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EPIDERMAL GROWTH FACTOR RECEPTOR LIGANDS IN MURINE MODELS FOR ERYTHROPOIETIC PROTOPORPHYRIA: POTENTIAL NOVEL PLAYERS IN THE PROGRESSION OF LIVER INJURY

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Abstract – Activation of the epidermal growth factor receptor (EGFR) plays an important role in liver regeneration and resistance to acute injury. However its chronic activation participates in the progression of liver disease, including fibrogenesis and malignant transformation. Hepatobiliary disease represents a constant feature in the clinically relevant $Fech^{m1pas}/Fech^{m1pas}$ genetic model of erythropoietic protoporphyria (EPP). Similarly, chronic administration of griseofulvin to mice induces pathological changes similar to those found in patients with EPP-associated liver injury. We investigated the hepatic expression of the EGFR and its seven most relevant ligands in $Fech^{m1pas}/Fech^{m1pas}$ mice bred in three different backgrounds, and in griseofulvin-induced protoporphyria. We observed that the expression of amphiregulin, betacellulin and epiregulin was significantly increased in young EPP mice when compared to aged-matched controls in all genetic backgrounds. The expression of these ligands was also tested in older (11 months) BALB/cJ EPP mice, and it was found to remain induced, while that of the EGFR was downregulated. Griseofulvin feeding also increased the expression of amphiregulin, betacellulin and epiregulin. Interestingly, protoporphyrin accumulation in cultured hepatic AML-12 cells readily elicited the expression of these three EGFR ligands. Our findings suggest that protoporphyrin could directly induce the hepatic expression of EGFR ligands, and that their chronic upregulation might participate in the pathogenesis of EPP-associated liver disease.

Key words: Erythropoietic protoporphyria, protoporphyrin, hepatobiliary disease, amphiregulin, betacellulin, epiregulin, epidermal growth factor receptor.

INTRODUCTION

Erythropoietic protoporphyria (EPP) is an autosomal dominant disease that results from deficient activity of ferrochelatase (Fech), the last enzyme of the heme synthesis pathway (2). The main clinical feature is cutaneous photosensitivity due to protoporphyrin accumulation in circulating erythrocytes and plasma. Protoporphyrin is so hydrophobic that it is excreted only in bile. In a minority of patients, bile canalicular sludging may result in progressive cholestasis, which is associated with marked protoporphyrin accumulation that can begin insidiously, and distorted lobular architecture in the hepatobiliary system ranging from mild inflammation to fibrosis and cirrhosis (3, 13, 36). Molecular analysis of the ferrochelatase mutations causing EPP has revealed no correlation between genotype and hepatobiliary complications. Concurrent viral hepatitis, alcohol abuse, iron deficiency, fasting or oral contraceptive steroids have appeared to contribute to exacerbate hepatic disease in some

Abreviations: EPP, erythropoietic protoporphyria; **AR,** amphiregulin; **HB-EGF,** heparin-binding epidermal growth factor-like growth factor; **TGFα,** transforming growth factor-alpha; **Btc,** betacellulin; **Ereg,** epiregulin; **EGFR,** epidermal growth factor receptor; **Q-PCR,** Quantitative polymerase chain reaction.

EPP patients (2, 3, 37). Liver transplantation is beneficial for such patients with end-stage protoporphyric liver failure (40). Long-term survival after liver transplantation for EPP has been documented. However liver transplantation does not correct the erythroid metabolic deficiency, and the new liver is susceptible to protoporphyrin-induced damage (2, 13, 40).

The liver displays a great capacity to defend itself and to regenerate after exposure to hepatotropic viruses, xenobiotics, or potentially toxic endogenous metabolites (4, 5, 11, 41). The regenerative response elicited by the destruction of parenchymal cells is complex, and is mediated by a network of cytokines, comitogens and growth factors in a coordinate multistep process. There are at least two growth factor signaling systems that appear to be critically involved in liver regeneration: the hepatocyte growth factor and its receptor c-Met, and the epidermal growth factor receptor (EGFR) axis (32). The EGFR is a transmembrane receptor endowed with tyrosine kinase activity that can be bound and activated by a broad family of ligands, and can also engage in extensive crosstalk with other signalling pathways (12). Besides the EGF, EGFR ligands include amphiregulin (AR), transforming growth factor α (TGFα), epigen (EPGN), betacellulin (Btc), heparin-binding EGF-like growth factor (HB-EGF) and epiregulin (Ereg)(11). The interaction of these ligands with the EGFR triggers intracellular pathways leading to the expression of a battery of genes involved in cell cycle progression, apoptosis resistance, differentiation, adhesion and cell migration.

