BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Amphiregulin: An Early Trigger of Liver Regeneration in Mice

CARMEN BERASAIN,* ELENA R. GARCÍA–TREVIJANO,* JOSEFA CASTILLO,* ELENA ERROBA,* DAVID C. LEE,† JESÚS PRIETO,* and MATÍAS A. AVILA*

*Division of Hepatology and Gene Therapy, Centro de Investigación Médica Aplicada, Facultad de Medicina, Universidad de Navarra, Pamplona, Spain; and †Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina

See editorial on page 504.

Background & Aims: **Liver regeneration is a unique response directed to restore liver mass after resection or injury. The survival and proliferative signals triggered during this process are conveyed by a complex network of cytokines and growth factors acting in an orderly manner. Activation of the epidermal growth factor receptor is thought to play an important role in liver regeneration. Amphiregulin is a member of the epidermal growth factor family whose expression is not detectable in healthy liver. We have investigated the expression of amphiregulin in liver injury and its role during liver regeneration after partial hepatectomy.** ***Methods:*** **Amphiregulin gene expression was examined in healthy and cirrhotic human and rat liver, in rodent liver regeneration after partial hepatectomy, and in primary hepatocytes. The proliferative effects and intracellular signaling of amphiregulin were studied in isolated hepatocytes. The in vivo role of amphiregulin in liver regeneration after partial hepatectomy was analyzed in amphiregulin-null mice.** ***Results:*** **Amphiregulin gene expression is detected in chronically injured human and rat liver and is rapidly induced after partial hepatectomy in rodents. Amphiregulin expression is induced in isolated hepatocytes by interleukin 1β and prostaglandin E_2, but not by hepatocyte growth factor, interleukin 6, or tumor necrosis factor α. We show that amphiregulin behaves as a primary mitogen for isolated hepatocytes, acting through the epidermal growth factor receptor. Finally, amphiregulin-null mice display impaired proliferative responses after partial liver resection.** ***Conclusions:*** **Our findings indicate that amphiregulin is an early-response growth factor that may contribute to the initial phases of liver regeneration.**

Cytoprotective and regenerating mechanisms are set in motion in the liver after loss of parenchymal mass due to partial hepatectomy (PH) or after tissue injury.[1–3] This complex response aimed at the restoration of liver-dependent homeostasis is mediated by a network of cytokines, comitogens, and growth factors in a coordinated multistep process.[2,3] Many of the mediators believed to be critical in the regenerative response to injury or resection in animals are also expressed in humans during liver regeneration, thus suggesting the preservation of fundamental mechanisms across species.[4]

Despite the intensive research performed in the past few decades, the molecules and mechanisms involved in the physiological adaptive response to liver injury are not completely known. We have recently observed that the expression of the Wilms' tumor suppressor 1 (*WT1*) gene is induced in the livers of patients with hepatocellular damage and in the livers of CCl_4-treated rats.[5] *WT1* encodes a zinc finger transcription factor that can regulate the expression of a diversity of growth- and differentiation-related genes.[6] One of the major physiological targets directly induced by WT1 is amphiregulin (AR).[7] AR is a polypeptide growth factor of the epidermal growth factor (EGF) family and a ligand of the EGF receptor (EGF-R) that was originally isolated from conditioned medium of MCF-7 human breast carcinoma cells treated with phorbol esters.[8] Activation of the EGF-R in the hepatocyte is thought to play an important role during the early stages of liver regeneration after

Abbreviations used in this paper: AR, amphiregulin; BrdU, 5-bromo-2′-deoxyuridine; CRE, cyclic adenosine monophosphate–responsive element; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; ERK, extracellular regulated kinase; FCS, fetal calf serum; HB-EGF, heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; IL, interleukin; JNK, c-Jun protein kinase; MEK, extracellular regulated kinase kinase; p38 MAPK, p38 mitogen-activated protein kinase; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PGE_2, prostaglandin E_2PH, partial hepatectomy; PI-3K, phosphatidylinositol 3-kinase; TGF, transforming growth factor; TNF, tumor necrosis factor; *WT1,* Wilms' tumor suppressor 1.

0016-5085/05/$30.00
doi:10.1053/j.gastro.2004.11.006

PH, and the levels of EGF-R ligands such as EGF and transforming growth factor (TGF)-α are increased after liver resection.[1–4] The expression of AR is tissue specific: it is most prevalent in human ovary and placenta but is undetectable in the healthy and quiescent liver parenchyma.[9] Like EGF and TGF-α, AR is synthesized as a transmembrane precursor that is proteolytically processed to the secreted mature form.[10] AR shows bifunctional properties, stimulating the proliferation of a variety of normal cells and inhibiting that of many tumor cell lines.[7,9,11] Together these observations prompted us to examine *AR* gene expression in liver injury and during experimental liver regeneration. We show here that the expression of *AR* is detected in the liver of cirrhotic patients and is rapidly induced in rodent liver after PH. In addition, we show that AR behaves as a primary mitogen for the hepatocyte. A role for AR in the early steps of liver regeneration is supported by the impaired proliferative response of AR-null mice to PH. Our findings identify a novel function for AR in the liver and suggest that this molecule may have therapeutic potential to stimulate liver regeneration in cases of acute liver injury.

