Novel Role for Amphiregulin in Protection from Liver Injury*

Clinical Role, the Fas and Fas ligand system plays a central role in the development of hepatocyte apoptosis, a process contributing to a broad spectrum of liver diseases. Therefore, the development of therapies aimed at the inhibition of hepatocyte apoptosis is a major issue. Activation of the epidermal growth factor receptor has been shown to convey survival signals to the hepatocyte. To define endogenous response of hepatocytes to growth factor receptor ligands during Fas-mediated liver injury we investigated the expression of epidermal growth factor, transforming growth factor α, heparin-binding epidermal growth factor-like growth factor, betacellulin, epiregulin, and amphiregulin in the liver of mice challenged with Fas-agonist antibody. Amphiregulin expression, barely detectable in healthy liver, was significantly up-regulated. Amphiregulin administration abrogated Fas-mediated liver injury in mice and showed direct anti-apoptotic effects in primary hepatocytes. Amphiregulin activated the Akt and signal transducer and activator of transcription-3 survival pathways, and up-regulated Bcl-xL expression. Amphiregulin knock-out mice showed signs of chronic liver damage in the absence of any noxious treatment, and died faster than wild type mice in response to lethal doses of Fas-agonist antibody. In contrast, these mice were more resistant against sublethal liver damage, supporting the hypothesis that chronic liver injury can precondition hepatocytes inducing resistance to subsequent cell death. These results show that amphiregulin is a protective factor induced in response to liver damage and that it may be therapeutic in liver diseases.

Hepatocyte apoptosis is a common mechanism to many forms of liver disease. It has been recognized to contribute to the pathogenesis of alcoholic liver disease, viral hepatitis, cholestatic liver disease, and ischemia/reperfusion injury (1–3). More recently hepatocyte apoptosis has been reported to occur in the liver of patients with nonalcoholic steatohepatitis, correlating with the severity of the disease (4). The consequences of hepatocellular apoptosis may extend beyond the mere loss of functional liver mass, because moderate and persistent apoptosis may contribute to the development of liver inflammation and fibrosis (5, 6). Apoptosis can be triggered by the activation of two molecular pathways: an intrinsic mitochondrially mediated cascade and a death receptor pathway. In the liver, the death receptor pathway appears to be predominant and involves the activation of cytokine receptors such as Fas, tumor necrosis factor receptor-1 and tumor necrosis factor-related apoptosis-inducing ligand receptors-1 and -2 (1, 2, 6). Upon activation by their respective ligands these receptors initiate intracellular signaling cascades that ultimately lead to the activation of death-inducing proteolytic enzymes (1, 2). Fas is abundantly expressed in the liver, and Fas-mediated apoptosis has been shown to significantly contribute to toxic or viral damage and acute liver failure (7–9). Interestingly, hepatocyte apoptosis observed in cholestasis is initiated by the ligand-independent activation of Fas by hydrophobic bile salts (1, 10). In addition, lack of Fas expression or RNA interference targeting of Fas results in significant protection from fulminant hepatitis (7, 11). Altogether these observations underscore the clinical relevance of the Fas pathway in liver disease.

The identification of agents that reduce hepatocellular apoptosis is thus of special significance for the development of hepatoprotective therapeutics (12, 13). Intensive research in this area has shown that cytokines such as interleukin-6 (IL-6), growth factors like hepatocyte growth factor, or the hepatocyte-derived insulin-like growth factor binding protein-1 display potent hepatoprotective effects, including the attenuation of apoptosis induced by Fas ligation (14–17). Activation of the epidermal growth factor receptor (EGF-R), a transmembrane protein endowed with tyrosine kinase activity, has been also recognized to convey survival and proliferative signals for the hepatocyte (18–24). Mature hepatocytes express the highest levels of EGF-R of any normal cell type, suggesting an important role for EGF-R signaling in liver function (25, 26). This receptor can be bound and activated by a broad family of ligands that, besides EGF, include transforming growth factor

