A Novel Herbal Formulation “LiverCare” Differentially Regulates Primary Rat Hepatocyte and Hepatocarcinoma Cell Proliferation *In Vitro*

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**ABSTRACT** Hepatocyte growth factor (HGF) plays an important role in hepatocyte proliferation. HGF expression is regulated by various signaling molecules and nuclear receptors. In the present study, LiverCare® (LC), a novel polyherbal formulation (The Himalaya Drug Company, Bangalore, India), was evaluated for its efficacy, using co-cultures of primary rat hepatocytes–non-parenchymal cells (NPCs) and human hepatocellular carcinoma cells (HepG2). The rate of primary hepatocyte co-culture proliferation was significantly and dose-dependently increased by LC as determined by[^3H]thymidine incorporation into newly synthesized DNA and cell proliferation assay. LC also increased HGF expression in primary hepatocyte co-culture. Albumin and urea content remained constant during proliferation of hepatocyte co-cultures in the presence of LC with decreased activity of alanine aminotransferase. It is interesting that LC inhibited incorporation of[^3H]thymidine into DNA in HepG2 cells. LC enhanced peroxisome proliferator-activated receptor-α expression during hepatocyte proliferation, whereas tumor necrosis factor-α expression remained unaffected. In conclusion, our study clearly showed that LC differentially regulates primary rat hepatocytes and human hepatocarcinoma cell proliferation. LC may be a promising candidate for treating degenerative liver diseases by enhancing liver regeneration.

**KEY WORDS:** hepatocyte growth factor • hepatocytes • LiverCare • peroxisome proliferator-activated receptor-α •[^3H]thymidine • tumor necrosis factor-α

**INTRODUCTION**

Liver is a remarkable organ, given its inherent capacity to fully restore itself after significant hepatic tissue loss from either infection or acute liver injury. Liver regeneration is mediated by proliferation of the mature resident liver cells, including hepatocytes, endothelial cells, biliary epithelial cells, hepatic stellate cells, and Kupffer cells. The hepatocytes are the first to proliferate and are the major target for parenchymal regeneration. After tissue loss or injury, hepatocytes enter the cell cycle from the quiescent state (G₀) to a prereplicative state (G₁), which is followed by DNA synthesis (S) and mitosis (M), with cell division completing the sequence.

Liver regeneration involves a signaling cascade mediated by various cytokines and growth factors. Complete mitogens are defined as substances that stimulate DNA synthesis and mitosis of cultured hepatocytes in serum-free medium. Co-mitogens have no direct proliferative effect on hepatocytes in culture but augment the stimulatory effect of complete mitogens and decrease the inhibitory effects of other factors. Among them, hepatocyte growth factor (HGF), a heterodimeric glycoprotein, also known as scatter factor, plays an important role in liver development and regeneration. The expression of HGF mRNA was rapidly up-regulated in various types of liver injuries in experimental animals. HGF exerts its mitogenic effect on hepatocytes by paracrine and endocrine mechanisms. Transgenic overexpression of HGF can result in the resolution of fibrosis and the improvement of survival rate in cirrhotic rats. Apart from HGF, other signaling molecules and nuclear receptors like interleukin-6, tumor necrosis factor-α (TNF-α), and epidermal growth factor (EGF) are directly implicated in hepatic regeneration. Similarly, various factors have been recognized as hepatocyte proliferative inhibitors. Among them, transforming growth factor (TGF)-β, a fibrogenic cytokine, is a potent inhibitor of hepatocyte proliferation and has been suggested as the main terminator of hepatic regeneration.

