The hepatic wound-healing response to chronic noxious stimuli may lead to liver fibrosis, a condition characterized by excessive deposition of extracellular matrix. Fibrogenic cells, including hepatic stellate cells and myofibroblasts, are activated in response to a variety of cytokines, growth factors, and inflammatory mediators. The involvement of members of the epidermal growth factor family in this process has been suggested. Amphiregulin (AR) is an epidermal growth factor receptor (EGFR) ligand specifically induced upon liver injury. Here, we have addressed the in vivo role of AR in experimental liver fibrosis. To this end, liver fibrosis was induced in AR+/+ and AR−/− mice by chronic CCl₄ administration. Histological and molecular markers of hepatic fibrogenesis were measured. Additionally, the response of cultured human and mouse liver fibrogenic cells to AR was evaluated. We observed that AR was expressed in isolated Kupffer cells and liver fibrogenic cells in response to inflammatory stimuli and platelet-derived growth factor, respectively. We demonstrate that the expression of α-smooth muscle actin and collagen deposition were markedly reduced in AR−/− mice compared to AR+/+ animals. AR−/− mice also showed reduced expression of tissue inhibitor of metalloproteinases-1 and connective tissue growth factor, two genes that responded to AR treatment in cultured fibrogenic cells. AR also stimulated cell proliferation and exerted a potent antiapoptotic effect on isolated fibrogenic cells. Conclusion: These results indicate that among the different EGFR ligands, AR plays a specific role in liver fibrosis. AR may contribute to the expression of fibrogenic mediators, as well as to the growth and survival of fibrogenic cells. Additionally, our data lend further support to the role of the EGFR system in hepatic fibrogenesis. (HEPATOLOGY 2008;48:1251-1260.)

Abbreviations: AR, amphiregulin; CHX, cycloheximide; CM, conditioned medium; CTGF, connective tissue growth factor; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular regulated kinase 1/2; FAK, focal adhesion kinase; FasL, Fas ligand; FBS, fetal bovine serum; HSCs, hepatic stellate cells; IL, interleukin; JNK, jun N-terminal kinase; KC, Kupffer cells; LPS, bacterial lipopolysaccharide; MFB, myofibroblasts; MEK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; PI-3K, phosphatidylinositol 3-kinase; αSMA, α-smooth muscle actin; TACE, tumor necrosis factor-α converting enzyme; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinases; TNFα, tumor necrosis factor-α.

From the 1Division of Hepatology and Gene Therapy, CIMA, University of Navarra, Pamplona, Spain, 2University Campus Bio-Medico of Rome, Rome, Italy, 3Institut National de la Santé et de la Recherche Médicale, Unité 841, Créteil, France, 4Université Paris 12, Faculté de Médecine, Créteil, France, 5Assistance Publique-Hôpitaux de Paris, Groupe Hospitalier Henri Mondor-Albert Chenevier, Service d’Hépatologie et de Gastroentérologie, Créteil, France, and 6Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, University Clinic, Pamplona, Spain.

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*These authors contributed equally to this work.
†Equal senior authors.

Address reprint requests to: Dr. Matias A. Avila or Dr. Carmen Berasain, Division of Hepatology and Gene Therapy, CIMA, University of Navarra, Avenida Pio XII, n°55, 31008 Pamplona, Spain. E-mail: maavila@unav.es (M.A.A.) and cberasain@unav.es (C.B.); fax: 34-948-194717.

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During chronic liver injury, the persistent wound-healing response of the organ may result in the progressive substitution of normal hepatic parenchyma by fibrous scar tissue, with the consequent alteration of the normal tissue architecture. In a significant number of patients, chronic liver injury progresses to liver cirrhosis characterized by massive deposition of extracellular matrix (ECM), the formation of nodules of regenerating hepatocytes, and a profound impairment of liver function. Current understanding of the pathogenesis of liver fibrosis indicates that ECM accumulation results from the disruption of normal matrix homeostasis in favor of net deposition of fibrillar collagen. Improving our knowledge of the mechanisms involved in hepatic fibrogenesis will increase the possibilities of therapeutic intervention.