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† The abbreviations used are: IL-6, interleukin-6; ALT, alanine aminotransferase; AR, amphiregulin; AST, aspartate aminotransferase; BTC, betacellulin; EGF, epidermal growth factor; EPR, epiregulin; ERK, extracellular regulated kinase; HB-EGF, heparin-binding EGF-like growth factor; MEK1, mitogen-activated protein kinase kinase 1; JNK, c-Jun N-terminal kinase; P38, MAP kinase; STAT3, signal transducer and activator of transcription 3; TGF, transforming growth factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling.
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**RESULTS**

Expression of EGF-R Ligands in Mouse Liver after the Administration of the Fas-agonistic Antibody Jo2—The expression of the EGF-R ligands EGF, TGFα, HB-EGF, BTC, EPR, and AR was studied by real-time PCR in the liver of control mice and in mice that received a dose of Jo2 antibody previously shown to cause significant liver damage and apoptosis (15). Liver samples were taken for analysis 2, 5, and 10 h after Jo2 administration. As depicted in Fig. 1A, serum levels of transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were still normal 2 h after Jo2 injection, increased remarkably between 5 and 10 h of treatment, and decreased thereafter. As shown in Fig. 1B we observed different patterns of response to Jo2 treatment among EGF-R ligands. HB-EGF gene expression showed a slight reduction early after Jo2 injection, but remained close to control values for the rest of the study. TGFα gene expression showed a peak 2 h after Jo2 administration and was rapidly down-regulated, reaching values below control levels by 10 h of treatment. EGF gene expression did not change significantly early after Jo2 injection, but dropped below control values 5 h after treatment. Similarly, BTC gene expression was significantly down-regulated between 2 and 5 h after Jo2 injection and remained so at the last time point tested. The two other EGF-R ligands examined, namely AR and EPR, showed a clear up-regulation in Jo2-treated mice. EPR gene expression was progressively induced, being maximal 10 h post-Jo2 administration, the latest time point tested. Interestingly, in agreement with other reports and our previous observations we noticed that AR gene expression was barely detectable in normal liver (35, 36); however, it was potently induced between 2 and 5 h after Jo2 injection.

**Measurement of Caspase-3 Activity**—Caspase-3 activity was measured in mouse hepatocytes and liver tissue lysates using the Caspase-3/CFPP93 Colorimetric Assay Kit (BioVision, Palo Alto, CA). Cells (5 × 10⁵) were plated in culture plates and cultured in growth media supplemented with 10% fetal calf serum. After 2 h incubation medium was removed, and cells were rinsed with PBS and centrifuged at 15,000 rpm for 10 min. Cell lysates and supernatants from liver homogenates (200 μg in 50 μl) were used to measure caspase-3 activity following the manufacturer’s instructions.

**Western Blot Analysis**—Homogenates from liver samples and isolated hepatocytes were subjected to Western blot analysis as described (30). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and visualized with anti-AR polyclonal antibody (BAF989) (BD Systems), actin as a loading control, and secondary antibodies conjugated to HRP. The membrane was exposed to X-ray film. Densitometric analyses were performed using the Gel-Pro analysis software.

**Statistical Analysis**—Data are means ± S.E. Statistical significance was estimated with the Mann-Whitney test. A p value of <0.05 was considered significant.
FIG. 1. A, serum levels of ALT and AST (10–1 IU/liter) at different time points after Jo2 antibody injection in mice. B, gene expression profiles of different EGF-R ligands in the liver of control mice (C), and at various times after Jo2 antibody injection as determined by real-time PCR (n = 4 animals per point). BTC, betacellulin; EPR, epiregulin; TGFα, transforming growth factor α; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF; AR, amphiregulin. Asterisks indicate p < 0.05 versus control mice.

FIG. 2. A, AR gene expression was assessed by RT-PCR in the liver of control mice and in the liver of mice treated with Jo2 antibody for 5 h. Western blot analysis of liver extracts from control and Jo2-treated mice performed with a biotinylated affinity purified anti-AR antibody. Samples were obtained after 10 h of treatment with Jo2 antibody. Arrowheads indicate the different AR forms. Three representative samples are shown per group.