Silymarin, Liv.52, sho-saiko-to, and Radix Polygoni Multiflori are some herbal drugs that have been widely used to treat hepatic disorders. LiverCare® (LC) (manufactured by The Himalaya Drug Company, Bangalore, India, and marketed by Himalaya Herbal Healthcare, Houston, TX, USA) is a novel herbal formulation (The Himalaya Drug Company, Bangalore, India) that has been shown to have beneficial effects on liver function.
USA) is an herbal formulation based on traditional Ayurvedic concepts for treating liver disorders and for protecting and rejuvenating the liver. The health benefits of LC constituents are well documented by clinical and experimental studies.19 Terminalia arjuna is known for its diuretic effect, and Cichorium intybus is known for anti-inflammatory and anti-immunotoxic effects.20 The antioxidative and hepatoprotective effects of esculetin and p-methoxybenzoic acid, the main constituents of C. intybus and Capparis spinosa, respectively, have been reported in experimental animals.21 Achillea millefolium contains several bioactive constituents, including flavanoids and terpenoids, with antioxidative and anti-inflammatory properties.22,23 The antioxidative property of Tamarix gallica and the protective effect of Solanum nigrum crude extract against free radical-mediated DNA damage are reported to increase the hepatoprotective effect.24 Similarly, arjunic acid and flavanoids present in T. arjuna are known to possess antioxidative and anti-liperoxidative effects and to increase glutathione content in liver.25 In this study, the role of LC in hepatocyte regeneration and proliferation was examined using primary rat hepatocytes and human hepatocarcinoma cells.

MATERIALS AND METHODS

Chemicals

RPMI medium, Dulbecco’s minimum essential medium, collegenase type IV, Bradford reagent, EGF, fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), TRI Reagent®; custom-prepared oligonucleotides, glucagon, insulin, and dexamethasone were obtained from Sigma (St. Louis, MO, USA). Penicillin, streptomycin, and amphotericin B were from Hi-media (Mumbai, India). MMLV reverse transcriptase, deoxynucleotide triphosphates, and Taq DNA polymerase were purchased from MBI Fermentas (Amherst, NY, USA). [3H]Thymidine was purchased from the Board of Radiation and Isotope Technology, Department of Atomic Energy, Government of India, Mumbai. All other reagents and chemicals used were of analytical grade.

Composition of LC

LC is an approved Ayurvedic herbal proprietary formulation of herbs and extracts of C. spinosa root (98 mg), C. intybus seed (98 mg), T. nigrum whole plant (50 mg), T. arjuna bark (50 mg), Cassia occidentalis seed (26 mg), A. millefolium aerial plant (26 mg), and T. gallica whole plant (26 mg). The Good Agricultural and Collection Practices were used at all the times during procurement and processing of the plant materials.26 Botanical identification and Ayurvedic criteria for quality complied with the guidelines of Pharmacopoeial Standards of Ayurvedic formulations.27

LC sample preparation

LC, obtained from The Himalaya Drug Company, was pulverized using a mortar and pestle and sieved at 0.5 mm to obtain fine powder. This powder was then extracted with distilled water (1:10 vol/vol) at room temperature, and the extract was centrifuged at 605 g. After centrifugation, water-soluble compounds in LC were decanted, lyophilized, and used in the experiments.

Animals

Animal experiments were carried out taking appropriate measures to minimize pain or discomfort in accordance with the guidelines of the U.S. National Institutes of Health and approval of the institutional Animal Ethics Committee. Eight-week-old male Wistar rats weighing 200–250 g were used in the experiments. The animals were fed ad libitum with commercial rodent diet and had free access to water with a 12-hour dark–light cycle. At the time of experiment the rats were anesthetized with ether, and the liver was perfused via the hepatic portal vein with Ca2+-free Krebs-Ringer phosphate buffer for 10 minutes, followed by Ca2+-free Krebs–Ringer hydrogen-carbonate buffer and then Krebs–Ringer hydrogen-carbonate buffer containing collagenase (type IV) and CaCl2·2H2O for 20 minutes.28 The hepatocytes and non-parenchymal cells (NPCs) were then fractionated via Percoll® (Pharmacia, Uppsala, Sweden) gradient centrifugation. The cell viability was typically >90% as measured by trypan blue dye exclusion assay.