Materials and Methods

Patients

Liver tissue samples were from 2 groups of subjects. The first group comprised control individuals (n = 26; 19 men; mean age, 50.8 years; range, 18–73 years) with normal or minimal changes in the liver. We collected tissue samples at surgery (16 cases) of digestive tumors or from percutaneous liver biopsy performed because of mild alteration of liver function tests (10 cases). The second group comprised subjects with liver cirrhosis (n = 29; 24 men; mean age, 56 years; range, 36–77 years) that was due to hepatitis C virus infection in 8 cases, alcohol abuse in 13 cases, hepatitis B virus infection in 3 cases, autoimmune hepatitis in 3 cases, and hemochromatosis in 1 case and that was cryptogenic in another case. Associated hepatocellular carcinoma was present in 10 cirrhotic patients. This study was approved by the Human Research Review Committee of the University of Navarra.

Animal Models

Liver cirrhosis was induced with CCl_4 in male Wistar rats (150 g) as previously described.[12] Two-thirds PH or sham operations were performed in male Wistar rats (150 g) and male $AR^{+/+}$ and $AR^{-/-}$ littermate mice[13] (20 g) as previously described.[14,15] The $AR^{-/-}$ mice, as described previously, are viable and display no overt phenotype.[13] One hour before being killed, mice were injected intraperitoneally with 50 mg/kg of 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO). This study was approved by our institution's Animal Welfare Committee.

Isolation, Culture, and Treatment of Rat Hepatocytes

Hepatocytes were isolated from male Wistar rats (150 g) by collagenase (Gibco-BRL, Paisley, UK) perfusion as described previously.[15] Cells were plated onto collagen-coated 6-well dishes (type I collagen; Collaborative Biomedical, Bedford, MA; 5×10^5 cells per well). Cultures were maintained in minimal essential medium supplemented with 10% fetal calf serum (FCS), nonessential amino acids, 2 mmol/L glutamine, and antibiotics (all from Gibco-BRL). After 2 hours, incubation medium was removed, and cells were refed the same medium with 0.5% FCS. Where indicated, hepatocytes were treated with interleukin (IL)-1β or tumor necrosis factor (TNF)-α from Roche (Mannheim, Germany), hepatocyte growth factor (HGF) or forskolin from Calbiochem (San Diego, CA), IL-6 from R&D Systems (Wiesbaden-Nordenstadt, Germany), or prostaglandin E_2 (PGE_2) from Alexis (Carlsbad, CA).

Transient Transfection of Rat Hepatocytes

We seeded rat hepatocytes 24 hours before transfection with the Tfx-50 reagent (Promega, Madison, WI) according to the manufacturer's instructions, and cells were transfected in duplicate with an equimolar mixture of pCB6 plasmids encoding the 4 isoforms of WT1 (characterized by the presence or absence of the splice inserts, exon 5, and lysine, threonine, serine [KTS]) or with an equivalent amount of the empty pCB6 vector, provided by Dr. Jochemsen (Leiden University Medical Center, Leiden, The Netherlands). Transfection efficiency was monitored by reverse-transcription polymerase chain reaction (PCR) analysis by using specific primers that discriminated these 4 isoforms.

Assay of DNA Synthesis

Rat hepatocytes were plated at a density of 3×10^4 cells per well in collagen-coated 96-well plates in minimal essential medium supplemented with 10% FCS. Five hours after plating, medium was changed, and cells were kept in the absence of serum for 20 hours more. DNA synthesis was measured in response to AR for 30 hours. A pulse of [^{3}H]thymidine (1 μCi per well; Amersham Biosciences, Piscataway, NJ) started 22 hours after AR addition. Cells were harvested, and [^{3}H]thymidine incorporation was determined in a scintillation counter. The effect of AR on DNA synthesis was also tested in the presence of TGF-β (Roche) or the extracellular regulated kinase kinase (MEK)-1 inhibitor PD98059, the phosphatidylinositol 3-kinase (PI-3K) inhibitor LY-294002, the c-Jun protein kinase (JNK) inhibitor SP600125, the p38 mitogen-activated protein kinase (p38 MAPK) inhibitor SB202190, and the EGF-R inhibitor PD153035, all from Calbiochem.

RNA Isolation and Analysis of Gene Expression

Total RNA was extracted by using the TRI Reagent (Sigma). We treated 2 μg of RNA with deoxyribonuclease I

(Gibco-BRL) before reverse transcription with Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) in the presence of RNaseOUT (Gibco-BRL). PCR products were electrophoresed in 2% agarose gels, stained with ethidium bromide, and quantitated with Molecular Analyst software (Bio-Rad, Hercules, CA). Data were normalized to β-actin messenger RNA (mRNA) levels. Real-time PCR was performed with an iCycler (Bio-Rad) and the iQ SYBR Green Supermix (Bio-Rad). To monitor the specificity, final PCR products were analyzed by melting curves and electrophoresis. The identity of the PCR products was confirmed by sequence analysis. The comparative Ct method for relative quantitation of gene expression was used ($2^{\Delta Ct}$, where ΔCt represents the difference in threshold cycle between the target and control genes). The primers used were designed according to published complementary DNA or genomic sequences.