Injection, preceding that of EPR. In concordance with the up-regulation of AR mRNA levels, Western blot analyses performed with a biotinylated affinity-purified anti-mouse AR antibody on liver samples obtained 10 h after Jo2 antibody administration allowed us to detect a set of proteins that were present in the liver of treated mice (Fig. 2). Four bands of ~50, 43, 28, and 19 kDa are consistent with the different forms of AR described in epithelial cells (36). The 50- and 28-kDa bands likely represent membrane-anchored forms of AR, whereas the 43- and 19-kDa bands may be proteolytically processed soluble forms of AR (36). The fact that AR gene expression becomes induced during the early time period following Jo2 treatment suggested that AR may be protective against Fas-mediated apoptosis. This observation led us to evaluate the in vivo hepatoprotective effects of this EGF-R ligand.

AR Administration Attenuates Fas-mediated Acute Liver Injury in Mice—To test if AR can limit the extent of liver injury we examined the effect of AR administration in mice challenged with Jo2 antibody. Serum levels of ALT and AST were greatly elevated 10 h after Jo2 injection (Fig. 3A). AR treatment potently suppressed liver damage, as indicated by the reduced levels of serum transaminases and histopathological analysis of liver tissue sections from AR-treated mice, showing how this growth factor prevented the destruction of the parenchymal architecture induced by Fas ligation (Fig. 3A). As mentioned above, apoptotic cell death is a major determinant in Fas-mediated liver damage. To confirm that the hepatoprotective effects of AR against Fas-mediated liver injury derived from an anti-apoptotic action, we performed TUNEL staining and measured caspase-3 cleavage and activity in mouse liver extracts. TUNEL staining showed profuse apoptotic injury in Jo2-treated mice (Fig. 3B), which was confirmed by cleavage and activation of caspase-3 (Fig. 3D). Bcl-2 family proteins inhibit apoptosis induced by variety of stimuli, including Fas-mediated apoptosis (37–39). We assessed the expression of the antiapoptotic protein Bcl-xL by Western blotting 10 h after the injection of anti-Fas antibody. Bcl-xL proteins levels were decreased in the liver of mice treated with Jo2; however, AR administration helped to maintain the Bcl-xL protein levels found in control mouse liver (Fig. 3D).

Interestingly, AR mRNA levels are also rapidly induced (between 2 and 3 h) in other models of hepatic injury such as acute CCl4 or bacterial lipopolysaccharide administration (data not shown). CCl4 causes necrosis and apoptosis in liver cells through the promotion of membrane lipid peroxidation and the production of high levels of tumor necrosis factor α (40, 41). The administration of AR 0.5 h prior and 12 h after CCl4 significantly prevented the development of tissue injury and the rise in serum transaminases: from 9193 ± 908 IU/ml ALT in CCl4-treated mice to 4688 ± 735 IU/ml ALT in CCl4 plus AR-treated mice, p < 0.05. In the same animals AST levels were reduced from 8579 ± 735 IU/ml in AR-treated mice, p < 0.05.

Direct Antiapoptotic Effect of AR on Primary Cultured Hepatocytes—To determine whether the in vivo antiapoptotic effects of AR could be mediated by a direct action of AR on the liver...
parenchymal cells, we used mouse hepatocytes in primary culture. It has been described that hepatocytes exposed to anti-Fas antibodies (Jo2) efficiently undergo apoptosis in the presence of actinomycin D (15, 18, 42). Hepatocytes were pretreated with different concentrations of AR for 3 h prior to actinomycin D and Jo2 antibody addition. Apoptosis, and apoptosis-related molecular events, were measured 18 h later. As shown in Fig. 4A, AR dose-dependently protected hepatocytes from apoptosis indicating a direct cytoprotective effect of AR in the prevention of Fas-mediated liver apoptosis. The antiapoptotic effect of AR was further demonstrated by TUNEL staining of isolated hepatocytes treated with Jo2 plus actinomycin D. As shown in Fig. 4B the number of TUNEL-positive cells was significantly reduced in AR-treated cultures. Interestingly, the cytoprotective activity of AR was also observed in apoptosis induced by other agents such as okadaic acid, TGFβ, and tumor necrosis factor α plus galactosamine (data not shown). In concordance with the antiapoptotic effect of AR, we observed that caspase-3 cleavage and activation induced by anti-Fas antibody were significantly inhibited by AR (Fig. 4C). In agreement with our previous observations performed in vivo, we found that, although the levels of the antiapoptotic protein Bcl-xL were reduced in hepatocytes undergoing apoptosis they were maintained in the presence of AR (Fig. 4C). On the other hand, Bcl-2 protein was not detected in any sample (data not shown).