Most EGFR ligands are upregulated after two-thirds partial hepatectomy, and it has been shown that the hepatic expression of HB-EGF, TGFα and AR is increased in mouse liver upon acute CCl_4 intoxication, while that of AR, Ereg and TGFα is also upregulated in the clinically relevant model of Fas-mediated acute liver injury (9). A number of studies have demonstrated the significant hepatoprotective and pro-regenerative potential of the EGFR axis. Evidence has been collected using different experimental approaches, including for instance transgenic overexpression (TGFα and HB-EGF)(25, 27), adenoviral gene transfer (HB-EGF) (26), or direct intraperitoneal administration of the recombinant growth factor (EGF and AR) (9, 19), in models of acute liver injury and regeneration.

A common observation made in the different models of acute liver injury is that upon cessation of the noxious stimuli the expression of the different EGFR ligands returns to the levels found in normal liver. While this system is considered as an important defence mechanism for the liver during acute tissue injury, accumulating evidence suggests that its chronic stimulation can participate in hepatic fibrogenesis and in the neoplastic conversion of the liver (17). In this respect up-regulation of the ligands AR, TGFα and HB-EGF has been detected in experimental models of chronic liver injury as well as in liver samples obtained from human liver tumours (8, 16, 27, 33, 39). Experimental studies provided more direct evidence of the actual implication of deregulated EGFR signalling in the progression of liver disease. For instance, the contribution of AR to CCl_4-induced hepatic fibrogenesis has been recently demonstrated using AR null mice, in which extracellular matrix accumulation was reduced as compared to wild-type animals (35). Moreover, persistent overexpression of EGFR ligands is increasingly being recognized as an important step towards development of liver cancer, and as a key player in the maintenance of the transformed phenotype of liver tumour cells (11).

Hepatobiliary disease represents a major and constant feature in the $Fech^{m1pas}/Fech^{m1pas}$ murine model of EPP (1, 18, 30, 31, 42). In the BALB/cJ background, the livers of EPP animals show protoporphyrin depositions, progressive inflammation, fibrosis and cholestasis at 6 weeks of age, and the appearance of cell dysplasia and Hepatocellular carcinoma foci after seven months of age (30). Similarly, chronic administration of the antifungal agent griseofulvin to mice induces pathological changes similar to those found in patients with EPP-associated liver injury (7, 23). The aim of this study was to investigate the expression profile of seven different ligands of the EGFR in the livers of $Fech^{m1pas}/Fech^{m1pas}$ mice bred under three different backgrounds, and in the pharmacological model of EPP induced by a diet containing griseofulvin.

MATERIALS AND METHODS

Animal models

Male $Fech^{m1Pas}/Fech^{m1Pas}$, EPP mice, bred in the BALB/cByJCrl (BALB/cJ) background were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animals carrying the $Fech^{m1Pas}$ mutation were identified by genomic DNA PCR followed by enzymatic restriction digestion with BspHI (14). Congenic strains on the C57BL/6JCrl (C57BL/6J) and SJL/JOrlCrl (SJL/J) inbred background were kindly provided by Drs. Marie Abitbol and Xavier

Montagutelli from the Institut Pasteur (Paris, France). These congenic strains were developed by repeated backcrossing of the BALB/cJ congenic strain with the C57BL/6J and SJL/J inbred strains for 10 generations. All mice were kept under pathogen-free conditions in air-filtered cages and provided with autoclaved food and water. The mice had unlimited access to water and standard laboratory feed and were subjected to 12-hour light/dark cycles.