Western Blot Analysis and Immunohistochemistry

Homogenates from liver samples and isolated hepatocytes were subjected to Western blot analysis as described previously.[15] Antibodies used were affinity-purified biotinylated anti-mouse AR polyclonal antibody (R&D Systems); phosphorylated Akt (Ser^{473}) and phosphorylated signal transducer and activator of transcription 3 (Tyr^{705}) (Cell Signaling, Beverly, MA); and extracellular regulated kinase (ERK)-1/-2, phosphorylated EGF-R (Tyr^{1173}), and signal transducer and activator of transcription 3 (Upstate Biotechnology, Charlottesville, VA). Antibodies for EGF-R, β-actin, phosphorylated ERK-1/-2 (Tyr^{204}), Akt, JNK, phosphorylated JNK (Thr^{183}/Tyr^{185}), proliferating cell nuclear antigen (PCNA), and cyclin D_1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Immunohistochemical detection and quantitation of BrdU incorporation into cellular DNA was performed in formalin-fixed and paraffin-embedded liver tissue as described previously.[16]

Statistical Analysis

Normally distributed data were compared among groups by using an independent Student *t* test and 1-way analysis of variance. Nonnormally distributed data were compared by using the Kruskal–Wallis and Mann–Whitney tests. Correlation was assessed by Spearman or Pearson correlation coefficients. Data are reported as means ± SEM or as medians and interquartile ranges. A *P* value of $< .05$ was considered significant.

Results

Amphiregulin Gene Expression Is Induced in Human and Experimental Liver Injury

We have previously shown that expression of the transcription factor WT1 is induced in almost all tested samples of cirrhotic human liver and always in CCl_4-induced cirrhosis in rats.[5] The fact that *AR* is a major transcriptional target for WT1[7] led us to examine the expression of this growth factor in the liver of cirrhotic patients. Although real-time PCR analysis showed barely detectable levels of *AR* expression in healthy human liver, *AR* gene expression was observed in approximately 75% of patients with cirrhosis (Figure 1*A*). It is interesting to note that the levels of *AR* gene expression directly correlated with those of *WT1* in the liver of control and cirrhotic subjects ($r = 0.752$; $P < .001$). In accordance with data in humans, *AR* gene expression was also observed in experimental cirrhosis induced in rats by CCl_4 administration (Figure 1*B*).

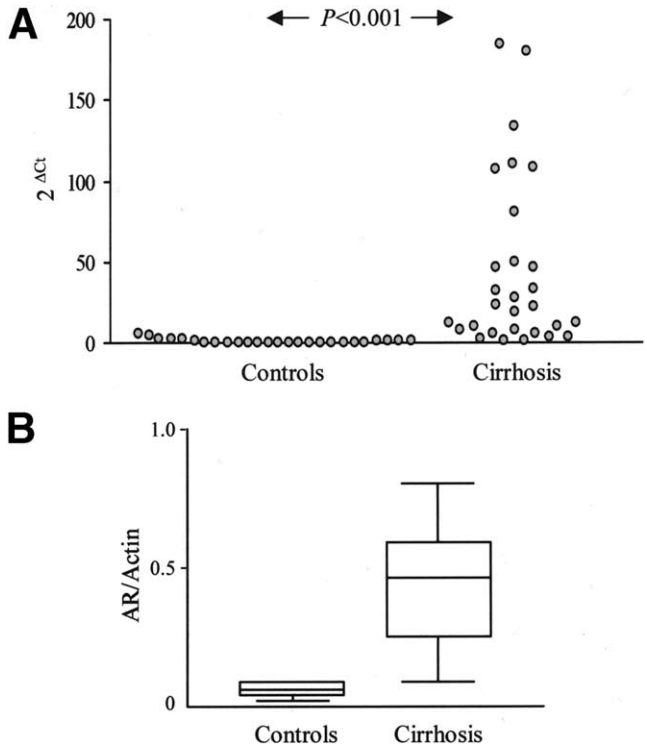

Figure 1. (*A*) *AR* gene expression in human liver cirrhosis. *AR* gene expression was measured by real-time PCR in liver samples from control individuals (n = 26) and cirrhotic patients (n = 29). (*B*) *AR* gene expression in control and in CCl_4-cirrhotic rat liver as determined by semiquantitative reverse-transcription polymerase chain reaction (n = 6 animals per group).

Amphiregulin Gene Expression in the Liver After Partial Hepatectomy

The fact that AR is a growth factor and a ligand of the EGF-R suggested that its induction in the damaged liver could be part of the regenerative response of the damaged organ triggered during chronic injury. To directly evaluate this possibility, we used the well-characterized model of rat liver regeneration after two-thirds PH.[4,14] As shown in Figure 2*A*, *AR* mRNA was absent in the rat liver before PH, became detectable as early as 0.5 hours after the intervention, peaked by 6 hours, and steadily decreased between 15 and 24 hours. In sham-operated rats, *AR* gene expression was transiently induced between 6 and 15 hours

