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Regulation of polyamine synthesis in human hepatocytes by hepatotrophic factor augmenter of liver regeneration

Rania Dayoub [a,b,1], Wolfgang E. Thasler [c,1], Anja K. Bosserhoff [d], Thomas Singer [e], Karl-Walter Jauch [c], Hans J. Schlitt [a,b], Thomas S. Weiss [a,b,*]

[a] Department of Surgery, University of Regensburg Hospital, Germany
[b] Center for Liver Cell Research, University of Regensburg Hospital, Germany
[c] Department of Surgery, LM University Munich, Hospital Grosshadern, Germany
[d] Institut of Pathology, University of Regensburg Hospital, Germany
[e] Hoffmann-LaRoche, Ltd. Non Clinical Safety, Basel, Switzerland

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Abstract

Different stages of liver regeneration are regulated by a variety of factors such as the liver growth associated protein ALR, augmenter of liver regeneration. Furthermore, small molecules like polyamines were proven to be essential for hepatic growth and regeneration. Therefore, using primary human hepatocytes in vitro we investigated the effect of ALR on the biosynthesis of polyamines. We demonstrated by HPLC analysis that recombinant ALR enhanced intracellular hepatic putrescine, spermidine, and spermine levels within 9–12 h. The activation of polyamine biosynthesis was dose dependent with putrescine showing the strongest increase. Additionally, ALR treatment induced mRNA expression of ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase, both key enzymes of polyamine biosynthesis. Further, ALR induced c-myc mRNA expression, a regulator of ODC expression, and therefore we assume that ALR exerts its liver regeneration augmenting effects through stimulation of its signalling pathway leading in part to enhanced polyamine synthesis.

Keywords: Liver regeneration; Polyamines; Ornithine decarboxylase; Hepatotrophic growth factor; Augmenter of liver regeneration; Immediate early gene; c-myc

The process of liver regeneration requires the regulatory action of several factors such as cytokines (IL-6, IL-1α, and TNF-α) and hepatotrophic growth factors (HGF and EGF). Using versatile animal models and primary hepatocytes in vitro the regeneration related effect of these molecules was demonstrated [1–3]. Additionally, a novel protein ALR, augmenter of liver regeneration [4] (hepatopoietin [5]), was discovered to be highly expressed in regenerating livers and significantly augmenting the progress of hepatic healing after resection or damage [6]. Interestingly, among all other factors ALR was shown to exhibit its action exclusively on regenerating livers and hepatoma cells [6–8]. ALR, was reported to accumulate in vivo after partial hepatectomy [5–7], under circumstances of liver regeneration [7] as well as in liver diseases such as liver cirrhosis and carcinoma [9,10]. Exogenously administered ALR was demonstrated to reveal a beneficial effect on the rate of liver regeneration [6], fulminant hepatic failure [11], and cirrhosis [12]. Additionally, recombinant ALR was shown to stimulate proliferation of hepatocytes as well as hepatoma cells in vitro and this hepatotrophic effect may be exerted through a specific receptor found on the plasma membrane [5,8,13,14]. Further, it was proposed that interaction of ALR with its receptor is responsible for enhanced

* Corresponding author. Fax: +49 941 9446838.
E-mail address: thomas.weiss@klinik.uni-regensburg.de (T.S. Weiss).
[1] These authors contributed equally to this work.

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proliferation and augmentation of liver regeneration by activating the mitogen-activated protein kinase (MAPK) cascade [13,14] and inducing the activity of NFκB [15].

Investigations on gene expression during hepatic regeneration have not only been restricted exclusively to study hepatotrophic factors. A number of studies focused on expression of ornithine decarboxylase (ODC), the rate-limiting enzyme in the pathway of polyamine biosynthesis. Polyamines, mainly putrescine (Put), spermidine (Spd), and spermine (Spm), are located in a variety of tissues and cell types, including hepatocytes and are considered essential components in several biological pathways comprising normal cell growth and differentiation, membrane functions and stability, stabilization of nucleic acid and subcellular organelles, activation of protein synthesis, and production of second messengers [16–18]. For a variety of cell types a role of both, enhanced ODC activity and high levels of polyamines, has been demonstrated inducing a proliferative response or to maintain cell differentiation [17]. Increases in ODC activity in concert with ODC mRNA levels are associated with post-partial hepatectomy regeneration [19]. It has also been shown that polyamines are essential for hepatic regeneration in that specific inhibition of their synthesis by chemical agents significantly impairs regenerative activity after partial hepatectomy [20,21]. Furthermore, polyamines improve the survival rate of rats after liver transplantation [22] and play an important role in growth factor induced DNA synthesis in cultured rat hepatocytes [23,24].