Primary rat hepatocyte–NPC co-culture

Hepatocytes (2×10⁶ cells/mL) were plated into 96-well culture plates with RPMI 1640 medium supplemented with antibiotic–antimycotic solution at 37°C with 5% CO₂ for 2 hours. After 2 hours of incubation, NPCs (1×10⁶ cells/mL) were added and co-cultured for an additional 3 hours.18 Then, the medium was changed to one without the serum, and the test samples (insulin [1 IU], EGF [1 ng/mL], glucocorticoid [1 µg/mL], LC [200 µg/mL], and glucocorticoid [10 µM]) were added and studied for their effects on cell proliferation and other parameters.

Human hepatocellular carcinoma cell (HepG2) culture

HepG2 cells obtained from the National Center for Cell Science, Pune, India, were maintained in culture using 25-cm² polystyrene flasks (Tarsons, Kolkata, India) with Dulbecco’s minimum essential medium containing 10% fetal bovine serum, 1% antibiotic–antimycotic solution, and 3.7 g/L sodium bicarbonate under an atmosphere of 5% CO₂ at 37°C with 95% humidity.

[3H]Thymidine incorporation assay

[3H]Thymidine was added to the cells at a dose of 0.2 µCi per well (1 µCi = 37 kBq) and incubated with or without test samples for different time intervals as described earlier.29 The radioactivity in the hepatocytes was measured after washing the cells with a solution containing 5% trichloroacetic acid and 95% ethanol. The radioactivity was measured using a Packard (Downers Grove, IL, USA) Top
Count-NXT™ liquid scintillation counter. All the samples were assayed in duplicate.

**MTT assay for determination of cell proliferation**

Cell proliferation was studied by following the cell viability at different time intervals by MTT assay. The amount of formazan formed gives the direct indication of proliferation rate of cultured cells and was used to estimate cell proliferation compared with the control as previously described.30

**RNA isolation and reverse transcription–polymerase chain reaction expression of TNF-α, peroxisome proliferator-activated receptor-α, and HGF mRNA**

Cells treated with samples and controls were washed with phosphate-buffered saline and homogenized in TRI Reagent, and RNA was extracted (following the manufacturer’s instructions) and stored at –70°C. cDNA was synthesized as described earlier.31 The following oligonucleotides for genes used in this study were according to reported cDNA sequences:31–33 TNF-α, sense 5’-ATGAGCAGTGAAACGATGATC-3’, antisense 5’TACAGGCAATGATCCTTCAAG TAGACCTGCC-3’; peroxisome proliferator-activated receptor (PPAR)-α, sense 5’-GGGACAGCTGATACGCAGGC-3’, antisense 5’-GACCACGGATAGTCACC-3’; HGF, sense 5’-CTCCCCCATGCCATCCC-3’, antisense 5’-CACCATGGCGCTGG-3’; and glyceraldehyde 3-phosphate dehydrogenase, sense 5’-GACCACAGTCCATGCCATCCC-3’, antisense 5’-TACCACACCTGTTGCTGT AG-3’. The desalted primers were synthesized by the solid-phase triester method by Sigma. Polymerase chain reaction was performed in a final reaction volume of 25 μL following the addition of specific primer pairs and Taq DNA polymerase using the conditions as described earlier.31–33 The expected polymerase chain reaction products of 453 bp (glyceraldehyde 3-phosphate dehydrogenase), 749 bp (HGF), 700 bp (TNF-α), and 252 bp (PPAR-α) were separated on 1.5% agarose gel by electrophoresis and visualized by ethidium bromide staining. The TNF-α, PPAR-α, and HGF expressions were confirmed against glyceraldehyde 3-phosphate dehydrogenase expression in all the experiments.

**Biochemical and enzyme analysis**

Cell supernatants were collected for analysis of albumin, urea nitrogen, and alanine aminotransferase at different time intervals and analyzed using an automated Nanolab chemistry analyzer (Trivitron Diagnostics Pvt. Ltd., Chennai, India) with commercial assay kits obtained from Diasys Diagnostics Systems (Holzheim, Germany) following the manufacturer’s instructions.

