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A Fresh Look at Augmenter of Liver Regeneration In Rats

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Abstract

Augmenter of liver regeneration (ALR) is a hepatotrophic protein originally identified by bioassay in regenerating rat and canine livers following partial hepatectomy and in the hyperplastic livers of weanling rats, but not in testing adult livers. The ALR gene and gene product were subsequently described, but little is known about the cellular/subcellular sites of ALR synthesis in the liver, or about the release and dissemination of the peptide. To obtain this information in rats, we raised antibodies in rabbits against rat ALR for development of an enzyme-linked immunosorbent assay (ELISA). ALR concentrations were then determined in intact livers of unaltered weanling and adult rats; in regenerating residual liver after partial hepatectomy; in cultured hepatocytes and nonparenchymal cells (NPCs); and in culture medium and serum. ALR in the various liver cells was localized with immunohistochemistry. In addition, hepatic ALR and ALR mRNA were assayed with Western blotting and reverse-transcriptase polymerase chain reaction (RT-PCR), respectively. The hepatocyte was the predominant liver cell in which ALR was synthesized and stored; the cultured hepatocytes secreted ALR into the medium in a time-dependent fashion. Contrary to previous belief, the ALR peptide and ALR mRNA were present in comparable concentrations in the hepatocytes of both weanling and resting adult livers, as well as in cultured hepatocytes. A further unexpected finding was that hepatic ALR levels decreased for 12 hours after 70% hepatectomy in adult rats and then rose with no corresponding change in mRNA transcripts. In the meantime, circulating (serum) ALR levels increased up to 12 hours and declined thereafter. Thus, ALR appears to be constitutively expressed in hepatocytes in an inactive form, and released from the cells in an active form by unknown means in response to partial hepatectomy and under other circumstances of liver maturation (as in weanling rats) or regeneration.

The control of hepatic growth and regeneration has interested experimentalists for much of the 20th century.[1] Soon after the classical description in 1931 by Higgins and Anderson[2] of liver regeneration in rats following 70% hepatectomy, a search began for growth factors within the liver itself. McJunkin and Breuhaus3 observed that the modest mitotic response to a 30% to 40% hepatectomy in rats was enhanced with an intraperitoneal injection 2 days postoperatively of homogenized homologous rat liver. Two decades later, Teir and Ravanti4 and Bioniquist5 noted that this “augmentation” effect was demonstrable only when the injected homogenates were prepared from regenerating liver fragments following hepatectomy or from weanling rat livers that have a naturally heightened mitotic index. Subsequently, LaBrecque and Pesch[6] reported the same prerequisite of a hyperplastic liver source for cytosol extracts containing a putative “hepatic stimulatory substance” (HSS).

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Importantly, however, a cocondition for demonstrating a mitosis-augmenting activity of cytosolic HSS[6] was its injection into test rats whose livers already were primed, *i.e.*, committed to an increased mitotic response induced by partial hepatectomy. Consequently, LaBrecque and Pesch standardized the minimum (40%) hepatectomy assay for HSS, a modification of which has been used to study HSS in dogs.[7] The assay also has been used increasingly to study other hepatic growth factors whose *in vivo* role in regeneration has been largely extrapolated from results with in vitro models.[8–12] The principal limitation of this assay is the variability of the mitotic response to the priming hepatectomy, and the additional variability of the mitosis augmentation.[7,8]

The far more sensitive canine Eck fistula assay that ultimately guided the steps in purification of HSS8 also is based on the priming principle, because portacaval shunt causes a tripling of hepatic cell renewal.13–15 In essence, this assay consists of performing a completely diverting portacaval shunt in dogs, and then infusing test substances into one of the detached main portal vein branches while simply ligating the other main branch, and then comparing the infused liver lobes With the noninfused (control) lobes. In 1975, it was demonstrated that a nonhypoglycemic infusion of insulin prevented the characteristic hepatocyte atrophy and organelle disruption caused by the portal diversion. In addition, the already-heightened rate of hepatocyte mitosis was quadrupled. [14,]15 Combined with previous evidence from a variety of experimental models, 16–22 it now had been established that portal venous blood contained factors, dominated by but not limited to insulin, that were essential for the maintenance of normal liver size, function, and the capacity for regeneration.

The spectacular augmentation of the mitotic response caused by insulin in the Eck fistula model[14,15] was consistent with earlier observations of Younger, King, and Steiner[23] in rats that were allowed to be alloxan-diabetic for 1 month before treating them with insulin. The livers of the diabetic rats already contained an abnormally high number of hepatocytes, but as in the hyperplastic Eck fistula livers, the proliferative response to insulin was as great as that following a 40% to 50% hepatectomy The insulin effects were so overwhelming that despite circumstantial evidence of weaker but cumulatively important additional splanchnic growth factors,[15,20–22] no other growth factors exclusively secreted by visceral organs have been identified.

The hepatotrophic effects of infused insulin were interpreted at first in terms of relief of the relative insulinoprival state caused by the portal diversion. This view was revised in 1979 when it was shown with the Eck fistula assay that HSS had hepatotrophic effects indistinguishable from insulin.[24] Because HSS activity was not species-restricted,[8,]25 and was so precisely measurable, increasingly purified rat HSS could be identified with certainty with a single canine Eck fistula experiment.8,25–27 Eventually, the peptide purified from HSS was renamed augmenter of liver regeneration (ALR)[8] and cloned.28,29 The cDNA for rat ALR encodes a protein containing 198 amino acid residues and has a molecular weight of about 22 kd.29,[30] Cloned mouse and human ALR genes[30] that have highly conserved nucleotide and predicted amino acid sequences have been mapped to allele-rich regions of mouse chromosome 17 and human chromosome 16.[30]

Meanwhile, the six additional molecules annotated in Table 1[6–9,12,]14,15,23–45 also had been shown to have priming-dependent hepatotrophic qualities comparable with insulin and ALR. Unlike insulin and ALR, two of these molecules (hepatocyte growth factor [HGF] and transforming growth factor [TGF-α]) initiate mitosis of cultured hepatocytes. Like an the others, however, they are only feebly mitogenic when injected into unaltered animals[9,]12 The small number of molecules with genuine hepatotrophic qualities (Table 1) as established by the criteria of the Eck fistula, minimum hepatectomy, or both assays, contrasts with the large

number of physical and chemical factors that can initiate mitosis of hepatocytes *in vitro* or *in vivo*.[10,11,46–49]

Epidermal growth factor does not fulfill hepatotrophic criteria even though it is mitogenic for cultured hepatocytes, has a 30% homology with the hepatotrophic TGF-α, and has the same receptor.[10,11] Moreover, the historical contention that glucagon augments the growth effects of insulin and is, therefore, itself hepatotrophic[50,51] is not consistent with the results of either the Eck fistula [14,15,32] or minimum hepatectomy assay.[9] The degree of congruence of the two assays also is evident by their parallel identification of the only two molecules known to have highly specific antihepatotrophic effects: TGF-β[32,36,52–54] and rapamycin (Table 1).[55]

The essentially identical hepatotrophic properties of the eight disparate molecules shown in Table 1 has suggested the possibility that one of them could be an initiator of, target of, or interacter with all the others. ALR has been a candidate for such a keystone role, in part because it is heavily expressed in the liver, more so than in any other tissue or organ, excepting only the testis.[28,30] Unlike the other hepatotrophic and antihepatotrophic agents, however, the paucity of information about ALR under normal or altered conditions, including even the location of its synthesis in the liver or its kinetics, has hindered evaluation of its physiological Significance.

Consequently, we have determined the localization and concentration of ALR and of ALR mRNA in adult, weanling, and posthepatectomy livers and of hepatocytes in culture, as well as the concentration of circulating ALR following partial hepatectomy. The unexpected results reported here mandate revision of previous assumptions about the metabolism of ALR and its potential role in the homeostasis of the liver or other tissues.