The ECM produced in chronic liver injury originates from myofibroblastic cells (MFBs) deriving from distinct cell populations, including hepatic stellate cells (HSCs) and portal fibroblasts. Activation of these matrix-producing cells occurs upon tissue injury through a complex interplay among different cell types. The profibrogenic mediators can be produced by hepatocytes, Kupffer cells (KCs) or endothelial cells, as well as by infiltrating nonhepatic cells, and act on MFBs in a paracrine fashion. Additionally, activated MFBs are capable of autocrine stimulation mediated by the concomitant expression of activating factors and their receptors. These factors include reactive oxygen species, inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, monocyte chemotactic protein type 1, and tumor necrosis factor-α (TNFα), vasoactive cytokines, and adipokines. Growth factors like platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), and transforming growth factor-β (TGFβ) are known to play central roles in the proliferation, survival, and acquisition of the myofibroblastic phenotype of fibrogenic cells.

Activation of the epidermal growth factor receptor (EGFR) on ECM-producing cells has also been recognized to contribute to their phenotypic transformation. EGFR ligands such as EGF and TGFα are released during liver injury and inflammation, and in vitro experiments have shown that these factors can stimulate the proliferation and migratory properties of fibrogenic cells. Besides EGF and TGFα, EGFR may be activated by heparin-binding EGF, epiregulin, betacellulin, and amphiregulin (AR). To the best of our knowledge, the relative contribution of the different EGFR ligands to liver fibrogenesis in vivo has not been evaluated so far. The expression of AR is hardly detectable in the healthy liver; however, it is readily induced upon acute injury.
injury and inflammation and remains elevated in cirrhosis. Here, we have evaluated whether AR participates in hepatic fibrogenesis. Our data show that AR gene expression is up-regulated during CCl4-induced liver fibrogenesis and that AR-deficient mice develop significantly less collagen accumulation, suggesting that this EGFR ligand plays a nonredundant role in hepatic fibrosis.

Materials and Methods

Experimental Model of Fibrosis and Histological Analyses. Male AR+/+ and AR−/− littersmates (20 g) (n = 4-5 per condition and time-point) were used. Liver fibrosis was induced by intraperitoneal injection of 0.6 μL/g of body weight of CCl4 twice a week. The CCl4 was diluted in olive oil (1:4), and control mice received the same volume of vehicle. Animals were treated for 4 or 6 weeks and were sacrificed 1 or 4 days after the last injection. Animals received humane care according to National Institutes of Health guidelines (NIH publication 86-23, revised 1985). Details about histological analyses and immunostaining are described in Supplementary Materials and Methods.

Isolation, Culture, and Treatment of Mouse KCs, HSCs, Hepatic MFBs, and Human LX-2 Cells. Details are described in Supplementary Materials and Methods.

Measurement of Apoptosis. Cell death enzyme-linked immunosorbent assays (ELISAs) were performed using the Cell Death Detection Assay (Roche). RNA Isolation and Gene Expression Analyses. RNA was extracted as described. Real-time polymerase chain reaction (PCR) was performed using an iCycler (Bio-Rad Laboratories, Hercules, CA) and the iQ SYBR Green Supermix (Bio-Rad). Gene expression was determined using the ΔCT calculation.
**Results**