Putrescine converted of ornithine and catalyzed by ODC is thought as the most essential polyamine for liver regeneration [25]. Thus, substances or factors which stimulate hepatic putrescine production seem to promote regeneration of the liver remnant. This assumption is comparable with observations that hepatic putrescine content is increased in proportion to the extent of partial liver resection, with significant correlations with restitution of liver mass and hepatic DNA and protein synthesis [26]. ALR paralleled these observations and therefore we investigated the effect of recombinant human ALR on primary human hepatocytes in vitro with regard to polyamine synthesis. Here, we demonstrate that ALR induces hepatic polyamine levels presumably due to its ability to increase ODC mRNA expression via c-myc induction.

Materials and methods

Reagents. Human recombinant HGF was purchased from R&D Systems (Wiesbaden, Germany). Collagenase (type IV), Hepes, and other buffer supplements were purchased from Sigma (Taufkirchen, Germany). Collagen I coated plates (Biocoat) were purchased from BD (Bedford, UK) and fetal calf serum was from Biochrom (Berlin, Germany). Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose was obtained from Biowhittaker (Verviers, Belgium), and all other media additives were purchased from Serva (Heidelberg, Germany).

Hepatocyte preparation and culture. Tissue samples from human liver resection were obtained from patients undergoing partial hepatectomy for metastatic liver tumors of colorectal cancer. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation HTCR (Human Tissue and Cell Research), with the informed patient's consent [27] approved by the local Ethical Committee of the University of Regensburg. Human hepatocytes were isolated using a modified two-step EGTA/collagenase perfusion procedure and maintained in culture as described previously [15,28]. Cells were plated on collagen pre-coated 6-well plates at a density of 5.0×10^4 cells/cm^2 in appropriate volume of culture media. The medium consisted of DMEM with 5% fetal calf serum, 2 mM L-glutamine, and supplements as follows: 1.7 mU/ml insulin, 3.75 ng/ml hydrocortisone, 100 μg/ml streptomycin, and 100 U/ml penicillin. After 16 h of plating, medium was replaced by medium without serum. Cells were incubated at 37 °C in a humidified incubator with 5% CO_2 and media were changed daily except otherwise stated. Viability of hepatocytes during culture period was monitored by cell morphology (light microscopy, image analysis) and determination of enzyme release into culture medium (AST activity).

Preparation of recombinant human ALR and treatment of culture. Recombinant human ALR (rhALR) was prepared as described recently [10]. Fractions containing rhALR protein were combined and dialyzed against dialysis buffer (25 mM Hepes, 0.1% Tween 20, and 1 mM EDTA, pH 8.2) at 4 °C, with a threefold buffer change. Afterwards, rhALR protein was concentrated using a 5 kDa cut-off ultrafree-15 centrifugal filter device (Millipore GmbH, Schwalbach, Germany).

HGF and ALR were prepared as sterile solutions in culture medium and were added to cultures for the indicated times. Treatments of cells started 24 h after plating followed changing culture medium. Controls were not treated and not stimulated cells.

Determination of hepatocyte proliferation and polyamine metabolism. [^{3}H]thymidine incorporation into DNA was performed as described elsewhere [29]. Briefly, 24 h after plating either HGF or ALR was added to the cultures for the following 48 h. For the final 20 h of culture the medium was replaced by fresh medium supplemented with 8.3 μM (2 μCi/ml) methyl-[^{3}H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, GB). On termination of the incubation, the cells were washed with cold 0.9% NaCl, treated with 10% trichloroacetic acid for 15 min and with 5% trichloroacetic acid for 5 min at 4 °C. The acid precipitable radioactivity was extracted into 0.5 ml of 0.3 N NaOH containing 0.1% SDS and counted. Intracellular polyamine concentrations were quantified by a HPLC-method as described earlier [30].