**Statistical analysis**

The results are expressed as mean ± SEM values. Statistical significance was determined by one-way analysis of variance and the Tukey–Kramer post hoc test using GraphPad Prism 4 (La Jolla, CA, USA). Results were considered to be significant at $P < .05$.

**RESULTS**

**Effect of LC on hepatocyte–NPC cytotoxicity and cell proliferation**

LC at concentrations of 50, 100, 200, 400, 600, 800, and 1,000 μg/mL in primary rat hepatocyte–NPC co-cultures did not cause cytotoxicity (Fig. 1A). Among the tested concentrations, 50, 100, and 200 μg/mL LC were evaluated for primary hepatocyte–NPC proliferation activity at different time intervals. LC at 200 μg/mL more effectively increased the rate of cell proliferation than all other concentrations tested at all time intervals (Fig. 1B). Hence 200 μg/mL LC was used in further experiments. Similarly, the effect on cell proliferating activity of co-mitogens and mitogens was evaluated in the co-culture at different time intervals. EGF combined with LC significantly enhanced the rate of cell proliferation compared with the control at all the time intervals (Fig. 1C). The cells treated with LC and other mitogens showed linear proliferation up to 24 hours but not thereafter. Insulin, glucagon, and glucocorticosteroid also enhanced cell proliferation, but less than LC and EGF.

**Effect of LC on DNA synthesis**

The effect of co-mitogens and mitogens on DNA synthesis was evaluated using primary hepatocyte–NPC co-cultures and HepG2 cells at different time intervals by following the incorporation of [3H]thymidine into DNA. The results showed that mitogens and LC significantly enhanced the rate of DNA synthesis compared with the control at all the time intervals in a linear fashion in the hepatocyte–NPC co-cultures (Fig. 2A), but in the HepG2 cells LC did not enhance the DNA synthesis; in contrast, insulin, EGF, and glucocorticosteroid increased the DNA synthesis at all time intervals (Fig. 2B).

**Effect of LC on biochemical markers in proliferating primary hepatocyte–NPC co-culture**

Alanine aminotransferase activity decreased with increasing incubation time in cells treated with EGF and LC (Fig. 3A). The amount of urea secreted in the cell supernatant decreased from 0 to 72 hours of incubation with EGF and LC treatment, but albumin secretion was increased by LC and EGF from 0 to 72 hours of incubation (Fig. 3B).

**Effect of LC on TNF-α, PPAR-α, and HGF gene expression in HepG2 cells**

LC did not enhance the TNF-α gene expression on incubation for 24 hours, but glucagon and insulin did enhance the TNF-α gene expression compared with the control and EGF. LC marginally enhanced PPAR-α gene expression compared with other mitogens and glucocorticoid (Fig. 4A and B).

LC promoted HGF gene expression in comparison with glucagon, insulin, and glucocorticoid, and EGF significantly enhanced HGF expression compared with the control (Fig. 4C).
DISCUSSION

The present study demonstrates that LC enhances primary rat hepatocyte–NPC proliferation by modulating HGF expression. Liver regeneration is controlled by various biochemical and signaling molecules, which are in turn regulated by various factors that may promote or inhibit liver regeneration. Several factors such as HGF, TGF-β, EGF, TNF-α, insulin-like growth factor, etc., promote hepatocyte regeneration. Insulin and glucagon are co-
FIG. 2. Effect of LC on $[^3H]$thymidine incorporation during DNA synthesis. (A) Primary rat hepatocytes–non-parenchymal cell co-cultures were incubated with mitogenic compounds and LC for 0–24 hours. (B) HepG2 cells were incubated with mitogenic compounds and LC for 0–24 hours. $[^3H]$Thymidine incorporation was determined at various intervals to study the synthesis of DNA as described in Materials and Methods. Data are mean±SD values of six samples from three independent experiments.