**AR Is Expressed During Mouse Liver Fibrogenesis.** After 4 weeks of 
CCl\textsubscript{4} treatment, the presence of fibrous septa was already 
ev
dent, progressing to extensive collagenous networks by 6 weeks (Fig. 1A). AR gene expression 
was significantly induced after 4 and 6 weeks of CCl\textsubscript{4} administration (Fig. 1A). We observed that AR is 
expressed in isolated mouse KCs, and that it is up-regulated upon treatment with lipopolysaccharide (LPS), TNF\textalpha, or 
IL-1\beta (Fig. 1B). Additionally, we also show that AR is 
expressed in primary mouse MFBs and human HSCs (LX-2) in response to EGF or PDGF (Fig. 1C). AR protein 
is produced as a membrane-anchored precursor that is released from the cell by the tumor necrosis factor-\alpha 
converting enzyme (TACE/ADAM17).\textsuperscript{18} Expression of TACE messenger RNA (mRNA) was observed in primary 
mouse MFBs and KCs, as well as in LX-2 cells (Fig. 1D). TACE mRNA levels correlated with the presence of 
TACE protein, detected as the full-length precursor (pro-TACE) and the mature form as described for other cell 
types\textsuperscript{18} (Fig. 1E). In agreement with these observations, AR protein was detected by ELISA in the cellular matrix 
of LX-2 cells stimulated with EGF or PDGF (Fig. 1F).

**CCl\textsubscript{4}-Induced Fibrosis Is Attenuated in AR\textsuperscript{−/−} Mice.** To evaluate the potential contribution of AR to 
liver fibrosis, AR\textsuperscript{+/+} and AR\textsuperscript{−/−} mice were treated 
with CCl\textsubscript{4} for 4 and 6 weeks. Animals were sacrificed at 
1 or 4 days (recovery phase), after the last CCl\textsubscript{4} injec-
tion. Sirius Red staining of liver sections revealed the 
presence of fibrosis in AR\textsuperscript{+/+} mice at 4 weeks of 
treatment, with evidence of bridging fibrosis after 6 weeks (Fig. 2A). Livers from AR\textsuperscript{−/−} mice showed significant 
attenuation of the fibrogenic response. Morphometric quantification of the Sirius Red–stained areas con-
firmed the reduced accumulation of cross-linked colla-
gen in AR\textsuperscript{−/−} mice and also showed an attenuated 
recovery reaction (Fig. 2B). To assess whether the re-
duced fibrogenic response in AR\textsuperscript{−/−} mice could be 
due to diminished liver injury we measured circulating 
aspartate aminotransferase and alanine aminotransfer-
ase levels. After 4 weeks of CCl\textsubscript{4} administration 
transaminase levels in AR\textsuperscript{−/−} animals were slightly 
higher than in AR\textsuperscript{+/+} mice, but this tendency was not 
appreciated at 6 weeks (Fig. 2C). Accordingly, hema-
toxylin & eosin staining revealed similar histological 
characteristics (hepatocellular damage and inflamma-
tory infiltration) in the two genotypes (not shown).

**Immunoblotting, AR ELISA, and Assay of Hepatic TGF\textbeta.** Details are described in Supplementary Material-
s and Methods.

**Statistical Analysis.** Data are the means ± standard 
error of the mean (SEM). Unless otherwise stated, ex-
periments were performed at least three times in duplicate. 
Statistical significance was estimated with the Mann-
Whitney test. A P value of <0.05 was considered signif-
icant.
We also observed comparable basal levels of glial fibrillary acidic protein and vimentin mRNAs (data not shown), both markers of HSCs, indicating that apparently there were no basal differences in the number or activation state of ECM-producing cells between both genotypes.