Quantification of ODC, AdoMetDC, and c-myc mRNA expression by real-time PCR. Integrity of the RNA was verified by agarose gel electrophoresis and by visualization of ribosomal RNA by ethidium bromide staining. First strand cDNA was synthesized using 1 μg of total RNA and the avian myeloblastosis virus-reverse transcription reaction (Promega, Madison, USA). Transcript levels of human ODC, AdoMetDC, c-myc, and β-actin were quantified using the real-time RT-PCR technology (Lightcycler, Roche, Penzberg, Germany). The "QuantiTect Primer Assay" for ODC (QT00076468) and AdoMetDC (QT00494487) was obtained from Qiagen (Hilden, Germany). The sense and reverse primers, respectively, were as follows: c-myc forward: 5′-GCT CCT GGC AAA AGG TCA GAG TCT GG and c-myc reverse: 5′-GGG GCT GGT GCA TTT TCG GTT GTT GC; β-actin forward: 5′-GCC GGG ACC TGA CTG ACT AC and β-actin reverse: 5′-TGC GGA TGT CCA CGT CAC. For PCR 1–3 μl cDNA preparation, 2.4 μl of 25 mM $MgCl_2$, 0.5 μM of forward and reverse primers, and 2 μl of SybrGreen LightCycler Mix (Roche, Mannheim, Germany) in a total of 20 μl were applied. The following PCR programs were performed, for ODC, AdoMetDC, and β-actin: 10 min 95 °C (initial denaturation); 20 °C/s temperature transition rate up to 95 °C for 15 s, 10 s 58 °C, 22 s 72 °C, 10 s 82 °C acquisition mode single, repeated for 40 times (amplification), and for c-myc: 10 min 95 °C (initial denaturation); 20 °C/s temperature transition rate up to 95 °C for 10 s, 10 s 62 °C, 10 s 72 °C, 88 °C acquisition mode single, repeated for 40 times (amplification). $MgCl_2$ concentration and annealing temperature were optimized for each primer set. The PCR was evaluated by melting curve analysis following the manufacturer's instructions and checking the PCR products on 1.8% agarose gels. Each quantitative PCR was performed at least in duplicate for three sets of RNA preparations.

Statistical analysis. Results represented in figures are of representative experiments ($n \geqslant 2$ different donors) and were run in triplicate. Data are

expressed as means ± SD and the statistical analysis was performed by analysis of variance (ANOVA) and Fisher's LSD test. Differences were considered significant when $p < 0.05$.

Results

ALR enhances [^{3}H]thymidine incorporation in primary human hepatocytes

The potential of ALR to augment liver regeneration was analyzed by determination of DNA synthesis in primary human hepatocytes upon treatment with rhALR in vitro. To perform DNA synthesis induction experiments, recombinant human ALR was expressed and purified protein was verified by SDS gel electrophoresis demonstrating a monomeric and homodimeric structure under reducing and nonreducing conditions, respectively (Fig. 1A). Stimulation of primary hepatocytes with the hepatotrophic factors ALR (50 nM) or HGF (10 ng/ml) for 48 h increased significantly [^{3}H]thymidine incorporation compared to nontreated cells (Fig. 1B).

Time and dose dependence of ALR-induced polyamine synthesis in primary human hepatocytes

Intracellular polyamine levels of putrescine, spermidine, and spermine were investigated in human hepatocytes upon ALR treatment. Hepatocytes treated with 50 nM ALR demonstrated increased polyamine levels within 9–12 h after application (Fig. 2). Particularly, putrescine reached a maximum at 9 h with about 190% over nontreated cells, whereas spermidine and spermine showed moderate enhanced levels after 12 h of treatment. Polyamine levels at $t = 0$ h (control) and $t = 12$ h correspond to concentrations as follows: 198.1 ± 23.8 pmol/μg DNA and 315.7 ± 29.4 pmol/μg DNA for putrescine, 425.1 ± 21.3 pmol/μg DNA and 491.8 ± 41.4 pmol/μg DNA for spermidine, and 372.4 ± 29.8 pmol/μg DNA and 480.4 ± 48.1 pmol/μg DNA for spermine. Furthermore, dose dependence of ALR-induced polyamine synthesis was analyzed with increasing concentrations of ALR (0.5–500 nM) reaching a maximum increase of polyamines at 50 nM ALR after a 12 h treatment (Fig. 3).