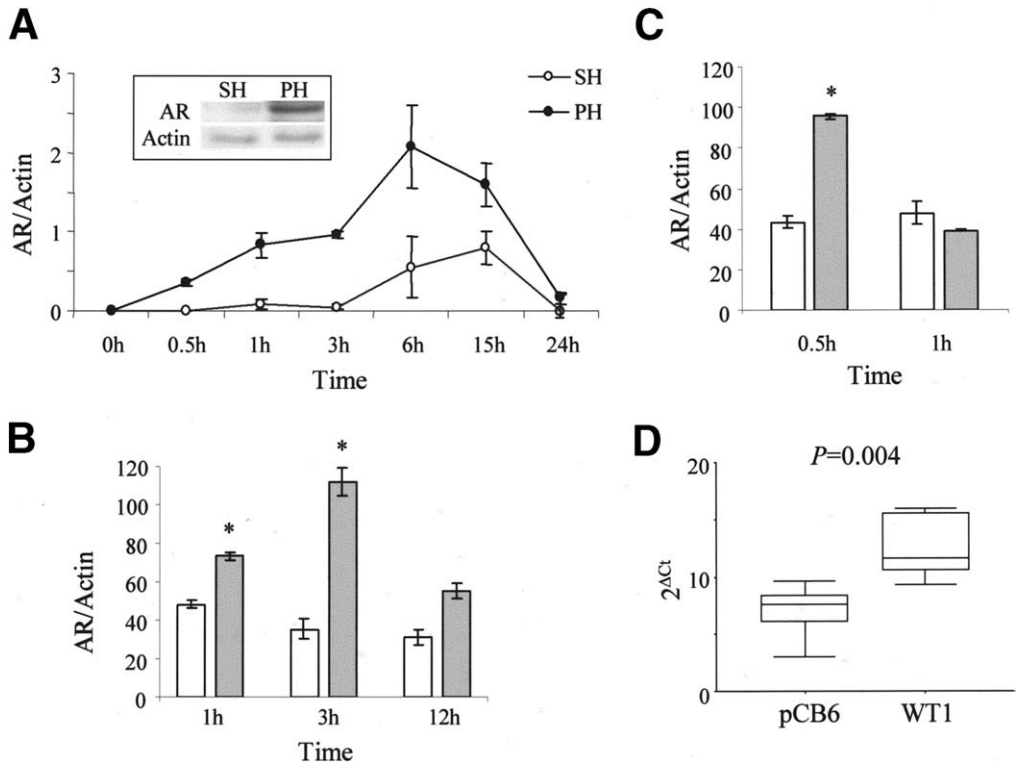

Figure 2. (*A*) *AR* gene expression was analyzed by semiquantitative reverse-transcription polymerase chain reaction in the remaining rat liver parenchyma at different time points after PH or sham operation (SH). Values are means ± SEM of 3 different animals. (*Inset*) The 50-kilodalton AR precursor protein as detected by Western blotting. Representative samples are shown. (*B*) Expression of *AR* in isolated rat hepatocytes treated with IL-1β (2 ng/mL) or (*C*) PGE_2 (10 μmol/L) for different periods of time. *AR* gene expression was analyzed by semiquantitative reverse-transcription polymerase chain reaction. $*P < .05$ vs controls (*open bars*). Values are means ± SEM of 3 experiments performed in triplicate. (*D*) Expression of *AR* in rat hepatocytes 24 hours after transfection with an equimolar mixture of pCB6 plasmids encoding the 4 isoforms of WT1 or with an equivalent amount of the empty pCB6 vector. *AR* expression was analyzed by real-time PCR. Values are means ± SEM of 3 experiments performed in triplicate.

but to a much lesser extent than in resected animals (Figure 2*A*). In mouse liver, *AR* gene expression was also readily induced after PH. *AR* mRNA levels were up-regulated 0.5 hours after PH, reached a peak between 24 and 48 hours, and subsequently decreased (data not shown). Using a biotinylated affinity-purified anti-AR antibody, we detected a band of approximately 50 kilodaltons that was induced in rat liver at 1 hour after PH (inset in Figure 2*A*). This band is consistent with the molecular weight of the membrane-anchored AR precursor previously described in epithelial cells.[10]

In an attempt to identify the mechanisms responsible for the induction of *AR* mRNA levels in the regenerating liver, we isolated rat hepatocytes and examined *AR* gene expression under different conditions. We tested the effect of several factors and mediators released early after PH that are believed to play a relevant role in liver regeneration, such as IL-1β, IL-6, TNF-α, HGF, and PGE_2.[1–4,17,18] Among the cytokines and growth factors tested, IL-1β was the only one that stimulated *AR* gene expression (Figure 2*B*). It is interesting to note that IL-1β treatment of isolated hepatocytes did not induce the expression of other EGF-R ligands produced by the hepatocyte, such as TGF-α (data not shown). In agreement with previous observations made in colon cancer cells,[19] we noticed that treatment of rat hepatocytes with PGE_2 resulted in the rapid induction of *AR* gene expression (Figure 2*C*). It has been postulated that PGE_2 stimulation of *AR* gene expression is signaled through the cyclic adenosine monophosphate (cAMP)/protein kinase A pathway acting on a cAMP-responsive element (CRE) in the *AR* gene promoter.[19] Consistent with this mechanism, we observed that the cAMP-inducing agent forskolin promoted *AR* gene expression in isolated hepatocytes (data not shown).