MATERIALS AND METHODS

The protocols of this investigation were approved by the IACUC, University of Pittsburgh, and the Veterans Administration Medical Center in accordance with the NIH guidelines.

Preparation of Recombinant Rat ALR

Rat ALR cDNA–transfected *Escherichia coli* were grown in a medium containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 100 μg/mL ampicillin (pH 7.2) by overnight incubation in an agitator at 37°C. The cell were washed, suspended in 10 mmol/L sodium phosphate (pH 8.0) containing 0.15 mol/L sodium chloride, and digested with (0.5 mg/mL) lysozyme (crystallized from chicken egg white; Sigma Chemical Co., St. Louis, MO) for 30 minutes in room temperature. The digested material was sonicated for 20 minutes in an ice bath (15-second bursts with 15-second intervals), centrifuged (12,500g for 20 minutes at 4°C), and the supernatant mixed with sodium acetate (final concentration, 100 mmol/L). ALR was then extracted by sequential acid treatment and ethanol precipitation.[8] For purification of ALR, the ethanol pellet was dissolved in 25 mmol/L sodium asetate (pH 4.65) containing 50 mmol/L NaCl. The solution was concentrated using a YCO5 Amicon Diaflo membrane in an ultrafiltration cell (Amicon Corp., Danvers, MA) at 4°C under nitrogen, filtered through a 0.2-μm cellulose acetate membrane (Gelman Corp., Ann Arbor, MI), and loaded on a SP 26/10 cation exchange column in an automated fast-protein liquid chromatography system (Pharmacia Biotechnology Corp., Piscataway, NJ). A linear gradient of 0 to 1,000 mmol/L NaCl was applied at a flow rate of 4 mL/min. As determined by enzyme-linked immunosorbent assay (ELISA), fractions eluting between 600 and 800 mmol/L NaCl contained ALR, which were combined and dialyzed twice against 50 mmol/L NaCl in 20 mmol/L Tris (pH 7.4) using a 1,000-mw cut-off SpectraPor6 dialysis tubing (Spectrum Industries, Houston, TX). For further purification by FPLC; the concentrated material, was loaded on a FF-Q column, and a linear gradient of 0 to 1,000 mmol/L NaCl was applied at a flow rate of 4 mL/min. Fractions

containing ALR (material eluting between 250 and 325 mmol/L NaCl) were combined, concentrated by YCO5 ultrafiltration, reconstituted in 20 mmol/L Tris-HCl (pH 7.4) containing 50 mmol/L NaCl at 1 mg of protein per milliliter, and stored in aliquots at −70°C.

Preparation of Antibodies for ALR

Standard methodology was employed for the development of antibodies against rat recombinant ALR (rrALR) and its peptide fragments (Covance, Inc., Denver, PA). Briefly, white New Zealand female rabbits (approximately 3 kg) were prebled and immunized with intradermal injections of the adjuvant. Three weeks after the initial immunization and every 3 weeks thereafter, mixtures of the conjugates of keyhole limpet hemocyanin with rrALR (250 μg) or its peptide fragments (250 μg) in phosphate-buffered saline (PBS) were injected subcutaneously at multiple sites. Ten to 12 days after each immunization, approximately 20 mL blood was drawn, and serum was separated by centrifugation.

For isolation of the IgG fraction, the serum was diluted with 10 volumes of 10 mmol/L sodium borate (pH 8.0) and loaded on a Protein A column (Pharmacia Biotechnology) equilibrated in the same buffer. The column was washed until the absorbance of the eluted fractions at 280 nm was similar to that of the borate buffer, and the IgG was eluted with Pierce gentle elution buffer. (Pierce Chemical Co. Rockford, IL). Fractions with absorbance greater than 0.05 as compared With the elution buffer were pooled arid dialyzed against two changes of Tris-buffered saline buffer in a 1,000-mw cut-off SpectraPor6 dialysis tubing (Spectrum Industries).

Western Analysis of ALR in Hepatic Extracts and Recombinant Protein

Native ALR was extracted from the liver and hepatocytes by homogenization in 100 mmol/L sodium acetate (pH 4.6).[8] After centrifugation, pH of the supernatant was neutralized, and solution containing 200 μg protein (Lowry's procedure) was loaded on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel After electrophoresis, the separated proteins were transferred to a Immobilon-P transfer paper (Millipore, Bedford, MA), and nonspecific sites were blocked with 1% nonfat milk in PBS (blocking buffer). The paper was washed in PBS and incubated with anti-rrALR antibody (2 μg/mL) in blocking buffer, followed by washing and incubation with donkey anti-rabbit horseradish peroxidase conjugate (Amersham, Arlington Heights, IL). The ALR-anti-ALR complex was detected using an ECL kit (Amersham) and chemoluminescence on Fuji X-ray film.

ALR ELISA

Immulon-1 flat-bottom 96-well ELISA plates (Dynatech Labs, Chantilly, VA) were coated with anti-rrALR antibody (1 μg/well) in 50 μL sample dilution buffer (0.1 mol/L Na_2Co_3 [pH 9.0]) by overnight incubation at 4°C. The medium was removed and the plates washed (4×) With the wash buffer (20 mmol/L Tris [pH 7.5] containing 0.5 mol/L NaCl and 0.05% Tween 20) After blocking the nonspecific sites with "Super Block" (Pierce Chemical Co.), the plates were washed (4×) with the wash buffer. Sample or standards dissolved in sample dilution buffer (20 mmol/L Tris [pH 7.5] containing 0.5 mol/L NaCl, 0.05% Tween 20) and 1% "Sea Block" (Pierce Chemical Co.) were added to the wells, and the plates incubated at 4°C overnight. The plates were washed (6×), incubated with biotinylated anti-rrALR antibody (prepared using a kit from Vector Laboratories, Burlingame, CA) (0.2 μg/well in 100 μL sample dilution buffer) for 1 hour at room temperature, and washed (6×). The plates were incubated with avidin alkaline phosphatase (Sigma Chemical Co.) (100 μL/well of 1:1,000; vol/vol in sample dilution buffer) for 30 minute at room temperature. After washing (8×), incubation was performed in the dark With alkaline phosphatase substrate (Sigma Chemical Co.) (1 mg/mL in 0.1 mol/L $NaHCO_3$ containing 1 mmol/L $MgCl_2$; 100 mL/well), and the developed color was read in a microplate spectrophotometer at 405 nm.

A linear relationship was observed between ALR concentrations of 3 to 500 pg and corresponding absorbance of the developed color (Fig. 1A). The selectivity of the assay for ALR was demonstrated by the lack of reactivity at concentrations in the micromolar range of several other proteins including albumin, glucagons, insulin, angiotensin, insulin-like growth factor-II, and HGF (results not shown).

Determination of ALR in Serum and Liver Tissue After Partied Hepatectomy

Partial hepatectomy (40% or 70%) was performed in rats (male Lewis 200–250 g; Harlan Sprague-Dawley, Indianapolis, IN) as described previously.2,26 At indicated times after the surgery, blood was drawn via the abdominal aorta, and serum separated by centrifugation. The liver was excised and snap-frozen in liquid nitrogen. Livers and blood from sham-operated rats were used as control. Serum was diluted with the sample dilution buffer (1:1, vol/vol), and 100-μL aliquots were used for the ELISA. The liver (1 g) was homogenized in ice-cold 100 mmol/L sodium acetate (pH 4.65), and the homogenate was centrifuged at 20,190g for 1 hour at 4°C after dilution to 50 mL. The supernatant was further diluted with sample dilution buffer (1:16; vol/vol), and 100-μL aliquots were used in the ELISA.

Preparation of Hepatocytes

Hepatocytes were prepared from male Sprague-Dawley rats (180–250g; Zivic-Miller, Zelienople, PA) by collagenase digestion of the liver as described previously.56 The Viability of the cells was determined by Trypan blue exclusion, and preparations with viability greater than 85% or higher were used. The cells were suspended in Williams' medium E containing 10% feral calf serum, 2 mmol/L L-glutamine, and 7 mg/L insulin (Eli Lilly Co., Indianapolis, IN) at 0.5×10^6 per milliliter. Aliquots of the cell suspension (2 mL/well) were placed in 6-well plates. The medium was renewed after 3 hours, and cells were used the following day.

