precipitable material after 24 hours of incubation with Pc SMC-conditioned medium. With prolonged incubation the rate of DNA synthesis continued to fall relative to control cells, so that at 48 hours DNA synthesis was 60% of the level observed in cells incubated in RPMI 1640 containing 1 mg/ml defatted BSA (Fig. 5(c)).

Table 1. Effects of Pc SMC-conditioned medium on Hep G2 cells

<table>
<thead>
<tr>
<th></th>
<th>RPMI 1640*</th>
<th>Pc SMC-conditioned medium*</th>
<th>Percentage change†</th>
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<tbody>
<tr>
<td><strong>LDL receptor activity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Binding and internalisation at 37°C (ng mg(^{-1}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>53 ± 1</td>
<td>140 ± 7</td>
<td>162 ( P &lt; 0.01 )‡</td>
</tr>
<tr>
<td>Low affinity</td>
<td>17 ± 1</td>
<td>28 ± 2</td>
<td>55 ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>High affinity</td>
<td>45 ± 1</td>
<td>112 ± 8</td>
<td>145 ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Degradation at 37°C (ng mg(^{-1}))</td>
<td>11 ± 1</td>
<td>23 ± 4</td>
<td>106 ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Binding at 4°C (ng mg(^{-1}))</td>
<td>23 ± 4</td>
<td>63 ± 5</td>
<td>185 ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>( B_{\text{max}} ) (ng mg(^{-1}))</td>
<td>16</td>
<td>46</td>
<td>187</td>
</tr>
<tr>
<td>Apparent ( K_d ) (ng l(^{-1}))</td>
<td>5.3\times10(^3)</td>
<td>5.2\times10(^3)</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol synthesis (cts min(^{-1}) h(^{-1}) mg(^{-1}))</td>
<td>44,800 ± 2000</td>
<td>19,800 ± 600</td>
<td>−56 ( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Cholesterol content (ng mg(^{-1}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>35.1 ± 7.2</td>
<td>34.9 ± 3.2</td>
<td>−1 \ NS</td>
</tr>
<tr>
<td>Cholesterol ester</td>
<td>9.3 ± 2.6</td>
<td>9.7 ± 0.8</td>
<td>4 \ NS</td>
</tr>
<tr>
<td><strong>Cell growth</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA synthesis (cts min(^{-1}) h(^{-1}) mg(^{-1}))</td>
<td>11,300 ± 1400</td>
<td>9,295 ± 334</td>
<td>18 ( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Cell number (cm(^{-2}))</td>
<td>177,000 ± 16,000</td>
<td>185,000 ± 15,000</td>
<td>4 \ NS</td>
</tr>
<tr>
<td>Cell protein (mg cm(^{-2}))</td>
<td>0.081 ± 0.004</td>
<td>0.102 ± 0.002</td>
<td>26 ( P &lt; 0.01 )</td>
</tr>
</tbody>
</table>

*Confluent Hep G2 cells were incubated for 24 hours with RPMI 1640 or Pc SMC-conditioned medium diluted two-fold with RPMI 1640. The activities listed were then measured as described in Materials and methods. Results represent the mean ± s.d. of triplicate observations.
†[(Level in Pc SMC-conditioned medium/level in RPMI 1640)\times100–100]%.
‡Student-Newman Keuls test. NS, not significant.

\( (a) \)

![Fig. 3.](image) The effect of Pc SMC-conditioned medium on \(^{125}\text{I}-\text{LDL} \) binding by Hep G2 cells at 4°C. Confluent Hep G2 cells were incubated for 24 hours with either 1 ml of RPMI containing 1 mg/ml defatted BSA (■) or 1 ml of Pc SMC-conditioned medium diluted two-fold with RPMI 1640 (▲). The binding of \(^{125}\text{I}-\text{LDL} \) to the cells was then measured at 4°C. (a) Represents the high affinity binding of \(^{125}\text{I}-\text{LDL} \) to the cells at 4°C and is given as ng of \(^{125}\text{I}-\text{LDL} \) apolipoprotein bound per mg cell protein at each \(^{125}\text{I}-\text{LDL} \) concentration. Results represent the mean ± s.d. of three determinations. (b) A Scatchard plot of the data presented in (a) to enable the LDL receptor number and its affinity for LDL to be determined for cells incubated in each condition.
Cell protein was approximately 26% higher in cells incubated for 24 hours with Pc SMC-conditioned medium than in control cells ($P < 0.05$, Table 1).