Enhanced thymidine incorporation as well as activated polyamine synthesis upon ALR application indicates a proliferative response as seen for molecules activating liver regeneration.

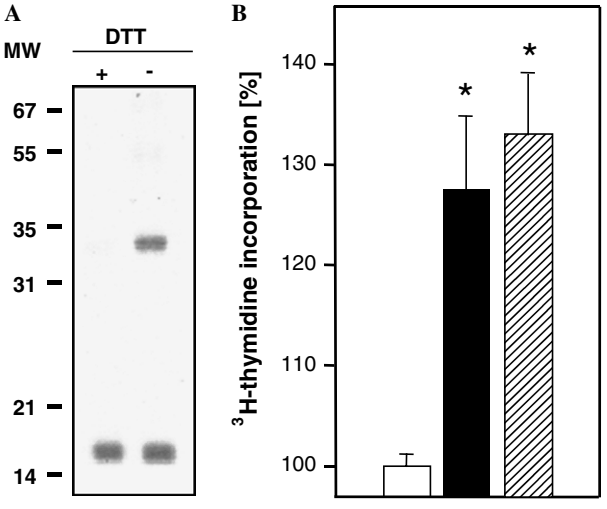

Fig. 1. Effect of recombinant human ALR on the proliferation rate of primary human hepatocytes in vitro. (A) Purified His-6-tagged recombinant human ALR (rhALR) was analyzed by SDS–PAGE under both reducing (+ dithioerythritol, DTT) and nonreducing conditions (− DTT) as a mixture of monomers and homodimers. (B) Human hepatocytes were cultured for 48 h with 50 nM ALR (black bar), 10 ng/ml HGF (hatched bar) or without supplements (control, open bar). [^{3}H]thymidine incorporation was measured within the last 20 h of culture. *$p < 0.05$, differs from control (three independent experiments in triplicate, ±SD).

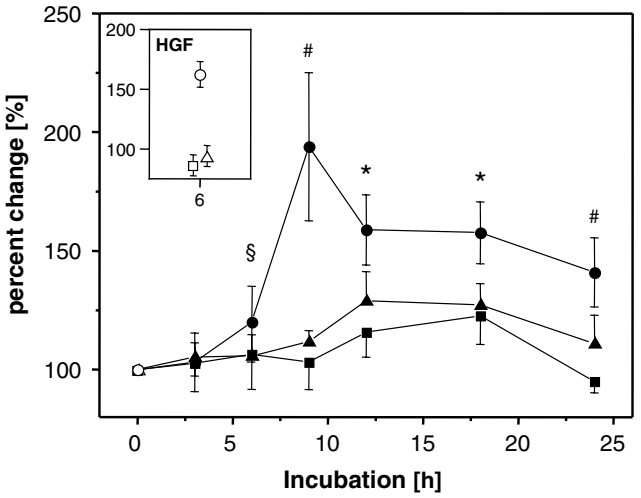

Fig. 2. Polyamine levels in primary human hepatocytes after treatment with ALR. After plating, cells were maintained for 24 h under serum free conditions following incubation with either 10 ng/ml HGF (see inset) or 50 nM ALR. Intracellular levels of polyamines (●, putrescine, ■, spermidine, and ▲, spermine) were determined and expressed as percentage compared to noninduced cells at the indicated times. $^{\S}p < 0.05$ putrescine level, $^{\#}p < 0.05$, putrescine, spermine levels. *$p < 0.05$, putrescine, spermine, and spermidine levels differ from control at 0 h incubation; (three independent experiments in triplicate, ±SD).

Intracellular polyamine levels of putrescine, spermidine, and spermine were investigated in human hepatocytes upon ALR treatment. Hepatocytes treated with 50 nM ALR demonstrated increased polyamine levels within 9–12 h after application (Fig. 2). Particularly, putrescine reached a maximum at 9 h with about 190% over nontreated cells, whereas spermidine and spermine showed moderate enhanced levels after 12 h of treatment. Polyamine levels at $t = 0$ h (control) and $t = 12$ h correspond to concentrations as follows: 198.1 ± 23.8 pmol/μg DNA and 315.7 ± 29.4 pmol/μg DNA for putrescine, 425.1 ± 21.3 pmol/μg DNA and 491.8 ± 41.4 pmol/μg DNA for spermidine, and 372.4 ± 29.8 pmol/μg DNA and 480.4 ± 48.1 pmol/μg DNA for spermine. Furthermore, dose dependence of ALR-induced polyamine synthesis was analyzed with increasing concentrations of ALR (0.5–500 nM) reaching a maximum increase of polyamines at 50 nM ALR after a 12 h treatment (Fig. 3).