C57BL/6J mice were fed with a diet containing 2.5 % of griseofulvin (Harlan, Barcelona, Spain) for 11 or 22 days. The control group received the same diet without the drug. All animals were fed *ad libitum* and were housed in filter-top cages to prevent cross contamination with griseofulvin.

Sacrifice and sample collection

Mice were sacrificed by cervical dislocation at the indicated ages or time of treatment. Liver was excised, rinsed in ice-cold phosphate-buffered saline solution, and divided into fractions to be frozen in liquid nitrogen. All animal experimentation was conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

In vitro assays

The immortalized normal hepatocyte cell line AML-12 (44) was routinely cultured in DMEM/F12 supplemented with 10% fetal calf serum, 1% insulin/transferrin/selenium, 0,2% dexametasone and antibiotics. When indicated the medium was changed to medium containing 2% fetal calf serum. Treatments were carried out in this culture medium supplemented with 1 mmol/l delta-aminolevulinate acid (ALA) (Sigma, St. Louis, MO, USA) and 2mmol/l melatonin (Sigma) as described (22). The ALA/melatonin solution was freshly prepared for each assay.

Molecular and biochemical analyses

Total RNA was extracted from liver tissues and cultured cells as previously reported (9). The steady state mRNA levels of the EGFR and its ligands EGF, AR, HB-EGF, TGFα, BTC, EPGN and Ereg, was analyzed by quantitative RT-PCR as previously described (9). Briefly, the amount of each transcript was calculated as the n-fold difference relative to the control gene actin ($2^{\Delta Ct}$, where ΔCt represents the difference in threshold cycle between the target and control genes). Porphyrin levels in liver tissues and cultured cells, and circulating transaminases were measured as reported before (21, 22). Serum alanine aminotransferase (ALT) was measured using a kit from Roche Diagnostics S.L with a Cobas Integra 400 (Roche Diagnostics S.L., Barcelona, Spain).

Statistical analysis

The Student's t-test was used for comparison of differences. When group variances were unequal, the data were transformed logarithmically [log(1+X)] before analysis. The null hypothesis was rejected when $P<0.05$. Pearson´s correlation coefficient (r) was computed to test the association between protoporphyrin levels and AR expression in the liver. Statistical analyses were carried out with GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

$Fech^{m1Pas}/Fech^{m1Pas}$ mutant mice from different strains congenic for the same ferrochelatase mutation ($Fech^{m1pas}$) manifest early liver damage (Table 1). At 6 weeks of age, the liver protoporphyrin accumulation in $Fech^{m1pas}/Fech^{m1pas}$ animals was mild in BALB/cJ, medium in SJL/J, and high in those animals bred into the C57BL/6J background (Table 1). The histological features of these mice have been described previously (1, 34). The expression of the different EGFR ligands was tested in the liver of EPP animals of different genetic backgrounds at six weeks of age (Figure 1A, B and C) and at 11 months of age in the BALB/cJ background. At 6 weeks of age, AR, BTC and Ereg mRNA levels were significantly increased in the EPP mice as compared to wild-type animals, while those of the other EGFR ligands tested remained unchanged (Figure. 1A, B and C). At 11 months of age, AR, Btc and Ereg expression remained increased in the BALB/cJ protoporphyric mice (Figure 1D). Interestingly, we found a significant direct correlation ($r=0.58$, $P<0.00001$) between the expression levels of AR and protoporphyrin contents in liver tissue samples from BALB/cJ EPP mice collected at different ages (from 4 weeks to 12 months, n=46) (Figure 2).

Young EPP mice from C57BL/6J and SJL/J backgrounds showed reduced mRNA steady state levels for EGFR when compared to wild-type animals, although statistical significance was reached only in SJL/J mice (Figure 1A and B). The expression of the EGFR was not changed in young animals from the BALB/cJ background (Figure 1C), but it was significantly compromised in the liver of old EPP mice (Figure 1D).