FIG. 3. Effect of LC on (A) alanine aminotransferase (ALAT) and (B) urea and albumin secretion into cell supernatant by primary rat hepatocytes–non-parenchymal cell co-cultures. The primary rat hepatocytes–non-parenchymal cell co-cultures were incubated with mitogenic compounds and LC for 0–24 hours, and ALAT activity and urea and albumin contents in cell supernatant were estimated as described in Materials and Methods. Data are mean±SD values of six samples from three independent experiments.
mitogens because they exert their mitogenic activity only in the presence of growth factors.6 TGF-β, interleukin-1, and other molecules inhibit the hepatocyte proliferation.35 LC increased proliferation of primary rat hepatocytes–NPCs but did not increase proliferation of HepG2 cells. This characteristic feature of LC is very important in view of liver regeneration without any risk of hepatocarcinogenesis, but the underlying mechanism governing the phenomenon deserves further study.

The cell proliferation rate was increased by LC as shown by the MTT assay. The cell proliferation was linear during the first 24 hours of incubation, and further growth was apparent but was not linear at 48 and 72 hours, thus establishing the capacity of LC to enhance hepatocyte proliferation. Insulin and glucagon were not more effective enhancers of DNA synthesis and cell proliferation than LC. EGF enhanced the DNA synthesis in hepatocytes after 12 hours, but LC enhanced DNA synthesis from 4 hours onward, suggesting that LC can initiate DNA synthesis more rapidly than EGF. The addition of glucocorticoid and dexamethasone enhanced the DNA synthesis in co-culture. Hepatocytes incubated without fetal bovine serum (control) did not show linear DNA synthesis. Studies have shown that DNA synthesis can be elicited in cultures of hepatocytes maintained in serum-free or serum-supplemented medium with combinations of the hormones insulin, EGF, and glucagon.36,37 It has been shown that EGF stimulates DNA synthesis and mitosis in the livers of intact animals or potentiates similar effects induced by insulin.36,39 EGF increases the number of hepatocyte nuclei that enter into DNA synthesis when it is added with insulin and glucagon in primary hepatocytes.35 It was previously demonstrated that hydrocortisone decreased the DNA synthesis, but similar effects did not occur in vivo.39 In the present study, dexamethasone, a synthetic glucocorticoid, induced DNA synthesis and increased hepatocyte proliferation. Taken together, the evidence demonstrates that LC might regulate and increase the rate of hepatic DNA synthesis and cell proliferation similar to previously reported hormones and growth factors.

The hepatocyte regeneration, which starts with the priming or initial stage and coincides with loss of growth inhibition, represents the G0 and G1 transition, whereas the progression phase acts to promote cell replication and represents the G1 to S transition. It is believed that a signaling molecule such as TNF-α induces priming events on G0 hepatocytes followed by the action of growth factors such as HGF and EGF, enabling hepatocytes to acquire proliferative competence for progression through the cell cycle.6

TNF-α is a pro-inflammatory cytokine secreted systemically by activated macrophages and in liver by biliary epithelial and venous endothelial cells.40 It has been shown to be an important mediator in both normal and pathological

![FIG. 4. Effect of LC on regulatory genes involved during hepatocyte proliferation: (A) tumor necrosis factor-α (TNF-α) and (B) peroxisome proliferator-activated receptor-α (PPAR-α) gene expression in HepG2 cells and (C) hepatocyte growth factor (HGF) gene expression in primary rat hepatocyte–non-parenchymal cell co-cultures. The hepatocytes were incubated with mitogenic compounds and LC for 24 hours as described, total RNA was extracted, and gene amplification was carried out as described in Materials and Methods. The relative levels of TNF-α, PPAR-α, and HGF gene expression were normalized to glyceraldehyde 3-phosphate dehydrogenase RNA, and values depict arbitrary units. Data are representative of three experiments. *P < .05.](image-url)
processes in liver.\textsuperscript{41,42} TNF-α levels are dramatically increased during liver regeneration and in patients with chronic hepatitis B and hepatitis C viral infections, alcoholic hepatitis, and inflammatory liver disease.\textsuperscript{41,43} The ability of TNF-α to act as a complete mitogen in primary rat hepatocytes was previously reported.\textsuperscript{12} We observed that LC, EGF, and dexamethasone did not significantly enhance the expression of TNF-α in HepG2 cells. Dexamethasone is known to inhibit TNF-α expression, and the results observed in this study are consistent with earlier reports.\textsuperscript{44} Glucagon and insulin significantly increased TNF-α expression in HepG2 cells in the present study. These discrepancies may be due to difference in response of TNF-α to human hepatocarcinoma cells and rodent hepatocytes because TNF-α stimulates nitric oxide synthase gene in rat and mouse but not in human hepatocytes.\textsuperscript{45}