Enhanced thymidine incorporation as well as activated polyamine synthesis upon ALR application indicates a proliferative response as seen for molecules activating liver regeneration.

ALR enhances mRNA expression of key enzymes of polyamine synthesis

Polyamine synthesis is regulated by key enzymes of the polyamine metabolism such as ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC). Therefore, we investigated the effect of ALR on ODC and AdoMetDC expression in human hepatocytes by quantitative RT-PCR. Treatment with ALR caused increased mRNA expression of ODC (Fig. 4A) and AdoMetDC (Fig. 4B) reaching the highest levels after 8 h compared to controls. Induction of mRNA expression showed a similar curve as for the hepatic mitogen HGF, but not at the same intensity. Polyamines as a result of active ODC (putrescine) and AdoMetDC (spermidine, spermine) were

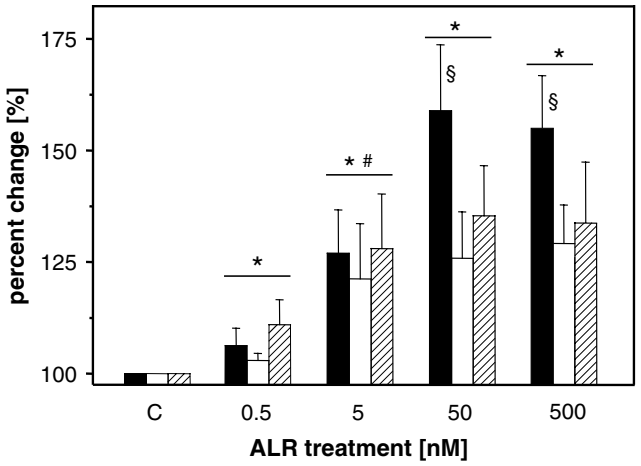

Fig. 3. Dose dependent polyamine synthesis in human hepatocytes upon ALR treatment. Cells were plated, kept for 24 h under serum free conditions followed by an incubation of 12 h with increasing concentrations of ALR (0.5–500 nM). Polyamine levels of putrescine (black bar), spermidine (open bar), and spermine (hatched bar) were expressed as percentage compared to noninduced (C, control) cells. $^{*}p < 0.05$, putrescine, spermine, and spermidine levels differ from control; $^{\#}p < 0.05$, putrescine, spermidine, and spermine levels differ from control and 0.5 nM ALR treatment; $^{\S}p < 0.05$ putrescine level differs from 5.0 nM ALR treatment; (three independent experiments in triplicate, ±SD).

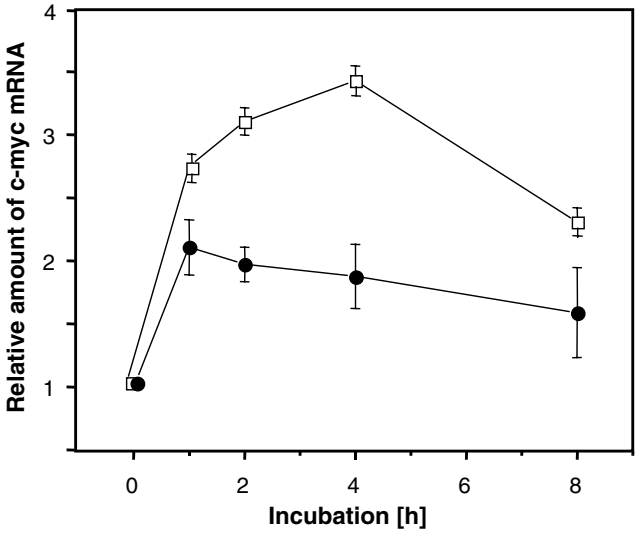

Fig. 5. Expression of transcription factor c-myc in human hepatocytes after hepatotrophic factor treatment. After plating, hepatocytes were kept for 24 h under serum free conditions followed by an incubation with either HGF (10 ng/ml, □) or ALR (50 nM, ●). Expression of c-myc mRNA levels at the indicated time points was determined by quantitative RT-PCR analysis (two independent experiments in triplicate, ±SD).