Mice fed with the griseofulvin diet showed increased hepatic protoporphyrin levels (Figure 3A) and circulating transaminases (Figure 3B). The expression of AR, Btc and Ereg was found to be significantly enhanced in the liver of mice fed with griseofulvin diet for 11 and 22 days (Figure 3C). Conversely, the expression of the EGFR was markedly reduced upon griseofulvin feeding (Figure 3C).

In order to test whether the expression of EGFR ligands in the liver of EPP and griseofulvin-fed mice could be related to the accumulation of protoporphyrin or its metabolic precursors in the parenchyma, we treated the non-transformed hepatocyte cell line AML-12 with the protoporphyrin precursor ALA as previously described (22). As expected AML-12 cells exhibited a rapid increased in intracellular porphyrin levels during ALA exposure (Figure

TABLE 1. Alanine Transaminase, hepatic levels of protoporphyrin and liver weight in Normal and Fech ^{m1Pas}/ Fech ^{m1Pas} Mice

BACKGROUND (age)	n	Wild-type	Fech^{m1Pas}/Fech^{m1Pas}	p
C57BL/6J (6 weeks old)				
ALT (IU/l)	3	46 ± 8	605 ± 167	p<0.001
Hepatic protoporphyrin (nmol/g Prot.)	3	49 ± 39	15772 ± 10099	p<0.001
Liver weight (% liver/ body weight)	3	5.2 ± 0.9	12.4 ± 2.85	p<0.01
SJL/J (6 weeks old)				
ALT (IU/l)	3	47 ± 7	483 ± 167	p<0.001
Hepatic protoporphyrin (nmol/g Prot.)	3	30.6 ± 10.6	6256 ± 1342	p<0.001
Liver weight (% liver/ body weight)	3	6.41± 0.45	11.5 ± 1.0	p<0.01
BALB/cJ (6 weeks old)				
ALT (IU/l)	7	52 ± 6	368 ± 363	p<0.01
Hepatic protoporphyrin (nmol/g Prot.)	7	14 ± 8	964 ± 177	p<0.001
Liver weight (% liver/ body weight)	7	5.1 ± 0.55	7.9 ± 1.1	p<0.05
BALB/cJ (11 months old)				
ALT (IU/l)	11	97 ± 27	166 ± 180	p<0.01
Hepatic protoporphyrin (nmol/g Prot.)	11	20 ± 10	179 ± 123	p<0.001
Liver weight (% liver/ body weight)	11	7.3 ± 1.6	9.2 ± 1.2	p<0.05

ALT, Serum alanine transaminase, PP: protoporphyrin. NS= not significant.

Data are expressed as mean ± standard deviation.