Preparation of Nonparenchymal Cells

Nonparenchymal cells (NPCs) were prepared by collagenase and protease digestion of the liver, and cultured as described previously.57–59 Stellate cells were separated from the other NPCs by centrifugation on a Nycodenz gradient.58 Kupffer cells and endothelial cells were separated on a metrizamide gradient, followed by centrifugal elutriation.57 Freshly isolated cells were assessed for Viability (which was always greater than 95%) with Trypan blue, and were used as such for extraction and analysis of ALR by ELISA. The purity of stellate cells was determine by vitamin A autofluorescence and immunolabeling for desmin.58 Antibodies to intracellular adhesion molecule-1 (clone 1A29; PharMingen, San Diego, CA)60 and ED-1 (clone IC7; PharMingen)61 were used to determine the purity of endothelial cells and Kupffer cells, respectively. By these procedures, the individual NPC preparations were found to be more than 95% pure.

Determination of ALR in Freshly Isolated and Cultured Cells

Freshly isolated hepatocytes, stellate tells, Kupffer cells, and endothelial cells were homogenized in 100 mmol/L sodium acetate (pH 4.65). After centrifugation at 13,000g for 1 hour at 4°C, the supernatant was used for determination of ALR by ELISA.

After an overnight incubation at 37°C in a humidified atmosphere containing 5% CO_2/95% air, cultured hepatocytes, stellate cells, Kupffer cells, and endothelial cells Were washed, treated with trypsin (0.05% in Hanks' balanced salt solution without Mg^{2+} and Ca^{2+} and containing 0.5 mmol/L ethylenediaminetetraacetic acid; GIBCO BRL, Gaithersburg, MD), and suspended in ice-cold PBS. Cells were counted and the suspension was centrifuged at 1,000g for 10 minutes at 4°C ALR from the cell pellet was extracted with 100 mmol/L sodium acetate (pH 4.65) and determined by ELISA.

Immunohistochemical Analysis of ALR in the Liver and Cultured Hepatocytes

A silce of the left lateral lobe of the liver was embedded in O.C.T. compound (Miles, Inc., Elkhart, IN) and frozen in liquid nitrogen. Sections (4-μm thickness) were cut, mounted on charged slides (Fisher Scientific, Pittsburgh, PA), and air-dried overnight at room temperature. Before immunostaining, sections were fixed in ice-cold acetone for 10 minutes and air-dried. Hepatocytes were cultured in chamber slides (Nalge Nunc International Corp., Napeville, IL), and after overnight incubation, they were washed twice with PBS and fixed in absolute ethanol for 10 minutes at room temperature. Immunostaining of both liver sections and cultured hepatocytes was performed as described previously[62] using anti-rrALR antibody. Rabbit IgG was used as an isotype control.

Determination of ALR mRNA by Reverse-Transcriptase Polymerase Chain Reaction

The relative levels of the ALR and α-actin cDNAs were assessed by semiquantitative reverse-transcriptase polymerase chain reaction CRT-PCR) as described by Dallman et al.[63] This technique was selected because of its ease and reproducibility in quantifying mRNA as compared with other methods such as Northern blotting, primer extension, and nuclease protection assays.[63,64] The level of β-actin mRNA was measured to determine the efficiency of cDNA synthesis and reverse transcription of different batches of mRNA. The PCR reactions were performed for 15, 20, 25, 30, and 35 cycles to quantify the product in the linear phase. The PCR primers specific for the rat ALR cDRNA were 5′ CGGACCCAGCAGAAGCGGGAC3′ and 5′TTAGTCACAGGAGCCGTCCTT3′,[28,30] and for β-actin cDNA, they were 5′TTCTACAATGAGCTGCGTGTG3′ and 5′ TTCATGGATGCCACAGGATTC3′. Details of the procedure are described elsewhere.[58]

[^{125}I] ALR Binding to Cultured Hepatoeytes

rrALR was radioiodinated by a lactoperoxidase procedure,65 and its purity was determined by fast-protein liquid chromatography (see above) and SDS–polyacryl-amide gel electrophoresis, By both procedures, radiolabeled rrALR was found to migrate as a single band. Specific activities of radioiodinated ALR preparations were between 750 and 900 Ci/mmol. For the binding assay, the cells were washed three times with Hanks' balanced salt solution containing 10 mmol/L HEPES (pH 7.4) and 0 1 % bovine serum albumin, and placed in this medium containing 0.3 mg/mL bacitracin (a blocker of internalization of pep tides and proteins66) and 20 pmol/L [^{125}I] rrALR + molar excess (100 nmol/L) of unlabeled rrALR. The reaction was terminated after a 3-hour incubation at room temperature by aspirating the medium, followed by four washes with Hanks' balanced salt solution/bovine serum albumin. Cells were digested with 0.75 N NaOH, and radioactivity in the digests was determined in a gamma-counter. Specific binding of [^{125}I]ALR was calculated as the difference between cell-associated radioactivity in the presence and absence of unlabeled ALR.

RESULTS

Serum and Hepatic Levels of ALR (With ELISA)

Unaltered Animals—Serum samples from unaltered adult rats contained readily measurable amounts of natural ALR (Fig. 1B). When the samples were spiked with predetermined concentrations of ALR, the intensity of the developed color increased proportionately (Fig. 1B), indicating the reliability and the sensitivity of the assay.

Because the finding of ALR in the control serum (see Fig. 1B, and time "0" in Fig. 2) and liver (Fig. 2) of adult rats was inconsistent with previous assumptions (based on bioassay) that ALR was present in weanling but not in adult livers, we compared the concentration of ALR in the

two age-defined categories. With the ELISA determinations, the ALR concentrations in the livers and sera were essentially the same in both (Table 2).

After 70% Hepatectomy—Serum ALR concentration rose steadily for 12 hours, and declined in. the ensuing 12 hours to near-basal concentrations (Fig. 2A). Serial ALR concentration in the residual liver fragment showed an inverse pattern. ALR declined for the first 12 hours following hepatectomy, and then gradually tended to return to the near-basal level by 40 hours (Fig. 2B).

After 40% Hepatectomy—In contrast, hepatic ALR levels did not change significantly after 40% hepatectomy (Fig. 2B), and the elevations of serum levels of ALR were smaller than after 70% hepatectomy. The peak of the rise occurred at 6 hours, and the concentration returned to basal levels at 12 hours (Fig. 2A).

Determination ALR by Western Analysis

In Extracts of Whole Liver—The results were congruent with the findings with ELISA (see above) in that the levels of the three immunoreactive proteins with approximate molecular weights of 36, 38, and 40 kd were very similar in the extracts from the weanling and adult livers (Fig. 3). Considering the multiple protein species reacting with the ALR antibodies (Fig. 3), it appears that ALR may exist as a dimer or even a multimer, as suggested previously.[28, 30] It should be noted that the 36-, 38-, and 40-kd proteins also exhibited immunoreactivity for the antibody developed against the C-terminus sequence of rrALR (CSRVDERWRDGWKDGS) and against MRTQQKRDIKFRED (amino acid residues 74–87) (results not shown).

In Extracts of Cell Populations—To determine which cell type(s) contained ALR, Western analysis was performed using protein extracts from freshly isolated hepatocytes and NPCs. All three anti-rrALR antibody–positive proteins found in the extracts of whole liver were also present in the extracts prepared from freshly isolated hepatocytes (Fig. 3); none were found in the equivalent amounts of extracts prepared from the NPCs (results not shown). Importantly, concentrations of the proteins reacting with the anti-rrALR antibody were higher in extracts prepared from isolated hepatocytes than in extracts prepared from the whole liver. The possibility that the lower concentrations of ALR in the whole liver together with the extracts from isolated hepatocytes resulted in part from dilution by extraparenchymal proteins was confirmed by the studies of ALR in intact livers (see below).