**Characterisation of Pc SMC LDL receptor stimulator**

In order to determine the nature of the mediator(s) of the increase in LDL receptor number preliminary characterisation studies were carried out (Table 2):

- Tryptic digestion completely destroyed the LDL receptor stimulator. Reductive alkylation with DTT and IAA resulted in a substantial loss of approximately 70% of the LDL receptor stimulator.
- The Pc SMC LDL receptor stimulator was stable to up to four cycles of freezing at $-20^\circ C$ and thawing at room temperature, and to acid and alkali treatment between pH 3 and pH 11 for 16 hours. Heat treatment destroyed little of the activity. Thus heating at 100$^\circ C$ for 5 minutes resulted in only a mean 13% reduction in LDL receptor stimulatory activity, although with prolonged incubation at 100$^\circ C$ for up to 1 hour 61% of the original activity was destroyed.
- Gel filtration of the Pc SMC-conditioned medium on a Superose 12 column failed to resolve the LDL receptor stimulatory activity; thus, significant activity was observed in all fractions containing proteins of $M_r$ greater than $5 \times 10^3$.

**Comparison of the effect of TGF-$\beta 1$ and Pc SMC-conditioned medium on LDL receptor activity in Hep G2 cells**

TGF-$\beta 1$ stimulated LDL receptor activity by 125 ± 29% ($P < 0.01$). Preincubation of TGF-$\beta 1$ with anti-porcine TGF-$\beta$ antisera resulted in a loss of 80% of the LDL receptor stimulatory activity. In contrast, preincubation of Pc SMC-conditioned medium with anti-porcine TGF-$\beta$ antisera resulted in a loss of only 16% of its LDL receptor stimulatory activity (Fig. 7).
Regulation of LDL receptor in Hep G2 cells

Discussion

The results clearly show that cell factors can regulate LDL receptor activity in Hep G2 cells. Thus, conditioned medium from seven of the fourteen cell types tested increased the binding and internalisation of $^{125}$I-LDL by Hep G2 cells after a 24 hour incubation. In further support of the hypothesis that paracrine factors may be important in regulating LDL receptor activity in the liver are the recent observations that the cytokines TGF-β1 and IL-1α stimulate the binding and internalisation of $^{125}$I-LDL at 37°C by Hep G2 cells (Moorby et al., 1992) and that Onco-statin M increases LDL receptor number in Hep G2 cells (Grove et al., 1991b).

The largest increase in binding and internalisation of $^{125}$I-LDL by Hep G2 cells observed in the present studies was produced by Pc SMC-conditioned medium, which was therefore, further analysed. The stimulation of LDL receptor activity induced by Pc SMC-conditioned medium in Hep G2 cells was originally detected by an increase in the $^{125}$I-LDL binding and internalisation at 37°C. Scatchard analysis of the binding of $^{125}$I-LDL at 4°C demonstrated that the increase in LDL receptor activity at 37°C was mediated entirely by an increase in LDL receptor number on the cell surface.

Many of the known physiological factors that stimulate LDL receptor activity act by increasing the demand of the cell for cholesterol either by depleting the cell of choles-

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Table 2. Preliminary characterisation of Pc SMC LDL receptor stimulator

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity remaining* (%)</th>
<th>Probability†</th>
</tr>
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<tbody>
<tr>
<td>Freeze/thawing</td>
<td>90 ± 7</td>
<td>NS</td>
</tr>
<tr>
<td>Heat stability</td>
<td>87 ± 7</td>
<td>NS</td>
</tr>
<tr>
<td>100°C for 1 h</td>
<td>39 ± 4</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>Dialysis</td>
<td>95 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Acid/alkali stability</td>
<td>84 ± 15</td>
<td>NS</td>
</tr>
<tr>
<td>pH 11 for 16 h</td>
<td>96 ± 12</td>
<td>NS</td>
</tr>
<tr>
<td>Reductive alkylation</td>
<td>30 ± 3</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>Trypsin sensitivity</td>
<td>9 ± 9</td>
<td>$P &lt; 0.01$</td>
</tr>
</tbody>
</table>

*The amount of LDL receptor stimulatory activity remaining after each treatment was estimated by comparing the level of binding and internalisation of $^{125}$I-LDL at 37°C after a 24 h incubation of confluent Hep G2 cells with the treated and untreated media. Results are the mean ± s.d. of triplicate observations and are expressed as:

\[
\frac{[\text{Level in treated Pc SMC-conditioned medium} - \text{level in RPMI 1640}]}{\text{[Level in untreated Pc SMC-conditioned medium} - \text{level in RPMI 1640}]} \times 100.
\]

†Student-Newman Keuls test. NS, not significant.