In agreement with histological data, expression of \( \alpha 1(I) \) procollagen mRNA was reduced in AR-deficient mice (Fig. 4A). In rodents, expression of the interstitial collagenase matrix metalloproteinase 13 (MMP13) and its inhibitor tissue inhibitor of metalloproteinase 1 (TIMP1) is induced during fibrogenesis. We observed that CCl4-treated \( AR^{+/+} \) mice displayed enhanced TIMP1 mRNA levels compared to \( AR^{-/-} \) mice (Fig. 4B). Similarly, the expression of MMP13 was higher in \( AR^{+/+} \) animals at earlier stages of fibrogenesis (4 weeks) (Fig. 4C). CTGF expression was also higher in \( AR^{+/+} \) than in \( AR^{-/-} \) mice (Fig. 4D). The expression of EGF, TGF\( \alpha \), and heparin-binding EGF in the livers of CCl4-treated \( AR^{+/+} \) and \( AR^{-/-} \) mice was not different (not shown). However, TGF\( \beta \) protein levels were significantly higher in \( AR^{+/+} \) than in \( AR^{-/-} \) mice when tested at 4 weeks of CCl4 treatment (430 ± 15 versus 250 ± 20 pg/mg of liver protein lysate, \( P < 0.05 \)).

**Direct Effects of AR on ECM-Producing Cells.** Human LX-2 HSCs, primary mouse MFBs, or quiescent mouse HSCs were treated with AR and the expression of key genes involved in liver fibrosis was measured. AR induced the expression of TIMP1 and also that of CTGF.
Interestingly, AR was also able to stimulate its own expression in ECM-producing cells (Fig. 5A-C). We further examined the effects of AR on freshly isolated mouse HSCs, and observed the specific activation of EGFR and downstream signaling through mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1 (MEK)/extracellular regulated kinase 1/2 (ERK1/2) and Akt (Fig. 6A). In these cells, AR stimulated the expression of cell proliferation–related early-response genes, such as c-fos and Egr-1, in an EGFR/MEK/ERK1/2-dependent manner (Fig. 6B). Accordingly, we observed that AR stimulated cell growth (Fig. 6C) and DNA synthesis in mouse HSCs. Treatment for 48 hours with AR at 50 nmol/L elicited a 147% increase versus controls in [3H]thymidine incorporation into DNA, whereas PDGF-BB treatment at 20 ng/mL resulted in a 142% increase, values that are consistent with previous observations in mouse HSCs.22

Survival of activated ECM-producing cells is essential for the progression of liver fibrosis.23 Therefore, we evaluated the effects of AR on apoptosis induced by serum withdrawal in LX-2 cells and mouse MFBs. AR significantly suppressed apoptosis in both cell types (Fig. 7A,B). In concordance with the antiapoptotic effect of AR, we observed that production of the active caspase-3 p17 subunit was inhibited by AR (Fig. 7A,B, lower panels). We could also demonstrate that AR protected LX-2 cells from apoptosis induced by potent proapoptotic stimuli such as TNFα or Fas ligand (FasL) in the presence of cycloheximide23,24 (Fig. 7C,D).

Next, we explored the signaling mechanisms involved in the antiapoptotic effects of AR. EGFR activation triggers intracellular pathways that may be relevant for the activation, proliferation, and survival of ECM-producing cells.11,14,25-28 We observed that incubation of LX-2 cells and mouse MFBs with AR rapidly induced the phosphorylation of the EGFR (Fig. 8A). Albeit with different kinetics, triggering of EGFR by AR was accompanied by the activation of Akt, ERK1/2, Jun N-terminal kinase (JNK), and focal adhesion kinase (FAK) phosphorylation in both cell types (Fig. 8A). We next tested the relative contribution of these signaling pathways to the antiapoptotic effects of AR on serum-starved MFB and LX-2 cells. Inhibition of EGFR tyrosine kinase by PD153035 resulted in the complete abolition of the cytoprotective effect of AR (Fig. 8B,C). Interestingly, the specific inhibition of phosphoinositide 3-kinase (PI-3K), MEK, JNK, or p38 (not shown) did not significantly impair the antiapoptotic effects of AR (Fig. 8B,C).

**Discussion**

EGFR ligands such as EGF and TGFα are known to stimulate the proliferation and activation of isolated hepatic ECM-producing cells.10,12-14,29 However, the relative contribution of the EGF-related factors to liver fibrogenesis is still unknown. Our study provides *in vivo*...
evidence showing that AR contributes to the pathologic accumulation of ECM after chronic injury.