Immunohistochemical Determination of ALR

In Intact Livers—In sections of both the weanling and adult livers, the major immunoreactivity for ALR detected with the anti-rrALR was in the cytosol of parenchymal cells (Fig 4A and 4B) NPCs in the sections of liver tissue also were found to immunostain with anti-rrALR antibody, but variably and to a lesser degree (Fig. 4B, inset). The immunostaining of the NPCs was not an artifact of the technique, because no labeling was apparent when isotype serum was used (Fig. 4A, inset).

In Isolated Cell Populations—ALR was a prominent constituent of the cytosol of cultured hepatocytes (Fig. 4C and 4D) Although freshly isolated stellate cells, Kupffer cells, and hepatic endothelial cells (in that order of frequency) also were found to contain ALR, the concentrations were much lower than those detected in hepatocytes (Table 3). Furthermore, on day 2 of culture, ALR could not be detected in any NPCs (Table 3).

To confirm that hepatocytes are capable of secreting ALR, we determined with ELISA the levels of the protein in the culture medium of hepatocytes at various times up to 24 hours. As

shown in Fig. 5, hepatocytes were found to secrete ALR into the medium in a time-dependent manner. At the end of the experiment, cellular damage was assessed by Trypan blue staining and the release of lactate dehydrogenase in the medium. More than 95% of cells excluded the stain, and the release of lactate dehydrogenase was not Significant during the entire time course, indicating that the accumulation of ALR in the medium was not caused by its leakage from the cells.

Expression of ALR mRNA in the Liver and Hepatocytes

The foregoing results suggested that hepatocytes are the primary sites of ALR synthesis in whole weanling and resting adult livers, as well as in cultured hepatocytes. To confirm this, ALR mRNA was determined in all three kinds of specimens using a semiquantitative RT-PCR procedure. Similar amounts of PCR products were generated from ALR messages present in the livers of weanling and adult rats, and from freshly isolated hepatocytes as shown from a representative experiment in Fig. 6. When the resolved PCR products were hybridized with radiolabeled probes after Southern transfer (Fig. 6A) and the net radioactivity of each band was determined, the ratio of the radioactivity generated from radiolabeling of ALR and β actin mRNAs also was similar in the three kinds of specimens (Fig. 6B). These results clearly demonstrated a similar extent of ALR mRNA expression in the adult and weanling rat livers, and in isolated hepatocytes. Hepatic ALR mRNA was determined after partial hepatectomy to ascertain changes in its expression after partial hepatectomy. No Significant alteration in ALR mRNA concentration was observed at any time up to 40 hours following 40% or 70% hepatectomy (results not shown)

Binding of Radiolabeled ALR to Hepatic Membranes and Cultured Hepatocytes

The inability of ALR to exert a mitogenic effect on hepatocytes *in vitro*, even in the presence of other mitogenic agents,[8] has been interpreted to mean that hepatocytes do not contain receptors for ALR. Consistent with this possibility, a competition binding assay using radioiodinated rrALR failed to show specific binding of ALR in the hepatocytes (Fig. 7).

However, because cultured hepatocytes synthesize and secrete ALR, the possibility remained that ALR receptors on the hepatocytes were down-regulated. Therefore, we treated cultured hepatocytes with acidic medium,[56] a procedure known to dissociate bound peptide ligand from its receptors.[56,67] Although this treatment improved nonspecific binding, it did not unmask specific binding of ALR (Fig. 7).

DISCUSSION

The current investigations were facilitated by the development of a sensitive ELISA that, combined with mRNA and other determinations, yielded new information about the synthesis and localization of ALR. Contrary to expectations, the findings were essentially the same in resting adult livers and in weanling livers. Conceivably, the ALR in the quiescent adult livers has different physiological properties than the ALR in proliferating (*i.e.*, weanling or posthepatectomy) livers. At present, however, a more likely explanation for the historical inability to demonstrate HSS in adult rat liver cytosol (see Introduction) may be the presence of an inhibitor, as was originally suggested by LaBrecque and Pesch.[6] This hypothesis has gained credibility with the discovery of TGF-β10،11،68 and other members of the TGF-β superfamily (*e.g.*, activin69).

In both the intact adult and weanling livers, as well as in freshly isolated cell subpopulations, the principal concentration of ALR peptide and essentially all of the mRNA transcripts were in hepatocytes, similar to TGF-α[34,35] but unlike the hepatotrophic HGF[37] and antihepatotrophic TGF-α[46,47] that are synthesized by NPCs (Table 1). ALR also was found by

immunolabeling in the NPCs of the intact livers, but the peptide was irregularly distributed and of lower concentration than in hepatocytes. When the isolated NPCs were cultured, all of the ALR initially found in them disappeared within 24 hours, In contrast, cultured hepatocytes had no loss of immunolabeling and actually secreted the ALR into the medium in a time-dependent manner.

ALR receptors could not be demonstrated either on the cultured parenchymal cells or on the NPCs. However, because of the entry of ALR into the NPCs, it remains possible that ALR affects hepatocytes indirectly via NPCs whose ALR receptors were down-regulated and therefore not demonstrable under the conditions of the *in vitro* experiments; this has been observed with other growth factors.[68,70] Another possibility is that the ALR molecule was inappropriately labeled for binding.

A further unexpected finding was the progressive decrease in the concentration of hepatic ALR after 70% hepatectomy in adult rats. This began to decline within 30 minutes, reached an estimated nadir of about two-thirds control concentration after 12 hours, and then returned toward, but not to, control levels in the ensuing 12 hours. The depletion was so much less marked with 40% hepatectomy that it could not be demonstrated, and therefore could only be assumed, As previously noted by Giorda et al,[30] in mouse hepatectomy experiments, there was no change in our current rat studies in the quantity of the ALR mRNA transcript after either 70% or 40% hepatectomy. Interestingly, it has been suggested recently that HGF also is not synthesized during regeneration, but is released at the time of regeneration from storage in intracellular matrix.[38–40]

Corresponding temporally to the decrease in hepatic ALR, the concentrations of circulating ALR followed an opposite pattern by rising 10-fold over the first 12 hours from the low concentrations consistently found in the control sera. These then returned to nearly control levels by 24 hours, the time when peak regeneration in the rat is known to occur.[11,68,71] The increases in circulating ALR were commensurately less and of shorter duration with 40% hepatectomy. Thus, the release of stored ALR from hepatocytes, rather than accelerated synthesis as previously assumed, probably accounts for most if not all of the posthepatectomy increase in the circulating peptide. It may be Significant that the changes in both the liver tissue and blood are already detectable within 30 to 60 minutes, earlier than TGF-α and at least as soon as HGF.[10,11]

The foregoing results have further established the association of ALR with liver regeneration, but they do not clarify its homeostatic role. Nor is the physiological role known of most of the other endogenous hepatotrophic molecules shown in Table 1, none of which (including HGF and TGF-α) substantially affect the liver *in vivo* unless the liver already is primed. The requirement of priming for demonstration of the growth effects of the hepatotrophic agents has increasingly raised the possibility that the initiation of mitosis by a wide range of nonspecific causes and the escalation of this early response to regeneration involve separate and distinct mechanisms.[8,10,32,72–74]

In addition, it may be prudent to reserve judgment about the relative physiological significance of either primary or augmenting mitogens. Although HGF and TGF-α (plus epidermal growth factor) are usually considered to be the principal liver growth factors,[10,11] this conclusion has been supported largely with evidence from circumstances that exist only in artificial laboratory systems. In-contrast, insulin, which frequently is described as “permissive” of regeneration, has been shown with a panoply of physiological evidence not remotely approached with any other molecule to play an indispensable role in liver growth and regeneration[22,23] and to have molecular biological mechanisms remarkably attuned to cellular growth control.[76,77]

Furthermore, Steiner[78] proposed a context of regeneration more than 20 years ago based on experiments with insulin in which mechanisms of signalling were described not only for more cells to maintain the appropriate liver/body weight ratio, but also for efficient removal of superfluous cells by apoptosis (see also discussion of article by H.K. Weinbren). The interaction and versatility of these mechanisms in regeneration have been recently demonstrated by Sakamoto and Demetris et al.,[79] who showed how a reduction in the rate of programmed cell death allowed nearly normal liver regeneration in rats despite a loss of efficient mitotic signalling in interleukin-6-deficient rats.