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Fig. 6. $M_r$ characterisation of Pc SMC LDL receptor stimulator. Pc SMC-conditioned medium was fractionated by gel filtration on a Superose 12 column as described in Materials and methods. Confluent Hep G2 cells were incubated with either 0.5 ml of each fraction or 0.5 ml of RPMI 1640 containing 1 mg/ml defatted BSA for 18 hours. The high-affinity binding and internalisation of $^{125}$I-LDL at 37°C was then measured. Results represent the mean ± s.d. of 4 experiments and are expressed as a percentage of the level observed in cells incubated in RPMI 1640 containing 1 mg/ml defatted BSA for 18 hours in the absence of TGF-β1. $M_r$ values ($\times 10^{-3}$) are indicated by arrows; Vo is void volume.

Fig 7. Comparison of the effect of TGF-β and Pc SMC-conditioned medium on LDL receptor activity. Confluent Hep G2 cells were incubated with 50 ng/ml TGF-β in RPMI 1640 containing 1 mg/ml defatted BSA, Pc SMC conditioned medium diluted two-fold with RPMI 1640 or RPMI 1640 containing 1 mg/ml defatted BSA for 18 hours in the absence (open) or presence (hatched) of 100 µl of anti-porcine TGF-β antiserum. The high-affinity binding and internalisation of $^{125}$I-LDL at 37°C to the cells was then measured. Results represent the mean ± s.d. of triplicate observations. * and ** Denote a significant difference to the level observed in cells incubated in RPMI 1640 containing 1 mg/ml defatted BSA, respectively ($P < 0.05$ and $P < 0.01$, Student-Newman Keuls test).
terol by acting as a cholesterol acceptor or by increasing the mitotic activity of the cell. No significant change in the total cell cholesterol or cholesterol ester content was observed, however, after a 24 hour incubation of Hep G2 cells with Pc SMC-conditioned medium at concentrations which produced a two-fold increase in LDL receptor activity. In contrast incubation of Hep G2 cells with concentrations of HDL₃ high enough to produce an equivalent 200% increase in LDL receptor activity produced a 25% decrease in cell cholesterol and a 50% decrease in cell cholesterol ester (data not shown). This suggests that the mechanism by which Pc SMC-conditioned medium raises LDL receptor activity in Hep G2 cells is not by depleting the cell of cholesterol. It remains possible that Pc SMC-conditioned medium may deplete a quantitatively small but nonetheless regulatory important pool of cholesterol.

The increase in LDL receptor activity induced by Pc SMC-conditioned medium could not be accounted for by a general increase in the mitotic activity of the cells as, for example, is the case for stimulation of LDL receptor activity in fibroblasts by PDGF and TNF (Chait et al., 1980; Harada et al., 1990). Indeed prolonged incubation with Pc SMC-conditioned medium actually inhibited the rate of [³H]thymidine incorporation into DNA.

Another set of circumstances where LDL receptor activity has been found to be enhanced is when cholesterol synthesis is inhibited, making uptake of lipoproteins via the LDL receptor the major source of cholesterol for the cell. This is the mechanism of action of certain 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMG-CoA reductase) inhibitors such as compactin (Cohen et al., 1984). It is, therefore, possible that the primary target of the Pc SMC-conditioned medium is the inhibition of cholesterol synthesis. This, however, seems unlikely for two reasons. Firstly, the time-course for the effect of Pc SMC-conditioned medium on these two activities in Hep G2 cells shows that the stimulation of LDL receptor activity precedes the inhibition of cholesterol synthesis (LDL receptor activity peaking between 6 and 18 hours whilst cholesterol synthesis is not maximally inhibited until 24 hours). Secondly, by analogy with drugs such as compactin a 50% reduction of cholesterol synthesis would be insufficient to account for the 267% increase in LDL receptor activity. Thus, for compactin a 50% decrease in the rate of cholesterol synthesis is correlated with only a 75% increase in LDL receptor activity in Hep G2 cells (Cohen et al., 1984). It, thus, appears that the effect of Pc SMC-conditioned medium on these two activities is independent.