As we previously mentioned, *AR* is a bona fide target for the transcription factor WT1.[7] We found that transfection of hepatocytes with an equimolar mixture of pCB6 plasmids encoding the 4 isoforms of WT1 resulted in the up-regulation of *AR* mRNA levels, as determined by real-time PCR (Figure 2*D*). This observation shows that WT1 can mediate *AR* expression in liver parenchymal cells, suggesting that WT1 may participate in the activation of the *AR* gene

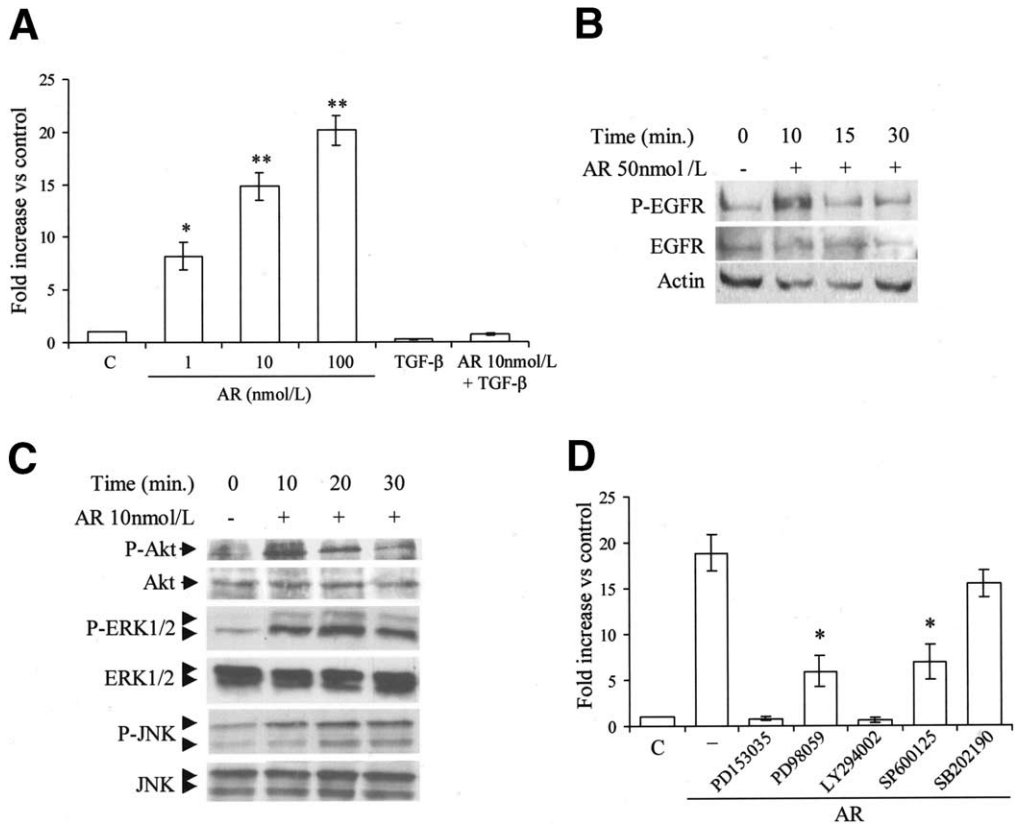

Figure 3. (*A*) Stimulation of DNA synthesis by AR in cultured rat hepatocytes. DNA synthesis was measured as [^{3}H]thymidine incorporation. The effect of TGF-β (8 ng/mL) on AR-induced DNA synthesis is shown. Values are means ± SEM of 3 experiments performed in quadruplicate. $*P < .05$; $**P < .01$ vs control. (*B*) Stimulation of EGF-R tyrosine phosphorylation by AR in cultured rat hepatocytes. Tyrosine-phosphorylated EGF-R (P-EGFR) and total EGF-R were detected by Western blotting. Representative blots of 3 experiments performed in duplicate are shown. (*C*) Phosphorylation of Akt, ERK-1/-2, and JNK in rat hepatocytes at different times after AR addition as assessed by Western blotting. Representative blots of 3 experiments performed in duplicate are shown. (*D*) Effect of AR (100 nmol/L) on DNA synthesis in rat hepatocytes in the presence of the EGF-R inhibitor PD153035 (1 μmol/L), the MEK-1 inhibitor PD98059 (10 μmol/L), the PI-3K inhibitor LY-294002 (20 μmol/L), the JNK inhibitor SP600125 (20 μmol/L), or the p38 MAPK inhibitor SB202190 (25 μmol/L). $*P < .05$ vs AR alone. Values are means ± SEM of 3 experiments performed in triplicate.

expression observed in chronic liver damage. However, the induction of *AR* gene expression in the regenerating liver after PH seems to be independent of WT1, because the expression of the latter gene is not induced after liver resection (data not shown).

Amphiregulin Induces DNA Synthesis in Isolated Hepatocytes

Having shown that AR gene expression is induced in the injured and regenerating liver, we next tested the effect of this growth factor on isolated parenchymal cells. As depicted in Figure 3*A*, AR behaved as a primary mitogen for the hepatocyte, stimulating [^{3}H]thymidine incorporation into DNA in a dose-dependent fashion. The effect of AR on DNA synthesis was abrogated by TGF-β, a growth factor implicated in the physiological termination of the liver regenerative response.[1]