Induction of DNA synthesis by peroxisome proliferators has been postulated to play an important role in non-genotoxic carcinogenesis.\textsuperscript{46} However, it has proved difficult to establish \textit{in vitro} models for the mitogenic response, and it is difficult to analyze the mechanisms that control PPAR agonist expression.\textsuperscript{47} In the present study, we demonstrated that LC enhances PPAR-α expression in HepG2 after a 24-hour incubation, and thus it may act as a PPAR-α agonist. Similarly, HepG2 cells treated with glucagon, insulin, EGF, and dexamethasone also induced PPAR-α gene expression. Human hepatocytes exhibit neither peroxisome proliferation nor induction of DNA synthesis in response to peroxisome proliferators \textit{in vitro}, and no increased incidence of hepatocarcinogenesis has been found in patients treated with hypolipidemic drugs.\textsuperscript{48,49} In the absence of such mechanism of action, the molecular basis for such marked species differences remains unclear, precluding accurate human risk assessment.

HGF is a potent mitogen for isolated rat and human hepatocytes and other epithelial cells and is important for hepatocyte proliferation during regeneration.\textsuperscript{7} We observed that LC enhanced the expression of HGF in hepatocyte–NPC co-cultures during a 24-hour incubation. It has been reported that plasma levels of HGF rise substantially within 1 hour after partial hepatectomy in both rats and humans. Also, the HGF mRNA level increases in hepatic stellate cells 3–6 hours after partial hepatectomy and peaks in 18–24 hours.\textsuperscript{50} Despite the marked induction of HGF in various tissues, mitogenic responses are found only in the liver or kidney, although it is an active mitogen for many different cell types. These observations show that HGF activation occurs in impaired organs rather than in normal organs.\textsuperscript{50} LC activated HGF expression in rat primary hepatocyte–NPC co-cultures and concomitantly enhanced DNA synthesis and cell proliferation, but did not enhance DNA synthesis in HepG2 cells as observed here. However, this study determined whether the expression of HGF is controlled and regulated by TNF-α and PPAR-α individually or in combination in primary hepatocytes. Whether mitogenic activation of hepatocytes by LC is due to additive or synergistic effects of its compounds would be interesting to study in the context of cell cycle regulation in primary hepatocyte and human hepatocarcinoma cell proliferation.

Albumin and urea are vital indicators of normal functioning of hepatocytes.\textsuperscript{28} LC helped maintain constant levels of urea and albumin levels. Similarly, activity of liver function enzymes such as alanine aminotransferase was not significantly affected by LC. This clearly indicates that the cultures were stable during the experiments.

In conclusion, LC when added exogenously brings about various alterations in cellular signaling during hepatocyte proliferation and acts as a mitogenic agent. The mitogenic activity of LC modulates the cell proliferation rate by altering the expression of multiple factors like TNF-α, PPAR-α, and HGF. In HepG2 cells, LC acts as a PPAR-α agonist without altering TNF-α expression. However, in primary hepatocyte–NPC co-cultures it effectively modulated the expression of HGF and enhanced the cell proliferation. Hence, LC may be beneficial during the treatment of hepatic disorders as it plays a vital role in increased hepatocyte proliferation and helps to restore normal liver function.

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**AUTHOR DISCLOSURE STATEMENT**

All authors are employees of The Himalaya Drug Company.

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