AR gene expression in the healthy liver is very low or undetectable; however, it is significantly elevated during acute damage and in liver cirrhosis.\(^\text{15}\) Now, we observed that AR was constantly expressed in the mouse liver parenchyma during CCl\(_4\)-induced fibrogenesis. Our experiments with isolated nonparenchymal liver cells show for the first time that AR can be up-regulated in response to the key profibrogenic factor PDGF, as well as through the activation of the EGFR, in mouse and human ECM-producing cells. Previously, we reported that AR expression was induced in mouse liver upon bacterial lipopolysaccharide (LPS) injection, and also in isolated hepatocytes treated with inflammatory cytokines like IL-1\(\beta\).\(^\text{16,17}\) Here, we show that TNF\(\alpha\) and IL-1\(\beta\) also stimulated AR expression in isolated KCs, as did the toll-like receptor-4 ligand LPS. These findings, together with the attenuated fibrogenic response of AR\(^{-/-}\) mice described here, suggest that AR may represent an additional link between hepatic inflammation and fibrogenesis, an association found in clinical and experimental fibrosis, the mechanisms of which are currently being exposed.\(^\text{2,8,9,30}\)

AR-deficient mice showed diminished collagen accumulation and a significant reduction in the number of fibrogenic cells as indicated by decreased expression and staining of \(\alpha\)SMA. These findings were paralleled by reduced levels of \(\alpha\)I(I)procollagen, TIMP1 and CTGF mRNAs, and the profibrogenic cytokine TGF\(\beta\) in AR\(^{-/-}\) mice as compared to normal mice undergoing CCl\(_4\)-induced fibrogenesis. The expression of MMP13 was higher in AR\(+/+\) mice at peak fibrosis, indicative of a strong tissue remodeling activity.\(^\text{21}\) Up-regulation of MMP13 participates in fibrosis resolution after prolonged CCl\(_4\) injury in mice.\(^\text{31}\) In spite of this, we observed enhanced collagen deposition in AR\(+/+\) mice. This can be explained in part by the increased expression of TIMP1, the principal inhibitor of MMP13,\(^\text{2,3,9,31}\) in the liver of wild-type animals. Additionally, the prosurvival effects of TIMP1 toward HSCs\(^\text{32}\) may also contribute to the increased ECM accumulation observed in AR\(+/+\) mice. Nevertheless, the impaired expression of MMP13 and the slow recovery from fibrosis displayed by AR\(^{-/-}\) mice may also suggest the implication of AR in the mechanisms involved in fibrosis resolution. This is consistent with the view of liver fibrogenesis as a chronic wound-healing response, in which the expression of ECM components and ECM-degrading enzymes is triggered almost concomitantly and in many cases by the same factors.\(^\text{1-4,21}\)

We also examined the effects of the direct interaction of AR with ECM-producing cells. We observed that AR could promote the expression of TIMP1 and CTGF...
genes. Although TIMP1 production during liver injury can be elicited by profibrogenic and inflammatory mediators such as leptin, IL-6 family members, and TGFβ21,22,34 our current findings suggest that the AR/EGFR system may also contribute to TIMP1 expression. The identification of CTGF as a direct target of AR effects is also relevant. Activation of CTGF expression in HSCs has been demonstrated in human and experimental fibrogenesis,1 and small interfering RNA–mediated knockdown of CTGF prevents liver fibrosis.35 So far, CTGF expression was known to be stimulated by TGFβ or PDGF in HSCs.36 Now, its reduced expression levels in AR−/− mice and its activation by AR in cultured ECM-producing cells, identifies a novel activation pathway for CTGF. Nevertheless, perhaps a more important finding was that these effects of AR on TIMP1 and CTGF gene expression could be recapitulated in freshly isolated quiescent mouse HSCs. This may indicate that AR, which is readily induced upon liver tissue injury,17 could be an early trigger of the hepatic wound-healing response, even before the ECM-producing cells become fully activated and responsive to other regulators like PDGF.37 Additionally, we also observed that AR treatment stimulated its own expression in ECM-producing cells. This suggests that the effects of AR synthesized in hepatocytes and KCs in response to inflammatory mediators may be amplified in fibrogenic cells through AR-mediated paracrine or autocrine signaling, important cell communication mecha-