To learn about the antiapoptotic signaling of AR, we examined the PI3K/Akt and ERK1/2 (extracellular-regulated kinase 1/2) pathways, general mediators of cell survival (24, 43–46). Mouse hepatocytes in culture treated with AR showed increased phosphorylation of Akt and ERK1/2 (Fig. 5A). Another key signaling molecule involved in the protection from apoptosis, including Fas-mediated apoptosis of murine hepatocytes, is signal transducer and activator of transcription 3 (STAT3) (24, 47). We observed that AR stimulated STAT3 phosphorylation (Tyr705), an indicator of STAT3 activation (Fig. 5B). Downstream of EGF-R, the activation of the PI3K/Akt pathway by AR was also necessary to prevent apoptosis, as demonstrated by the marked inhibitory effect of the PI3K inhibitor LY294002 (Fig. 5B). However, the MEK1 inhibitor PD98059 did not interfere with the antiapoptotic effect of AR (Fig. 5B).

The activation of the EGF-R by AR seems to be essential in the mediation of the antiapoptotic effect of this growth factor on Fas-induced cell death. This became evident when mouse hepatocytes were pretreated for 1 h with the EGF-R inhibitor PD153035 before addition of AR, and the protection afforded by AR was lost (Fig. 5B). Downstream of EGF-R, the activation of the PI3K/Akt pathway by AR was also necessary to prevent apoptosis, as demonstrated by the marked inhibitory effect of the PI3K inhibitor LY294002 (Fig. 5B). However, the MEK1 inhibitor PD98059 did not interfere with the antiapoptotic effect of AR (Fig. 5B).

Response of AR−/− Mice to Anti-Fas Antibody Administration—Once established the antiapoptotic effects of AR on liver parenchymal cells and to further define the role of AR expression in liver damage, we examined the response of AR−/− mice to Fas ligation. Unexpectedly, serum transaminases measured at different time points after Jo2 antibody administration were significantly higher in wild type (AR+/+) mice when compared with the knock-out littermates (AR−/−) (Fig. 6A). In agreement with the higher degree of liver injury found in AR+/+...
mice, we also observed that the extent of caspase-3 activation 6 h after Jo2 administration, as determined by the appearance of its p17 cleavage product, was more pronounced in AR+/+ mice than in the AR−/− counterparts (Fig. 6B). In addition, the induction of stress-activated pathways, such as that triggered by Jun N-terminal kinase (JNK) in response to Fas ligation (48), was markedly blunted in AR−/− mice (Fig. 6C). The increased resistance to Jo2 cell death in these knock-out mice was not due to a decreased expression of Fas receptor in liver tissue (Fig. 6D). Interestingly, the reduced vulnerability to liver injury of AR−/− mice was not restricted to Fas-mediated liver damage, because it was also observed for other hepatotoxins such as CCl4 (data not shown).

Although to a lesser extent than wild type mice, AR−/− mice are also vulnerable to liver damage-inducing agents. To further demonstrate the protective effects of AR in Fas-mediated liver injury, AR−/− mice were treated with AR and challenged with Jo2 antibody. As shown in Fig. 6E, AR treatment attenuated liver injury as judged by serum AST levels measured at 12 h after Jo2 administration. In concordance with the reduced AST levels, prevention of caspase-3 cleavage by AR was also observed (Fig. 6F). These observations suggest that exogenous AR can enhance the protective mechanisms that apparently operate in AR−/− mice.

Finally, we tested the response of AR+/+ and AR−/− mice to a lethal dose of Jo2 antibody (0.3 mg/kg, intraperitoneal) (n = 11 mice per genotype). Although we observed similar lethality for both strains as assessed 24 h after antibody injection (8/11 for AR+/+ and 9/11 for AR−/−), AR+/+ mice showed a survival advantage over AR−/− animals. Most (8/11) of the AR−/− mice that died in response to Jo2 challenge did so in the first 6 h post antibody injection, whereas by that time only one wild type mouse had died. These data suggest that the persistent liver injury observed in AR−/− mice, which can confer a certain degree of resistance to sublethal liver damage, does not afford enhanced survival in life-threatening liver injury.