The implication of such observations is that the context developed for research in liver regeneration during the last two decades may need major correction. In 1995, the series of reviews on this subject was introduced with the opinion that the liver does not exhibit true regeneration (*i.e.,* that exhibited by amphibians), but rather undergoes compensatory hyperplasia to establish the optimal mass for body size.[80] From this perspective, it would be logical to view the process of regeneration as a "cascade" into which newly discovered growth and antigrowth molecules could be neatly fit. Instead, we envision liver regeneration as a genuine recapitulation of the organogenesis of otogeny in which the appearance, proliferation, and disappearance of cells is governed by mesenchymal/epithelial molecular signalling.[30]

It remains puzzling that the diverse hepatotrophic molecules all appear to have the same effects on the primed liver. This could be explained most readily if these effects were mediated by a molecule within the liver itself that serves as a "master switch." In view of its independence from an acute increase in synthesis and its consequent rapid response time, ALR might be a candidate, working through immunologic pathways. Vujanovic et al.[81] recently reported that natural killer (NK) cells in the liver fragment left after 70% hepatectomy (but not in peripheral lymphoid organs) underwent a sequential loss and then restoration of cytotoxic and cytolytic function, while the susceptibility of hepatocytes to NK cell-induced lysis declined during the early phases of regeneration, with subsequent return to basal values.

Such data suggested that NK cells may be involved not only in the termination of regeneration as previously proposed by Itoh et al.,[82] but in its initiation.[81] The hypothesis that NK cells may be regulated in turn by hepatotrophic factors[81] has been tested in normal rats by administration for 24 hours of three hepatotrophic molecules: ALR, insulin-like growth factor-II, and HGF.[83] When given to the intact animal, each of these molecules mimicked the changes in NK cell function that occur after partial hepatectomy. In contrast, none had an effect when added individually or in combination to the media of cultured NK cells, demonstrating the apparent need for an additional as-yet-unknown intermediary mechanism of NK cell regulation.

It is self-evident that all of the hepatotrophic and antihepatotrophic molecules listed in Table 1 can induce profound changes in the immune system, and that most of these molecules are primarily associated either with normal immune function or its therapeutic alteration: *i.e.,* the three T-lymphocyte-directed immunosuppressants (cyclosporine, tacrolimus, rapamycin); three cytokines (HGF, TGF-α, TGF-β); and a ubiquitous immunophillin (FKBP12) to which tacrolimus binds and which in turn binds[84,]85 to TGF-β family Type I receptors86 with inhibition of TGF-β signalling pathways.87 Thus, it would be premature to conclude that regeneration is not a complex immunologic event of very early evolutionary development as we have suggested previously,[30] or that ALR is not a combined immune- and growth-regulatory gene.

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Abbreviations

HSS	hepatic stimulatory substance
ALR	augmenter of liver regeneration
HGF	hepatocyte growth factor
TGF	transforming growth factor
ELISA	enzyme, linked immunosorbent assay
rrALR	rat recombinant ALR
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulfate
NPC	nonparenchymal cell
RT-PCR	reverse-transcriptase polymerase chain reaction
NK cells	natural killer cells

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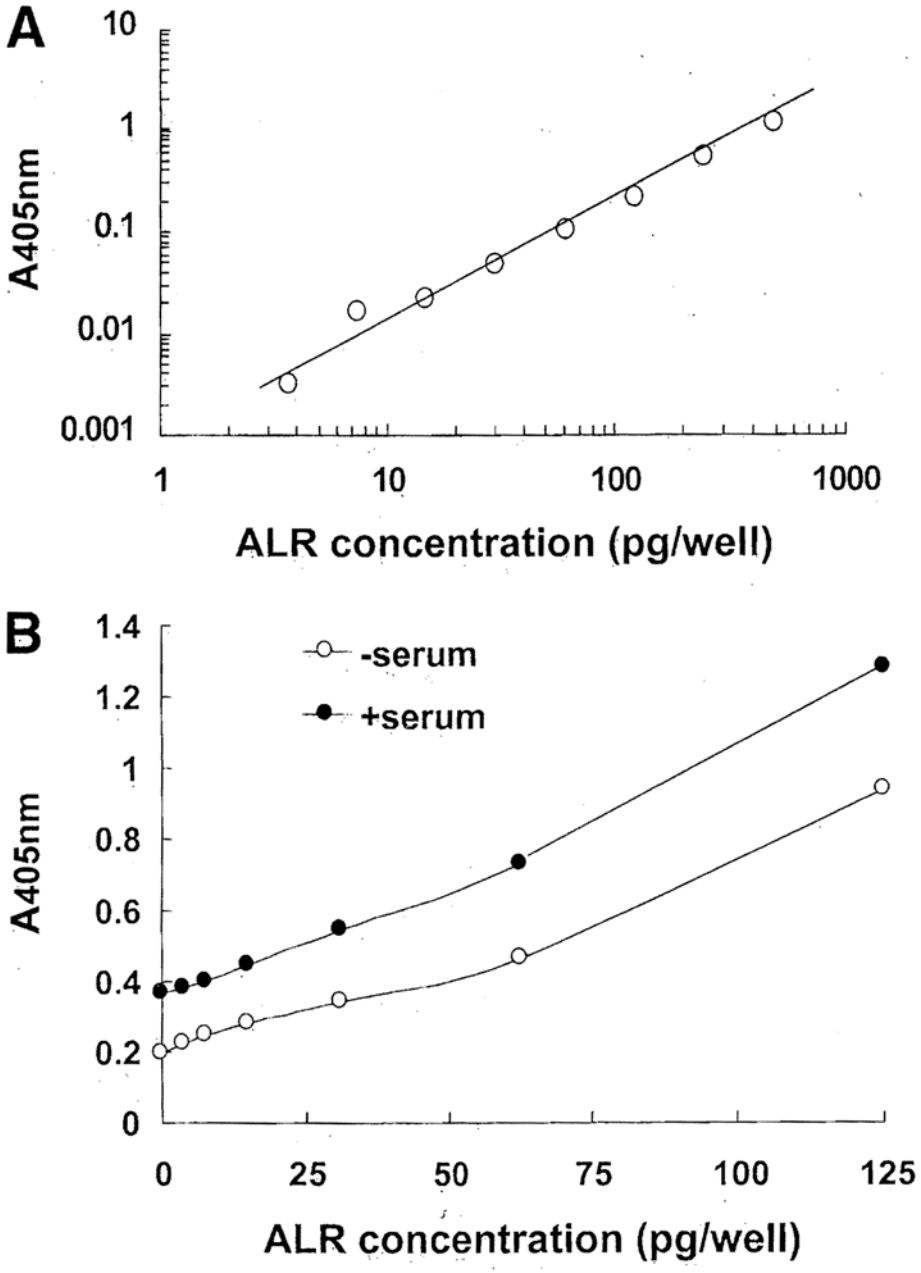

Fig. 1.

ALR ELISA. (A) Indicated concentrations of rrALR were used to develop a standard curve as described in Materials and Methods. Each value represents average of duplicate determinations. (B) One hundred microliters of the rat serum was spiked with indicated concentrations of rrALR. For control, the same concentrations of ALR were mixed With 100 μL PBS. ELISA was then performed as described in Materials and Methods. Valves represent averages of duplicate determinations of the experiment performed at least three times with essentially similar results.