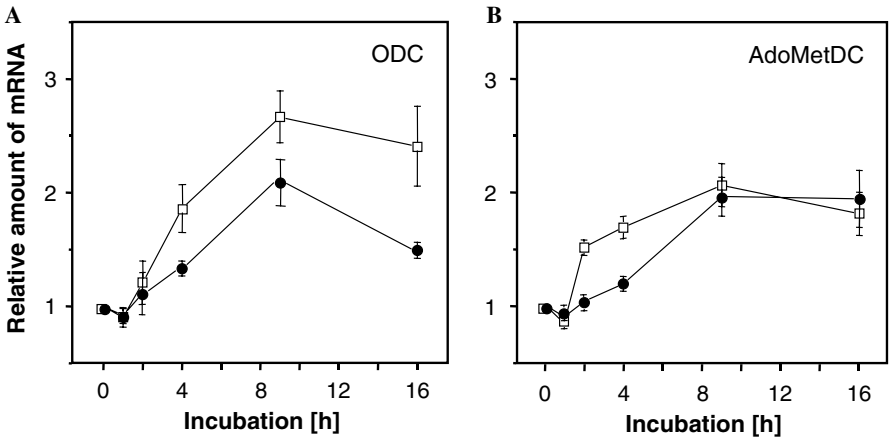

Fig. 4. Expression of key enzymes of polyamine metabolism in human hepatocytes after hepatotrophic factor treatment. After plating, hepatocytes were kept for 24 h under serum free conditions followed by an incubation with either HGF (10 ng/ml, □) or ALR (50 nM, ●). At the indicated time points, cells were harvested and used for total RNA extraction to perform quantitative RT-PCR analysis for ODC (A) and AdoMetDC (B) mRNA expression (three independent experiments in triplicate, ±SD).

enhanced and increased mRNA levels might partly explain these increased concentrations.

ALR enhances mRNA levels of transcription factor c-myc

To further elucidate the regulation of polyamine synthesis in human hepatocytes after ALR application analysis of c-myc expression, an immediate early gene, was performed (Fig. 5). Levels of c-myc mRNA strongly increased within 1 h after ALR treatment and kept significantly elevated compared to control. Growth factor treatment with HGF induced c-myc even more intense with a steep climb after 1 h and reaching a maximum at 4 h. HGF was shown to induce ODC mRNA by regulating c-myc and therefore enhanced c-myc mRNA expression after ALR treatment might be involved in ODC induction and elevated polyamine levels in human hepatocytes.

Discussion

The aim of this study was to elucidate the effects of ALR on the polyamine levels of primary human hepatocytes in vitro and which molecular mechanisms are involved in the regulation of polyamine metabolism upon stimulation with the hepatotrophic factor ALR.

The study is based on the findings that hepatocytes in a normal healthy liver are in a quiescent state, but get easily activated (''primed'') to enter the cell cycle for proliferation after tissue loss e.g., hepatectomy [3]. Further, it was shown that by performing cell isolation procedures primary human hepatocytes get into an activated status and cells leave the G_0 phase during culture to enter the cell cycle. This ''priming'' step ends at the so-called restriction point in the G_1 phase rendering the hepatocytes competent to respond to mitotic factors and crossing from G_1 to S phase only occurs in the presence of growth factors like HGF and EGF [1,2,31]. It became evident that an increase in DNA synthesis of hepatocytes in vitro upon growth factor treatment is dependent on culture time (lag period after cell plating) and duration of application [31,32]. Additionally, activating proliferative pathways led to modulation of hepatic metabolism suggesting a cross-linking of these pathways as shown for HGF [32] and EGF [31]. We have shown recently that reduction of cytochrome P450 expression by ALR is most effective for a 24–72 h treatment [15] and this is in agreement with a maximum in DNA synthesis in this study (data not shown). Using a human in vitro model, we could confirm the proliferation stimulatory effect of recombinant ALR on primary hepatocytes demonstrated by a thymidine incorporation assay as reported recently [13]. ALR acts in coincidence with action of growth factors in the priming period and, therefore, it might be concluded that ALR activates specific pathways for proliferation and hepatic gene regulation.