Lack of AR Results in Chronic Liver Damage: Potential Mechanisms for Cell Death Resistance—Our primary observations showing the hepatoprotective potential of AR on Fas-mediated liver damage seemed to be at odds with the resistance
to apoptosis and necrosis-like cell death observed in AR−/− mice challenged with anti-Fas antibody or CCl4. However, there is accumulating evidence showing that a pre-existing liver damage can precondition hepatocytes and result in a certain degree of cell death resistance. This phenomenon is widely recognized in the field of liver surgery and transplantation, where acute cell stress or injury induced by ischemic preconditioning can protect against subsequent reperfusion injury (49, 50). More recently, the protective effects of a pre-existing chronic liver disease have also been identified in a murine model of hereditary tyrosinemia (48). Considering this hypothesis, we went on to examine whether AR−/− mice displayed signs of chronic liver damage that might precondition hepatocytes against cell death. As shown in Fig. 7A, the basal levels of serum transaminases were significantly higher in AR−/− mice as compared with wild type animals. We also observed that the hepatic mRNA levels of cistathionine β-synthase and the modifier subunit of γ-glutamylcysteine synthetase, two enzymes that are induced under oxidative stress (34, 51), were up-regulated in AR null mice (Fig. 7B). Furthermore, analysis of caspase-3 activation, by detection of its p17 cleavage product, showed that mice lacking AR, but not wild type mice, displayed a basal activation of this apoptosis-related event (Fig. 7C). As previously mentioned, the STAT3 pathway is a major hepatoprotective effector activated in the liver as defense mechanism against apoptotic cell death (24, 47, 52). We observed that AR−/− mice showed enhanced levels of STAT3 phosphorylation (Tyr705) in the liver (Fig. 7D), suggesting the existence of an endogenous protective response against the ongoing liver damage. Cytokines and growth factors with hepatoprotective activity such as IL-6, cardiotrophin-1, and EGF, are known to activate and likely mediate their survival effects through the STAT3 pathway (52–55). Because AR is a member of the EGF-R family of ligands, we first tested the expression of other EGF-R ligands in the liver of AR−/− mice that could compensate for the lack of AR and play a role in the resistance to cell death. We did not observe any significant differences in the basal expression levels of EGF, TGFβ, EPR, HB-EGF, or BTC mRNAs between the two strains of mice (data not shown). Similarly the expression of the IL-6 family member cardiotrophin-1 was not different between AR wild type and knock-out mice (data not shown). However, the basal mRNA levels of IL-6 in the liver of AR−/− mice were significantly higher (2.5-fold) than in wild type mice (Fig. 8). IL-6 is a critical proregenerative factor and a major regulator of the acute phase response (52), its up-regulation in AR−/− mice may be indicative of an ongoing stress condition and may play an important role in the enhanced resistance of these mice to acute liver damage. In line with the essential functions of IL-6 we observed that its expression was rapidly and markedly induced in the liver of wild type mice shortly after the injection of Jo2 antibodies (Fig. 8). Interestingly, the up-regulation of IL-6 gene expression in the
Expression levels were determined by quantitative real-time PCR. *, knock-out mice determined by quantitative real-time PCR. *p < 0.05 with respect to AR+/+ mice. C, Western blot detection of caspase-3 p17 cleavage product in the liver of AR knock-out mice. D, basal levels of STAT3 Tyr175 phosphorylation in the livers of wild type and AR knock-out mice. Representative blots are shown.

**FIG. 7.** AR−/− mice show signs of chronic liver injury. A, basal levels of ALT were elevated in AR null mice. *p < 0.05. B, expression of cystathionine β-synthase and γ-glutamylcysteine synthetase genes in the liver of wild type and AR knock-out mice determined by quantitative real-time PCR. *p < 0.05 with respect to AR+/+ mice. C, Western blot detection of caspase-3 p17 cleavage product in the liver of AR knock-out mice. D, basal levels of STAT3 Tyr175 phosphorylation in the livers of wild type and AR knock-out mice. Representative blots are shown.