As previously mentioned, AR is a ligand of the EGF-R, a receptor that is highly expressed in adult hepatocytes.[20,21] In agreement with this, treatment of isolated rat hepatocytes with AR induced rapid and transient tyrosine phosphorylation of the EGF-R (Figure 3*B*). The MEK/ERK and PI-3K pathways are viewed as the main signaling cascades in the mitogenic response of hepatocytes to growth factors.[22–25] More recently, JNK activation has been shown to significantly contribute to hepatocyte proliferation after PH.[26] We observed that stimulation of isolated rat hepatocytes with AR rapidly induced ERK-1/-2 and Akt phosphorylation (Figure 3*C*). In addition, we observed that JNK was phosphorylated in response to AR treatment (Figure 3*C*). To evaluate the relative contribution of these signaling pathways to AR-induced DNA synthesis in rat hepatocytes, we made use of different specific inhibitors. As depicted in Figure 3*D*, the EGF-R tyrosine kinase inhibitor PD153035 completely prevented AR-stimulated DNA synthesis. A similar degree of inhibition was observed for the PI-3K inhibitor LY-294002, whereas the MEK-1 PD98059 and JNK SP600125 inhibitors reduced AR effects by 70% (Figure 3*D*). Although we did not test the activation of p38

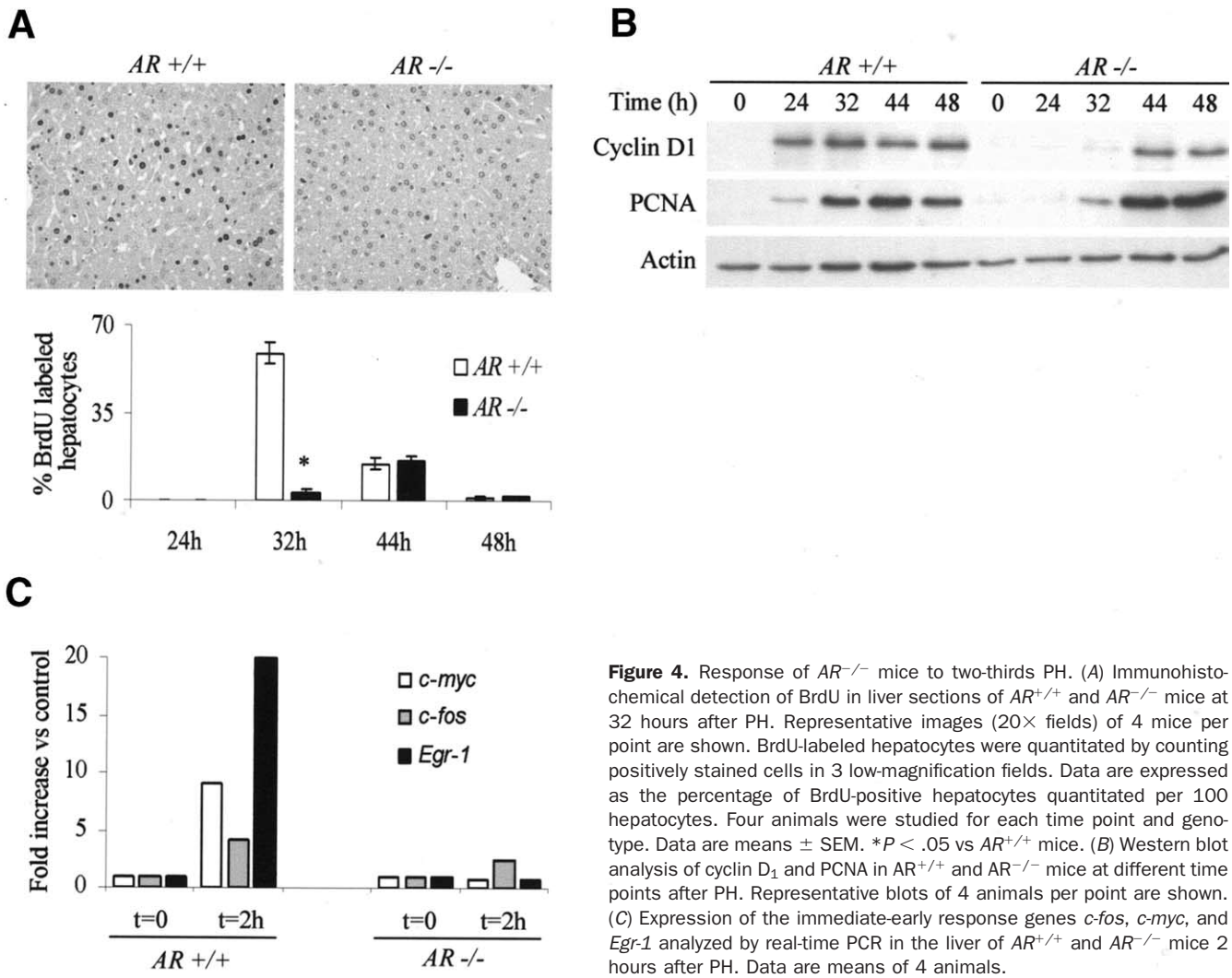

Figure 4. Response of $AR^{-/-}$ mice to two-thirds PH. (*A*) Immunohistochemical detection of BrdU in liver sections of $AR^{+/+}$ and $AR^{-/-}$ mice at 32 hours after PH. Representative images (20× fields) of 4 mice per point are shown. BrdU-labeled hepatocytes were quantitated by counting positively stained cells in 3 low-magnification fields. Data are expressed as the percentage of BrdU-positive hepatocytes quantitated per 100 hepatocytes. Four animals were studied for each time point and genotype. Data are means ± SEM. $*P < .05$ vs $AR^{+/+}$ mice. (*B*) Western blot analysis of cyclin D_1 and PCNA in $AR^{+/+}$ and $AR^{-/-}$ mice at different time points after PH. Representative blots of 4 animals per point are shown. (*C*) Expression of the immediate-early response genes *c-fos*, *c-myc*, and *Egr-1* analyzed by real-time PCR in the liver of $AR^{+/+}$ and $AR^{-/-}$ mice 2 hours after PH. Data are means of 4 animals.