Whatever the mechanism by which the Pc SMC-conditioned medium stimulates LDL receptor activity in Hep G2 cells it should be noted that Pc SMC-conditioned medium failed to increase LDL receptor activity in human skin fibroblasts. This indicates that the active factor(s) present in medium conditioned by Pc SMC act(s) in a tissue-specific manner and suggests that cytokines may be responsible, at least in part, for the differential uptake of LDL in the liver and extrahepatic tissues that is known to occur in vivo.

The identity of the factor(s) in Pc SMC-conditioned medium that produce the stimulation of LDL receptor activity is not known. The activity requires disulphide bond(s) and was both heat- and pH-stable, suggesting that it is probably due to a protein(s) of low $M_r$. Gel filtration of the Pc SMC-conditioned medium on a Superose 12 column failed to resolve the LDL stimulatory activity adequately and thus it remains uncertain whether it is due to one or multiple factors.

In extrahepatic cells several different cytokines have been previously shown to modulate LDL receptor activity. Identity of the Pc SMC LDL receptor stimulator(s) with one of these is unlikely in the majority of cases because either: (i) they appear to act by increasing the mitotic activity of the cells whereas Pc SMC-conditioned medium inhibits DNA synthesis in Hep G2 cells; (ii) they have been shown to stimulate LDL receptor activity in fibroblasts, whereas we have shown that Pc SMC-conditioned medium had no significant effect on LDL receptor activity in McK fibroblasts; or (iii) their physical characteristics differ from those of the Pc SMC LDL receptor stimulator.

The physical characteristics of the Pc SMC LDL receptor stimulator(s) correlate quite closely with those described for TGF-β (Sporn et al., 1986). Despite the fact that TGF-β has been shown to inhibit LDL receptor activity in bovine adrenocortical cells, in contrast to the stimulation of LDL receptor activity by Pc SMC-conditioned medium, it was thought to be a possible candidate for the Pc SMC LDL receptor stimulator(s), as it is known that TGF-β has different effects on different cell types (Hotta and Baird, 1987; Sporn et al., 1986). Indeed TGF-β1 from human platelets was found to increase LDL receptor activity in Hep G2 cells (Moorby et al., 1992). However, experiments with anti-TGF-β antibodies, which almost completely blocked the stimulation of LDL receptor activity by human TGF-β, produced only a 16% inhibition in the stimulation of LDL receptor activity by Pc SMC-conditioned medium. Thus we conclude that the Pc SMC-derived factor(s) is not TGF-β.

During the preparation of this manuscript, Grove et al. (1991b) reported that Oncostatin M also potently stimulates LDL uptake in Hep G2 cells. Like the Pc SMC cell-derived factor(s) Oncostatin M inhibits cholesterol synthesis by approximately 50% and has only a small inhibitory effect on DNA synthesis (Grove et al., 1991a). Oncostatin M differs from the Pc SMC LDL receptor stimulator(s), however, in its sensitivity to heat treatment. Oncostatin M activity being almost completely destroyed by heat treatment at 100°C for 10 minutes (Zarling et al., 1986), whereas approximately 39% of the Pc SMC LDL receptor stimulatory activity remained even after prolonged heating at 100°C for 1 hour. It thus appears that Pc SMC LDL receptor stimulator(s) does not share identity with Oncostatin M. It will be important, however, to determine whether there is any homology between Oncostatin M and the Pc SMC LDL receptor stimulator(s) as they have such similar effects on LDL receptor activity in Hep G2 cells.

In summary, we have shown that LDL receptor activity in Hep G2 cells can be potently stimulated by Pc SMC-conditioned medium. Further work will be necessary to determine the identity of this factor(s) and to establish if this or a related molecule(s) is produced in liver by non-parenchymal cells that may modulate the expression of LDL receptor in hepatocytes in a paracrine manner.
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