**FIG. 8.** Expression of IL-6 mRNA in the liver of AR+/+ and AR−/− mice before and after the intraperitoneal injection of Jo2 antibody. Expression levels were determined by quantitative real-time PCR. *, indicates p < 0.05 with respect to untreated AR+/+ mice.

liver of AR−/− mice after Jo2 administration was markedly blunted (Fig. 8). The attenuation of this protective response may be a consequence of the pre-existing hepatoprotective mechanisms triggered in AR knock-out mice.

**DISCUSSION**

The identification of endogenous protective mechanisms triggered upon liver injury is important not only for the study of the pathophysiology of this organ, but also for the design of more effective therapies that potentiate the natural defensive responses (13, 24, 56). The EGF-R is highly expressed in the hepatocyte and seems to be a relevant mediator of survival and proliferative responses (18–20, 25–27). Activation of the EGF-R by EGF administration or the overexpression of TGF-α in transgenic mice has been shown to efficiently protect hepatocytes from apoptotic cell death (19, 21). However, the expression profiles of these and other EGF-R ligands during acute liver damage induced by Fas ligation have not been defined. In the present work we observed that the mRNA levels of TGF-α and AR were up-regulated early after Fas-agonist antibody administration, suggesting that these factors may be protective against Fas-mediated apoptosis. Particularly noteworthy was the marked up-regulation of mRNA and protein levels of AR, a growth factor that in contrast to the other EGF-R ligands is barely detectable in the healthy normal human and rodent liver (35). Recently we have shown that the expression of AR is induced in chronic experimental liver damage and in human liver cirrhosis and is readily detected in rodent liver after partial hepatectomy (31). Moreover, AR seems necessary for adequate DNA synthesis during mouse liver regeneration (31). Now we observe that AR is rapidly induced in the mouse liver upon Fas-mediated acute liver damage.

The biological significance of AR up-regulation under these circumstances can be inferred from our experiments in which treatment with recombinant AR significantly prevented Fas-mediated hepatocellular death in vivo. Furthermore, the hepatoprotective effect of AR could be extended to acute liver damage mediated by CCl4. We observed that AR administration blocked the Fas-mediated signal transduction pathway upstream of caspase-3 activation. This effect could be mediated in part by its ability to preserve the expression of the antiapoptotic protein Bcl-xL, which has been involved in the suppression of liver apoptosis driven by Fas antibody injection (14). Experiments conducted in isolated mouse hepatocytes evidenced that AR can directly interact with the parenchymal liver cells and trigger survival signals. We have recently shown that AR can induce the synthesis of DNA in isolated hepatocytes through the activation of the EGF-R (31), and our current in vitro experiments show that the activation of the EGF-R by AR is also necessary to convey its antiapoptotic effect. The ability to induce cellular proliferation is often correlated with the promotion of survival (24), and the downstream PI3K, ERK1/2, and STAT3 pathways have been shown to be major regulators of cell proliferation and survival in response to growth factors (24, 43–47, 57). Our data indicate that the antiapoptotic activity of AR on Fas-mediated cell death is independent of the ERK1/2 pathway but dependent of PI3K. Additionally, we also demonstrate that AR activates STAT3, a factor that might also be involved in the hepatoprotective effects of AR.

To gain further insight into the role of AR production during liver damage we made use of AR null mice (32). Unexpectedly, we found that AR−/− mice were clearly more resistant to liver damage induced by Fas ligation, and CCl4 administration, than their wild type counterparts. These observations seemed to be at variance with our previous evidences showing that AR administration abrogated Fas-mediated cell death both in vivo and in vitro and prevented CCl4-mediated liver damage. Enhanced resistance to liver injury has been described in animals
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threating Fas stimulation. These observations, together with the hepatoprotective effect of exogenous AR in AR−/− mice, further underscore the essential role of AR in the protection from liver injury. In summary, our present observations reveal a novel hepatoprotective role of AR and suggest that this factor may be of therapeutic value for patients with liver disease.

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