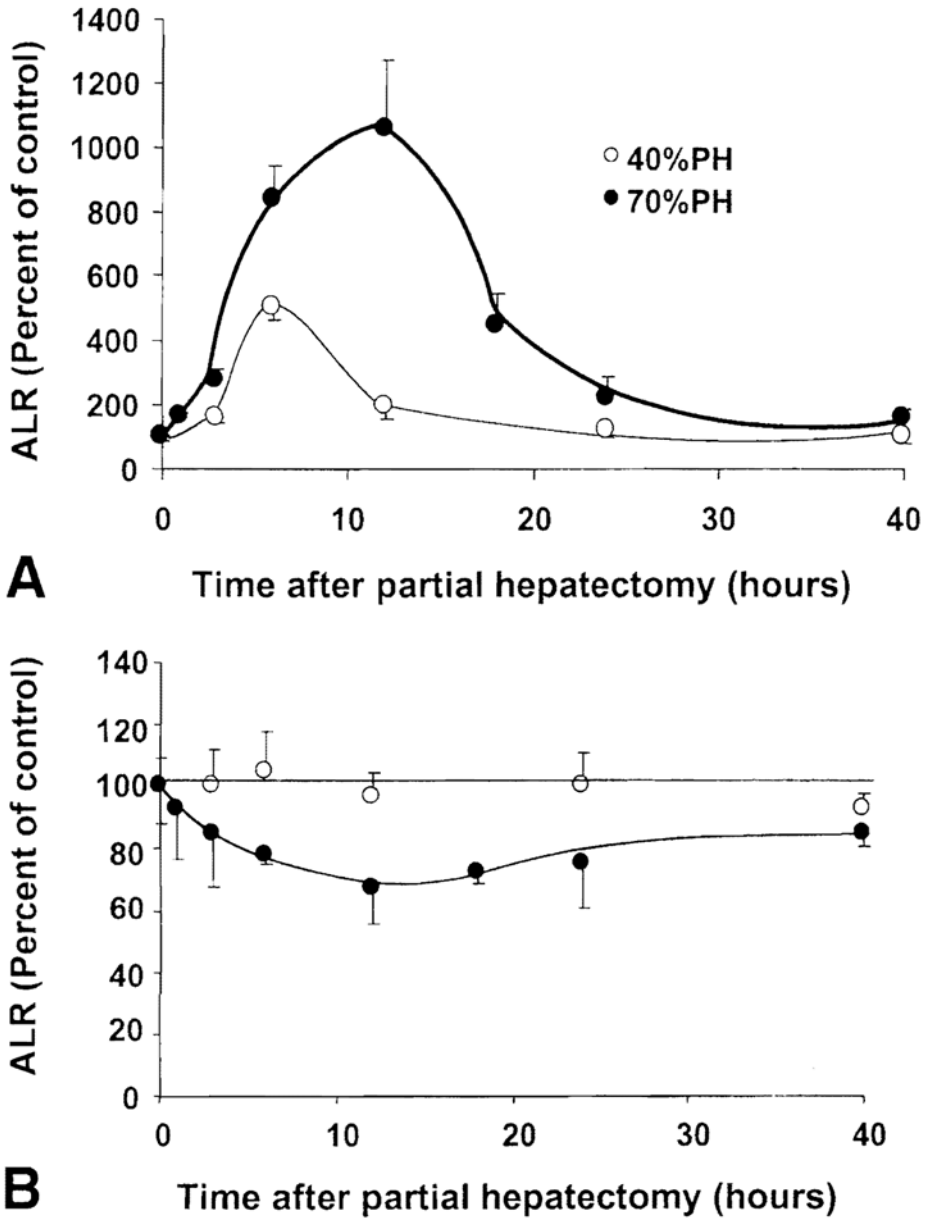

Fig. 2.

Serum (A) and hepatic (B) ALR levels following partial hepatectomy. Following 40% or 70% hepatectomy, the rats were killed at indicated times. Serum and the liver samples were processed for determination of ALR by ELISA as described in Materials and Methods. Each value represents the average of triplicate determinations ± SD from a representative experiment repeated two or three times.

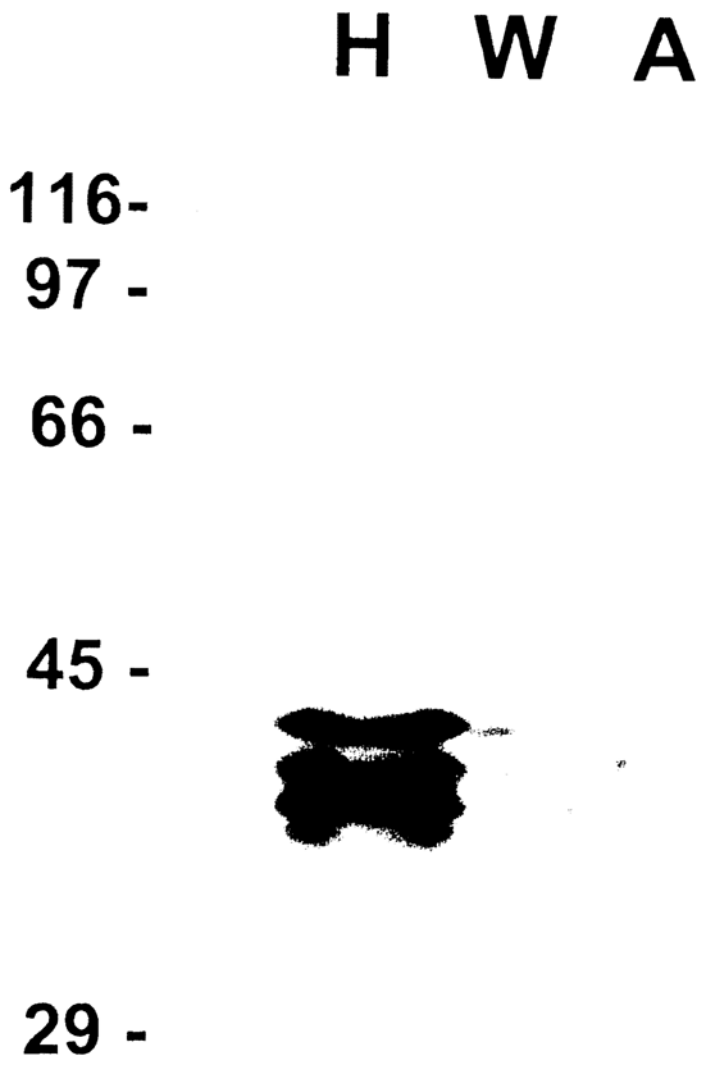

Fig. 3.

Western analysis of ALR in hepatocyte and the livers of weanling and adult rats. Extracts of the livers and hepatocytes were mixed with 2× loading buffer 0.125 mol/L Tris-HCl (pH 6.8) containing 4% SDS, 20% glycerol, 5 mmol/L ethylenediaminetetraacetic acid, and 0.05% bromophenol blue (1:1; vol/vol), heated in a boiling water bath for 10 minutes, and proteins separated by electrophoresis on a 15% SDS-polyacrylamide gel. The proteins were transferred to the Immobilon-P transfer paper and immunolabeled with anti-rrALR antibody. Details are described in Materials and Methods. H, hepatocytes from adult rats; W, weanling rat liver; A, adult rat liver.