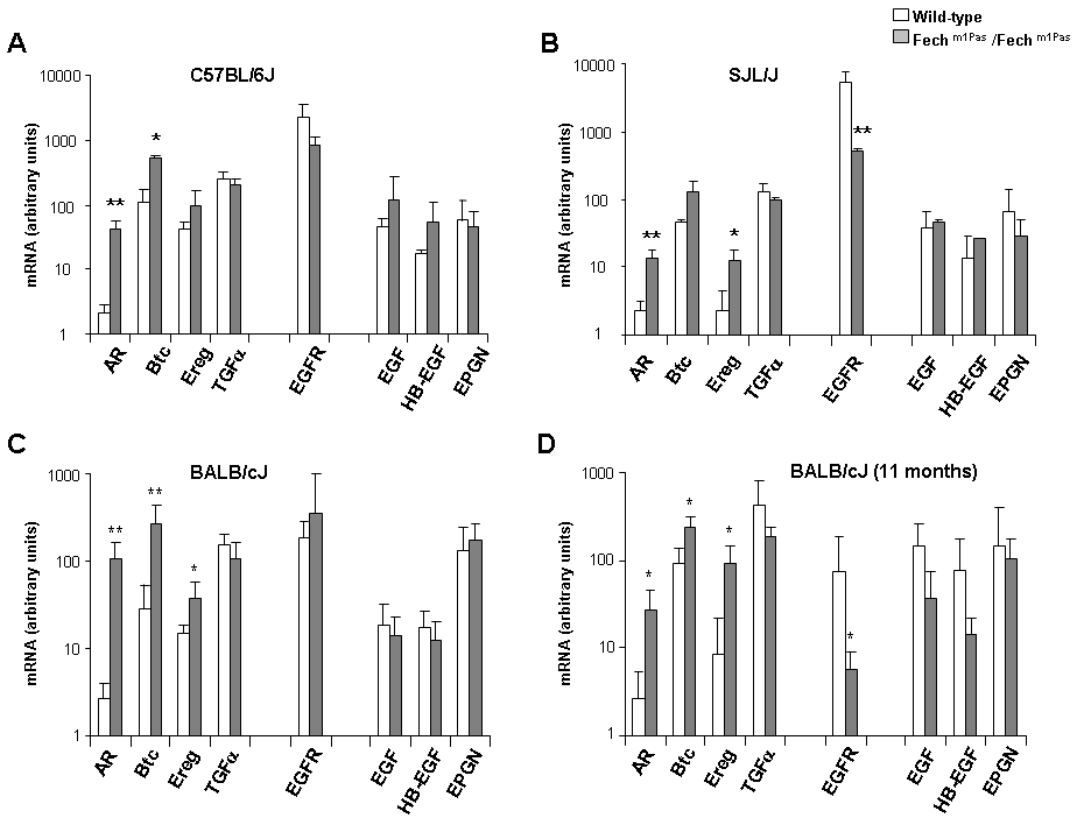

Figure 1. Hepatic expression of the EGFR and different EGFR ligands in the liver of 6 weeks old wild-type (open bars) and Fech^{m1pas}/Fech^{m1pas} mice (closed bars) with C57BL/J (n=3 mice per group) (A), SJL/J (n=3 mice per group) (B), and BALB/cJ backgrounds (C) (n=3 mice per group). Expression of EGFR and ligands was also measured in 11 months old EPP mice bred into BALB/cJ background (D) (n=4 mice per group). Amphiregulin (AR), betacellulin (Btc), epiregulin (Ereg), transforming growth factorα (TGFα), epidermal growth factor receptor (EGFR), epidermal growth factor (EGF), heparin-binding epidermal growth factor-like growth factor (HB-EGF), epigen (EPGN). mRNA levels were assayed by real time PCR. * P< 0.05, ** P<0.01, *vs* wild-type animals.

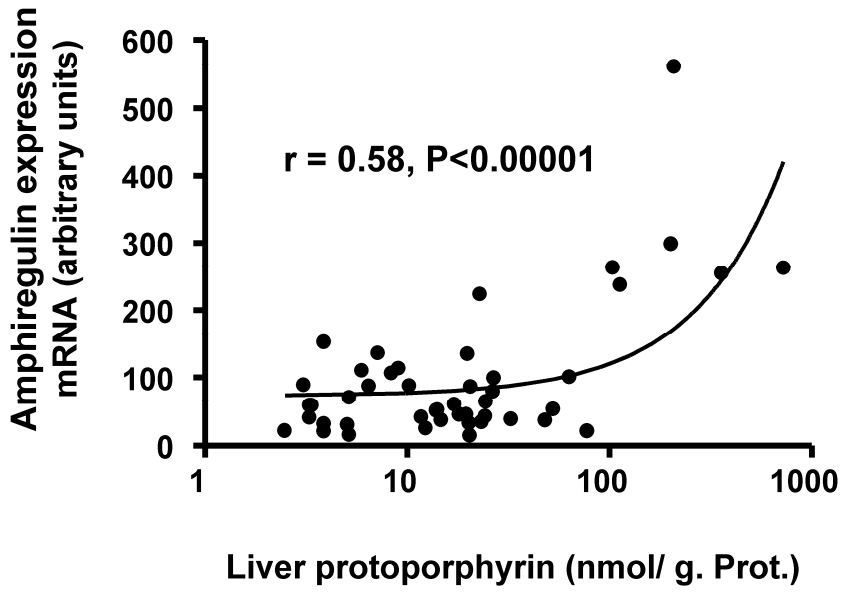

Figure 2. Correlation between hepatic levels of AR mRNA and protoporphyrin contents in Fech^{m1pas}/Fech^{m1pas} mice bred into BALB/cJ background (n=46, from 4 weeks to 12 months of age).