MAPK by AR treatment, the p38 MAPK inhibitor SB202190 had no significant effect on AR-stimulated DNA synthesis (Figure 3*D*).

Role of Amphiregulin in Liver Regeneration After Partial Hepatectomy

To directly evaluate the significance of *AR* gene expression during liver regeneration after PH, we used $AR^{-/-}$ mice.[13] There were no significant differences in survival after PH between $AR^{+/+}$ and $AR^{-/-}$ mice. We first tested the DNA synthetic response in $AR^{+/+}$ and $AR^{-/-}$ animals after two-thirds PH. BrdU incorporation was detected by immunohistochemistry to evaluate the number of cells in S phase at different times after PH. As shown in Figure 4*A*, BrdU incorporation into DNA was significantly reduced and delayed in *AR*-null mice, reflecting impaired DNA synthesis. In agreement with this observation, the induction of PCNA expression, a landmark of S phase, was delayed in $AR^{-/-}$ mice after PH (Figure 4*B*). It is interesting to note that although delayed, the induction of PCNA expression in $AR^{-/-}$ mice seemed higher than in $AR^{+/+}$ mice (Figure 4*B*). A similar feature has also been observed recently in conditional *Met* mutant mice during liver regeneration.[27] These mice display a delayed but more pronounced increase in PCNA levels after liver resection, and this situation accompanies a severely impaired DNA synthetic response.[27]

To assess whether the observed decrease in DNA synthesis was the consequence of impaired progression of hepatocytes through the G_1/S boundary, we measured the protein levels of cyclin D_1. We observed that the expression of cyclin D_1 was similar in $AR^{+/+}$ and $AR^{-/-}$ animals; however, its induction was significantly delayed in the $AR^{-/-}$ mice (Figure 4*B*). $AR^{-/-}$ mice also showed differential immediate-early gene activation 2 hours after PH when compared with wild-type animals, as evidenced by the reduced levels of *c-fos*, *Egr-1*, and *c-myc* mRNAs (Figure 4*C*). Impairment in the activation of these immediate-early genes is consistent with the in-

duction of *AR* gene expression shortly after liver resection. Together these observations suggest that the early induction of *AR* gene expression after PH is necessary for the normal onset of liver regeneration and cannot be compensated for by other EGF-R ligands.

Discussion

The loss of functional liver mass induced by toxins, viral infections, or tissue resection triggers a well-orchestrated regenerative response that involves a number of cytokines, growth factors, hormones, and metabolites.[1–4] The identities of many of these factors have been established over the past decades, but the complexity of this response likely involves additional players. Here we provide several lines of evidence supporting AR, a ligand of the EGF-R, as a new factor involved in liver regeneration.

Our previous findings showed that expression of the transcription factor WT1 was induced in human and experimental liver damage.[5] The fact that AR is probably the best-characterized physiological target activated by WT1[7] prompted us to examine whether AR expression, which is barely detectable in the healthy liver, could be up-regulated in the injured liver. We observed that *AR* gene transcription was induced in human and experimental liver cirrhosis, correlating with the expression of *WT1*. Because AR is a ligand of the EGF-R, we speculated that its expression in chronic liver injury could be part of the compensatory response of this organ aimed at the restoration of liver mass. To investigate whether the presence of AR was related to the regenerative process, we examined the expression of this gene in the well-established model of liver regeneration after two-thirds PH. We noticed that AR gene expression was induced very quickly after tissue resection both in rats and mice. The expression of *AR* before DNA synthesis after PH suggests that this factor could play an early role in liver regeneration.

Experiments conducted in isolated rat hepatocytes evidenced that AR can interact directly with the parenchymal liver cells and trigger growth signals. Our in vitro experiments also showed that activation of the EGF-R by AR was necessary to convey this mitogenic effect. Hepatocyte proliferation in response to other EGF-R ligands, such as EGF and TGF-α, involves the activation of different signaling pathways, including members of the MAPK family, such as ERK-1/-2, JNK, and p38, and the PI-3K-FK506-binding protein-12-rapamycin associated protein (FRAP)/mammalian target of rapamycin (mTOR) pathway. However, some controversies exist as to their relative contribution to DNA replication in hepatocytes.[22–26] In agreement with previous reports on EGF-induced hepatocyte proliferation,[22–26] we observed that AR stimulated ERK-1/-2, JNK, and Akt phosphorylation (a downstream target of PI-3K) and that in the presence of specific inhibitors of these pathways, AR-induced DNA synthesis was decreased. Although we did not test p38 activation by AR treatment, stimulation of DNA synthesis by AR was not affected by a p38 MAPK inhibitor, thus suggesting that this kinase is not involved in AR mitogenic effects.