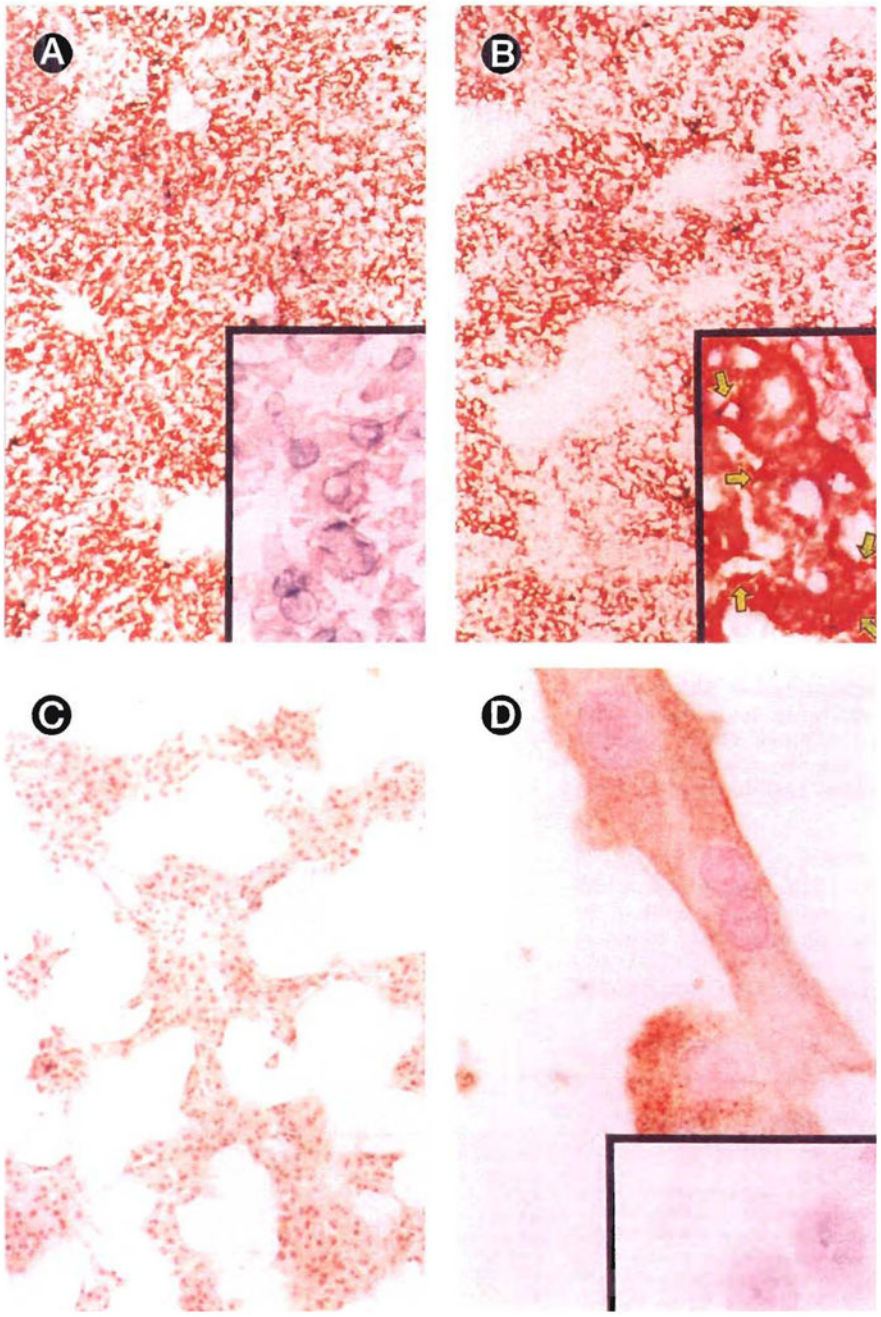

Fig. 4.

Immunohistochemical localization of ALR in the livers of weanling and adult rats, and cultured hepatocytes. (A) Weanling rat liver OCT-frozen liver sections were prepared and immunolabeling was performed as described in Materials and Methods. The majority of hepatocytes exhibiting immunolabeling for ALR can be seen (original magnification × 100) *Inset:* isotype control with rabbit IgG showing lack of any positive staining (original magnification × 1,000) (B) Adult rat liver the majority of hepatocytes are positive for ALR. *Inset:* Immunostaining (*arrows*) corresponding to the localization of nonparenchymal cells can be seen (original magnification × 1,000). Cultured hepatocytes: (C) (All of the hepatocytes are positive for ALR immunostain (original magnification × 100) (D) Hepatocytes are seen to

exhibit cytosolic localization of ALR immunostain; a binuclear cell is also shown (original magnification ×1,000). *Inset:* Isotype control with rabbit IgG showing lack of any positive staining

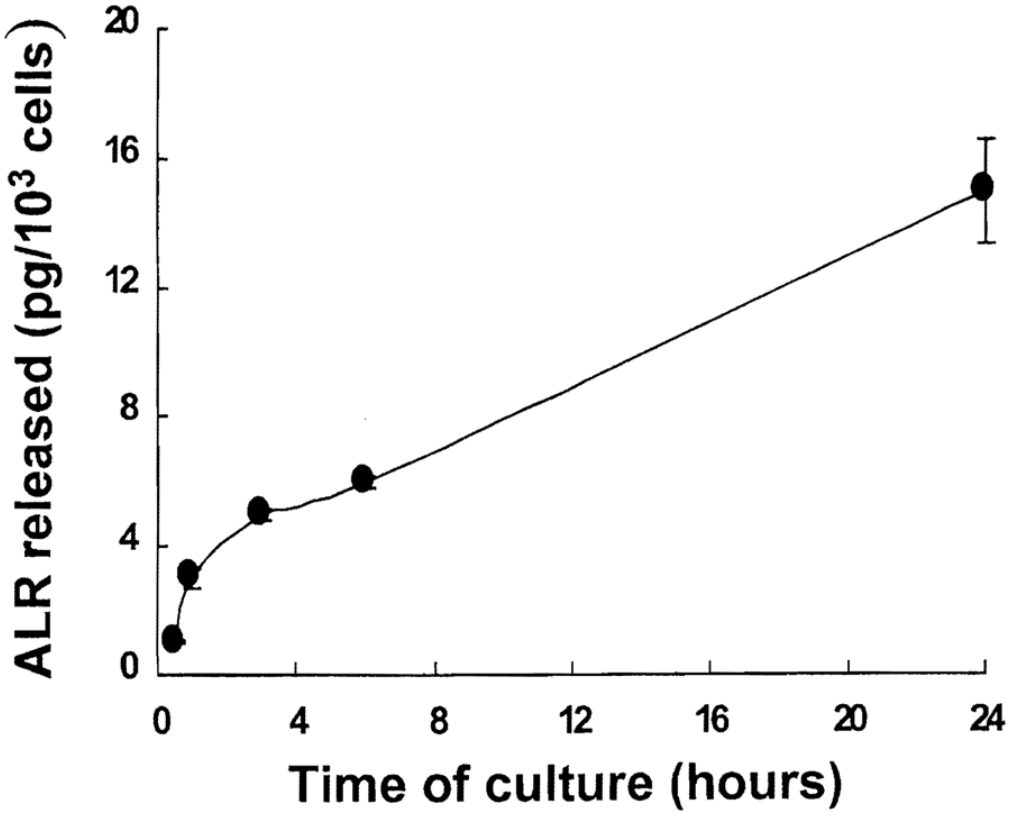

Fig. 5.

Time course of the release of ALR into the culture medium of hepatocytes. Hepatocytes were isolated and cultured as described in Materials and Methods. After 3 hours, the medium was renewed, and serum-free condition was introduced following an overnight incubation. At indicated time points, the medium was aspirated and ALR levels were determined by ELISA. Values are means ± SD of triplicate determinations of a representative experiment repeated three times with essentially similar results.