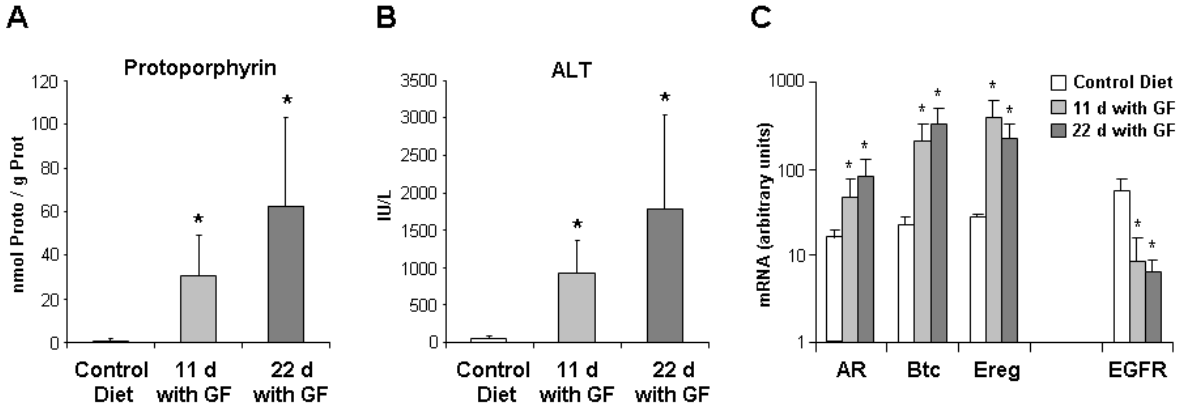

Figure 3. Hepatic protoporphyrin levels (A), serum alanine transaminase (B), and expression of the EGFR and different EGFR ligands (C) in the livers of C57BL/J mice fed control or griseofulvin-containing diet (GF) during 11 and 21 days (n=5 mice per group). * P< 0.05 *vs* controls. The mRNA levels were assayed by real time PCR.

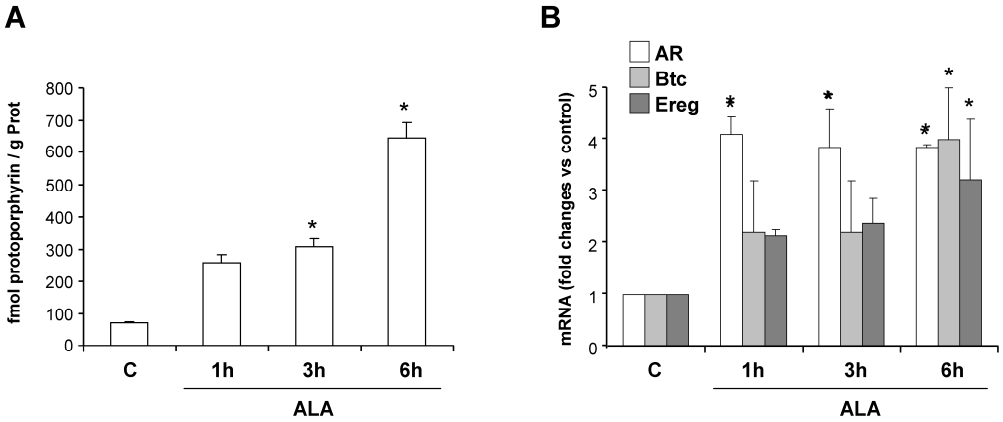

Figure 4. Intracellular protoporphyrin levels (A), and expression of different EGFR ligands (B), in control (C) or ALA treated AML-12 murine hepatic cells assayed at the indicated time points. Experiments were performed in triplicates. * P< 0.05 *vs* controls. The mRNA levels were assayed by real time PCR.

4A). The expression of the EGFR ligands AR, Btc and Ereg was significantly induced in ALA treated cells when compared to non-exposed cells, with AR showing the fastest response (Figure 4B).