Fig. 7. Antiapoptotic effects of AR on liver fibrogenic cells. LX-2 cells (A), or mouse MFBs (B) were cultured in serum-free medium for 12 hours in the absence or presence of AR (50 nmol/L). Apoptosis was determined by ELISA determination of soluble histone-DNA complexes (*P < 0.05 versus untreated cells). Lower panels show the Western blot analyses of the active caspase-3 p17 subunit. (C) Cells were pretreated with AR (50 nmol/L) for 3 hours, then CHX (20 μg/mL) was added to the cultures for 30 minutes, and subsequently cells were treated with TNFα (20 ng/mL) or FasL (50 ng/mL) for 12 hours. Apoptosis was measured by ELISA determination of soluble histone-DNA complexes (*P < 0.05 with respect to AR-untreated cells). (D) Western blot analyses of the active caspase-3 p17 subunit in samples described in (C).
nisms proposed by early research to participate in HSC activation.12,29

Activation of EGFR tyrosine kinase on ECM-producing cells has been essentially associated with the stimulation of cell proliferation.10-13 We observed that AR treatment triggered signaling pathways such as ERK1/2,38,39 FAK,28 PI-3K/Akt,25,28,39 and JNK26 that are connected with cell proliferation in human and rodent liver fibrogenic cells. Consistently, through the EGFR/ERK1/2 pathway, AR up-regulated the expression of growth-related transcription factors such as c-fos and Egr-1, and stimulated the growth and proliferation of mouse HSCs. However, intracellular signals emanating from the EGFR, being elicited by AR or other EGFR ligands, can also generate potent antiapoptotic stimuli, as previously shown in other cell types including hepatocytes.15,17 Here we demonstrated that AR can overcome apoptosis induced by FasL or TNFα in LX-2 cells, or by serum starvation in both LX-2 and mouse MFBs. In agreement with our previous observations on isolated hepatocytes,17 the antiapoptotic effects of AR on fibrogenic cells were mediated through the activation of the EGFR. However, downstream of the EGFR, the independent inhibition of highly protective pathways like JNK,26 PI-3K/Akt,39,40 or ERK1/226,40 did not abolish the antiapoptotic effects of AR. This is in contrast to what has been recently observed for the fibrogenic cytokine leptin, of which its prosurvival effects on HSCs were strictly dependent on PI-3K/Akt activation.39 These findings indicate that AR is a potent survival factor for ECM-producing cells, able to elicit redundant antiapoptotic mechanisms that deserve further consideration. In the in vivo setting, AR-mediated escape from apoptosis, together with its promitogenic effects, may be important mechanisms in the promotion of fibrosis.2,7,24,32 However, and in agreement with the potent effects of AR on
freshly isolated nonactivated HSCs, these actions of AR may be relevant mainly during the early stages of the fibrogenic process, because we did not find significant differences in the relative numbers of proliferating and apoptotic α-SMA–positive cells between AR+/+ and AR−/− mice after 4 or 6 weeks of CCl4 treatment (data not shown).

Persistent activation of the EGFR has been cogently demonstrated to participate in the pathogenesis of tissue fibrosis in different organs, including the lung and kidney.41,42 Furthermore, the EGFR inhibitor gefitinib is a safe and effective inhibitor of experimental kidney fibrosis43 and hepatocarcinogenesis.44 Our current findings, in addition to furthering our understanding of the mechanisms underlying hepatic fibrogenesis, also suggest that the AR/EGFR signaling system could be a new target in the prevention of liver fibrosis.

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