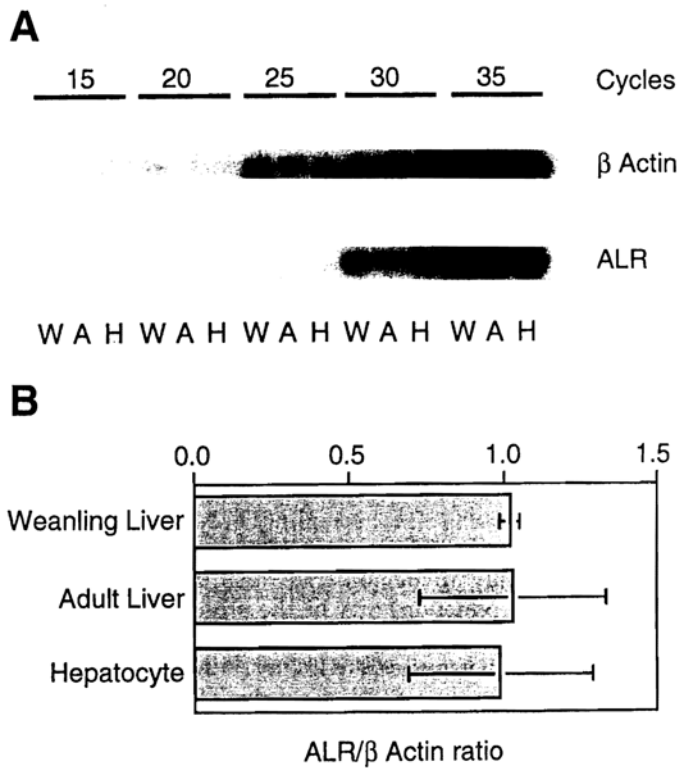

Fig. 6.

Analysis of ALR mRNA expression by RT-PCR. The extent of the expression of ALR and β actin mRNAs in the livers of weanling and adult rats, and hepatocytes was determined by RT-PCR as described in Materials and Methods. (A) Southern blot of the PCR products generated after 15, 20, 25, 30, and 35 cycles from equal amounts of cDNAs derived from the livers and hepatocytes were resolved in a 1.2% agarose gel, then transferred to Nylon membrane. The blot was hybridized with radiolabeled DNA probes specific for ALR and β actin. (B) The ratio of radioactivity associated with the PCR products generated from ALR and β actin cDNAs of the same samples. The results shown are representative of an experiment repeated three times. W, weanling rat liver; A, adult rat liver; H, hepatocytes from adult rats.

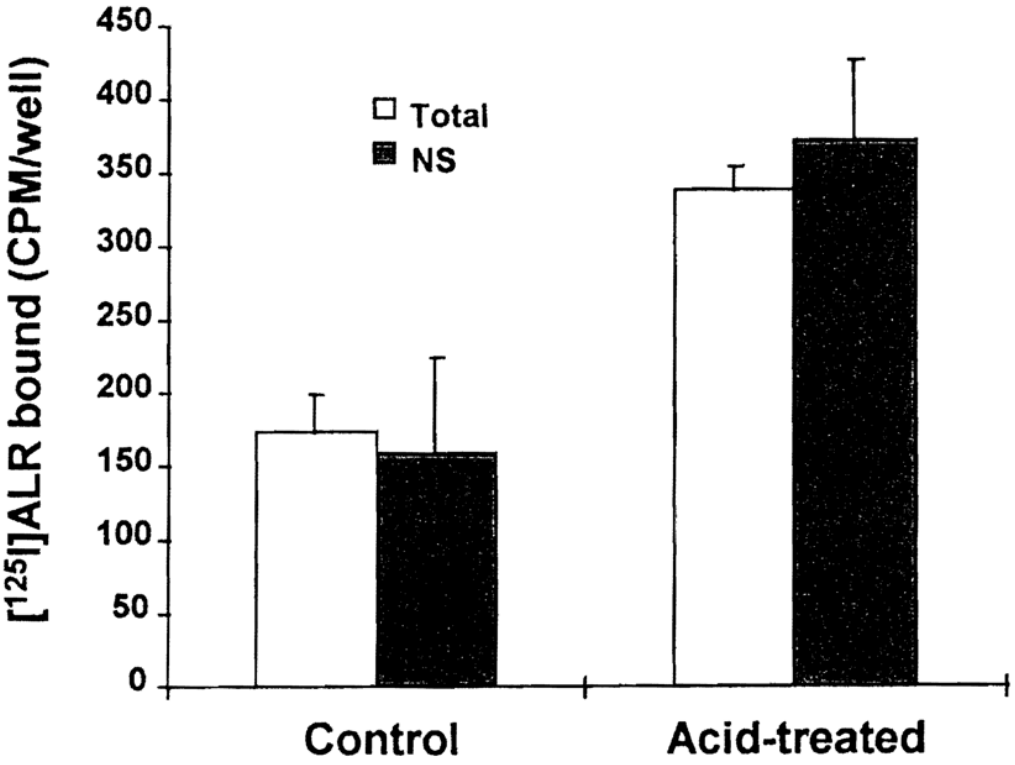

Fig. 7.

Binding of ALR to cultured hepatocytes. Hepatocytes were isolated and cultured as described After an overnight incubation, cells were washed and [^{125}I]ALR binding assay was performed before and after treatment of cells with acidic medium. Results are averages ± SD of triplicate determinations of an experiment that was performed at least 5 times. Total and nonspecific (NS) binding of radioiodinated ALR is shown. For details, see Materials and Methods.

Table 1

Growth Factors Revealed by *In Vivo* Studies

Growth Factors	Endogenous Origin	Eck's Fistula Assay	Minimum Hepatectomy (or other) *In Vivo* Assay
Hepatotrophic			
Hormones			
Insulin	Pancreas (B cells)	14, 15	23
Growth factors			
HSS or ALR	Ubiquitous; regenerating or hyperplastic livers (previous view) (6, 7, 24, 25)	24, 25, 28	6–8, 25–27
IGF-II.	Ubiquitous; liver Kupffer endothelial and stellate cells (31)	32	ND
TGF-α*	Ubiquitous; liver Kupffer cells (33); stellate cells (34) hepatocytes (34, 35)	32, 36	9, 12
HGF*	Ubiquitous; liver stellate cells (37) and liver matrix (38–40)	32, 36	9, 12
Immunosuppressants			
Cyclosporine	NA	41	42
FK506		43	44
Immunophilins			
FKBP12	Ubiquitous	45	ND
Antihepatotrophic			
Growth factors			
TGF-β†	Ubiquitous NPC	32, 36	54
Immunosuppressants			
Rapamycin†	NA	55	55

Abbreviations: NA, not applicable (xenobiotic); ND, not done.

*Mitogenic in tissue culture.

†Inhibitory in tissue culture.

Table 2

Concentration of ALR in the Serum and Liver of Adult and Weanling Rats

	Liver (pg/mg wet weight)	Serum (pg/mL)
Adult rat	3710 ± 810	280 ± 70
Weanling rat	3410 ± S60	210 ± SS

NOTE. Blood of adult (150–200 g) and weanling (40–50 g) rats was drawn via the abdominal aorta, after which the livers were excised, rinsed in ice-cold PBS, and snap-frozen in liquid nitrogen. Concentrations of ALR in the liver and the serum were determined by ELISA. For details, see Materials and Methods.

Table 3

Concentration of ALR in Freshly Isolated and Cultured Hepatic Cells

	ALR Concentration (pg/10^4 cells)	
	Freshly Isolated	Cultured
Hepatocytes	800 ± 130	600 ± 150
Stellate cells	8 ± 3	Undetectable
Kupffer cells	3 ± 0.5	Undetectable
Endothelial cells	1 ± 0.5	Undetectable

NOTE. Hepatocytes, stellate cells, Kupffer cells, and endothelial cells were prepared, counted, and a portion was snap-frozen in liquid nitrogen and stored at −70°C. The rest of the cells were placed in culture. The culture medium was renewed after allowing the cells to attach (3–6 hours). After overnight incubation, the cells were detached from the plates by trypsin treatment and suspended in ice-cold PBS. A portion of the suspension was used for counting, and the rest was centrifuged at 1,000g for 10 minutes at 4°C. ALR was extracted from the pelleted cultured cells and freshly isolated (snap-frozen) cells, and analyzed by ELISA. Values shown are averages ± SD of triplicate determinations from an experiment repeated three times.