Second, we found that ALR treatment enhanced dose dependent intracellular concentrations of polyamines, mainly putrescine, indicating an activation of hepatic polyamine metabolism. Polyamines are thought as a prerequisite for proliferation and differentiation of a variety of cells and it was further shown that polyamine metabolism plays an essential role in hepatic regeneration [33]. An association with liver regeneration was particularly not only shown for putrescine [23,25] but also for spermidine and spermine [18,24]. A depletion of cellular polyamines in the liver either by chemical agents or by transgenic rats was shown to be responsible for an impairment to initiate liver regeneration, but this was reversible by exogenous substitution [18,25]. Among several factors, which are able to stimulate polyamine metabolism, HGF and EGF were found responsible for increased putrescine levels under circumstances of hepatic regeneration in vivo as well as in vitro [25]. ALR, another member of hepatotrophic factors, is highly expressed during liver regeneration [7] and was shown to have beneficial effects on regeneration as well as in liver diseases [6,12]. Additionally, we found that ALR treatment enhanced hepatic polyamine levels in vitro and therefore, this might at least in part represent a cellular mechanism by which ALR exerts its proliferation augmenting effect during liver regeneration.

ODC is a key enzyme of polyamine metabolism and its regulation within the cell can result from complex and variable effects on gene transcription, stability and translation of mRNA, and stability and modification of enzyme. In many growth related systems enhanced ODC activity upon activation correlates well with increases in the level of ODC mRNA [34]. Therefore, the demonstrated increase of ODC as well as AdoMetDC mRNA upon ALR treatment might be responsible for enhanced polyamine levels rather than a decreased catabolism. Further, it could be shown that growth factor treatment of human hepatocytes in vitro produced cell cycle associated proteins like cyclin A and B [1,2] as well as increasing expression of immediate early genes such as c-myc [2]. Additionally, it was demonstrated that HGF induced c-myc and ODC mRNA in hepatoma cells [35]. ODC, a delayed-early gene involved in control of the cell cycle, is considered to be a target gene of Myc/Max transcription factor [36] and therefore we assume a role of ALR in regulating ODC mRNA by inducing c-myc expression. Another possible explanation for ODC regulation comes from a report showing ODC as a target gene of NFκB in HGF treated tumor cells [37]. Further, there is some evidence for a cooperate action of NFκB and ERK (extracellular signal regulated kinase) leading to induction of ODC [38]. On the other hand, we have recently shown that ALR application caused a significant NFκB activation in human hepatocytes and reached almost 40% of TNF-α induction [15]. Additionally, it was reported that ALR upon binding to its own specific receptor [5] acts through stimulation of the tyrosine phosphorylation of the epidermal growth factor receptor and activates the MAPK cascade indicated by phosphorylated MEK (MAPK kinase) and ERK [13]. We, therefore, may speculate that ALR can directly regulate ODC induction via ERK/NFκB activation as well as via c-myc, which is also known to be controlled by NFκB [39].

Polyamines are not only important for physiological growth but also exhibit a protective effect against acute liver injury caused by hepatotoxins [40], which have also been shown for HSS, a factor similar to ALR [11]. On the other hand polyamines are known to contribute to carcinogenesis and tumor progression by increased levels due to the overexpression of ODC [41] and further to be correlated with the degree of malignancy in hepatocellular carcinomas [42]. Also, increased ALR levels were detected for various types of acute liver disease [9], and in addition, we found increased expression of ALR in livers from patients with cirrhosis, hepatocellular, and cholangiocellular carcinoma [10]. Future studies have to be performed investigating a putative role of ALR in hepatocellular carcinoma by regulating polyamine metabolism.

In conclusion, we showed that recombinant ALR can moderately induce cell proliferation and significantly enhance intracellular polyamine levels of human hepatocytes in vitro and therefore the liver proliferation augmenting effect of ALR, observed in vitro as well as in vivo, could be mediated by an activated polyamine metabolism regulated by increased c-myc as well as ODC and AdoMetDC mRNA expression.

Acknowledgments

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