Using primary cultured hepatocytes, we have addressed the potential signaling mechanisms responsible for the induction of *AR* gene expression in the liver. We tested the effect of different cytokines, growth factors, and mediators involved in the early phases of liver regeneration after injury and PH. We observed that IL-1β treatment significantly increased *AR* mRNA levels in isolated hepatocytes. The induction of the *AR* gene expression by IL-1β in cultured cells is consistent with the early production of this cytokine in different experimental models of liver injury and regeneration in which we observed *AR* up-regulation.[17,28] In contrast, IL-6, TNF-α, and HGF failed to activate *AR* gene expression in isolated hepatocytes. It is interesting to note that TNF-α[29] and HGF,[30] but not IL-1β (unpublished data), are able to induce the expression of other EGF-R ligands, such as TGF-α, in hepatocytes. Together these observations attest to the diversity of signals involved in the regulation of the apparently redundant pathways involved in liver regeneration.

Prostaglandins are produced in the liver very early after PH and are necessary for a successful regenerative response.[18] It has been recently reported that the expression of *AR* in colon cancer cells is induced by PGE_2 generated by cyclooxygenase 2.[19] Now we show that PGE_2 can also stimulate the expression of AR in the hepatocyte. The effect of PGE_2 on *AR* gene transcription in colon cancer cells is mediated through the cAMP/protein kinase A pathway acting on a CRE in the *AR* promoter.[9,19] In line with these observations, we noticed that increased intracellular cAMP levels also promoted *AR* gene expression in hepatocytes. It is interesting to note that transfection of hepatocytes with an expression vector harboring *WT1* complementary DNA promoted the accumulation of *AR* mRNA. It is worth mentioning that WT1 seems to activate the same CRE site in the *AR* promoter that is involved in PGE_2-mediated AR transcription.[9,19] Altogether, these experiments lend support to the hypothesis that in the chronically injured liver, the induction of *AR* gene expression can be mediated in part by *WT1* up-regulation, whereas in situations of acute liver injury or liver regeneration after PH (in which *WT1*

expression is not detected; data not shown), other mediators, such as IL-1β and PGE_2, may promote *AR* gene expression. Finally, our observations regarding the inducibility of *AR* gene expression in isolated hepatocytes are in agreement with a previous report showing the production of AR by spontaneously growing hepatocytes in hepatic organoid cultures.[31]

Rapid tyrosine phosphorylation of the EGF-R occurs shortly after PH in rats.[1] This fact, together with the mitogenic potential of EGF-R ligands for hepatocytes in culture, suggests a significant role for EGF-R activation in the early stages of liver regeneration.[1–4] The expression of other EGF-R ligands and hepatocyte mitogens, such as TGF-α and heparin-binding EGF-like growth factor (HB-EGF), has been previously examined in rat liver after PH.[32,33] Maximal *TGF-α* expression occurs 24 hours after PH, whereas peak *HB-EGF* mRNA levels are detected 6 hours after liver resection. Similar to what has been described for HB-EGF, we have found that *AR* gene expression is rapidly induced in the liver of rats after PH. Experiments performed in *TGF-α*–null mice indicated that this factor was not necessary for liver regeneration.[34] The normal response to PH observed in these mice was attributed to the compensatory increase in other members of the EGF-R family of ligands, such as EGF.[34] Another good candidate besides AR would be HB-EGF; however, the physiological role of HB-EGF in liver regeneration has not yet been tested in the *HB-EGF*–null mouse.[35] Our present findings indicate the lack of AR results in deregulation of cell cycle–associated mechanisms resulting in impaired DNA synthesis after liver resection. The blunted induction of immediate-early genes in $AR^{-/-}$ mice reflects the involvement of this growth factor in the very early steps of hepatocyte proliferation. It is interesting to note that the induction of immediate-early genes such as *c-fos* is not altered in the liver of a conditional *Met* mutant mouse model after PH.[27] The existence of other growth factor signaling pathways cooperating with HGF/c-met in the early control of hepatocyte proliferation has been also suggested in an independent study with conditional *Met* mutant mice.[36] Our present data suggest that AR, probably together with other cytokines such as IL-6,[3] could be responsible for this effect. Taken together, our observations indicate that AR is a new player in liver regeneration and has a prominent role in triggering the early phases of this process.

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Received August 16, 2004. Accepted October 27, 2004.

Address requests for reprints to: Jesús Prieto, MD, Division of Hepatology and Gene Therapy, Department of Medicine, Clínica Universitaria, University of Navarra, 31008 Pamplona, Spain. e-mail: jprieto@unav.es; fax: (34) 948-29-6785.

Address correspondence to: Matias A. Avila, PhD, Division of Hepatology and Gene Therapy, CIMA, Universidad de Navarra, 31008 Pamplona, Spain. e-mail: maavila@unav.es; fax: (34) 948-194717.

This work was supported by the agreement between Fundación Investigación Médica Aplicada (FIMA) and the "Unión Temporal de Empresas (UTE) project CIMA," Grants C03/02 and G03/015 from Instituto de Salud Carlos III (C.B., E.R.G.-T., J.P., M.A.A.), Grant SAF-2004-03538 (M.A.A.), National Institutes of Health Grants CA49793 and CA43793 (D.C.L.), and Grant FISPI040819 from Ministerio de Sanidad (to C. B.). E.E. is supported by a fellowship from Gobierno de Navarra, Spain.

J.P. and M.A.A. share senior authorship.

The authors thank Dr. P. Dempsey, Pacific Northwest Research Institute (Seattle, Washington), for his help with amphiregulin immunoblots, and Eva Petri for technical assistance.