DISCUSSION

$Fech^{m1Pas}/Fech^{m1Pas}$ mutant mice show increased protoporphyrin levels and severe liver dysfunction starting at a very early age and during all the life of the animal (1, 14, 21, 42). Mice from different strains congenic for the $Fech^{m1Pas}$ mutation manifest variable degree of liver injury (1, 34). The three congenic strains do not significantly differ in liver ferrochelatase activity, however, the development of liver disease and biliary protoporphyrin excretion is different between the three strains (1, 34). This feature makes $Fech^{m1pas}/Fech^{m1pas}$ mouse a suitable model for the study of chronic hepatic disease related to alterations in protoporphyrin metabolism. In the BALBC/cJ background, protoporphyrin overproduction by red blood cells and increased biliary protoporphyrin excretion result in a rapid and profound impairment of the liver excretory function due to bile duct obstruction, with moderate pigment deposits in the liver (1, 21, 34). EPP mice bred into C57BL/6J background show the most pronounced liver accumulation of protoporphyrin (34), associated with more severe hepatocyte damage, parenchymal cell loss and chronic hepatitis. However, the liver excretory function was almost unaffected (1). EPP animals from SJL/J background exhibit higher protoporphyrin concentration in red blood cells and liver, but mild liver lesions with low protoporphyrin deposits (1, 34).

The induction of Btc, Ereg and, in particular, AR gene expression observed in EPP mice from BALB/cJ, SJL and C57BL/6J backgrounds most likely represents a defensive mechanism linked to increased liver regeneration in response to the ongoing parenchymal injury. Among the different EGFR ligands tested, AR showed the most prominent changes and was present in all of three backgrounds. The expression of AR, which as opposed to other EGFR ligands is low or undetectable in the healthy liver, is readily induced during experimental acute liver damage (11, 15). The important role of AR expression in the injured liver has been demonstrated using AR null mice. These animals are viable and display no overt hepatic phenotype (38). However, when liver regeneration was induced through partial hepatectomy, AR null mice showed an impaired proliferative response when compared with their wild-type counterparts (11, 28). Conversely, Ereg knockout mice show no apparent defects in liver regeneration upon partial hepatectomy (29), while no data are available on Btc null mice. These findings suggest that the expression of AR during liver injury serves protective and pro-regenerative functions that cannot be fully compensated for by other EGFR ligands (24, 29). Although speculative, a potential mechanism that may account for the distinct role of AR can be found in how they bind and activate their receptor (11, 20). It has been shown that the tendency of AR to induce formation of heterodimers between EGFR and other members of the EGFR family is different from that of other EGFR ligands (10). In addition, AR differs from other ligands such as EGF in the kinetics of ligand-induced EGFR turnover, which may influence intracellular downstream signaling (43).

In the genetic model of EPP, protoporphyrin accumulation and liver disease start in the first week of life, and it is difficult to establish their direct implication in the enhanced expression of EGFR ligands. Nevertheless, we observed a direct correlation between the tissue levels of protoporphyrin and AR gene expression in the liver of BALB/cJ EPP mice. To further examine the influence of hepatic protoporphyrin accumulation on the expression of EGFR ligands, and to evaluate the potential direct effect of protoporphyrin and/or related metabolites on the expression of these growth factors in liver cells, we performed additional *in vivo* and *in vitro* assays.

Griseofulvin is an antifungal agent that when administered chronically to normal mice produces a liver pathology similar to that found in human EPP (23). One of the earliest features of griseofulvin exposure is the accumulation of protoporphyrin due to inhibition of hepatic ferrochelatase, probably caused by a griseofulvin adduct of protoporphyrin produced in a cytochrome P450-mediated suicide reaction (7). Given that this condition is successfully reproduced, this model is ideal for studying the early hepatic responses to protoporphyrin accumulation. In our animals, griseofulvin diet administration induced the over-expression of

AR, Btc and Ereg in the liver. Changes in the expression of these growth factors in the griseofulvin model were consistent with those observed in $Fech^{m1Pas}/Fech^{m1Pas}$ mutant mice.

As previously indicated, the expression of EGFR ligands in the liver is induced during injury and regeneration, and this response can be mediated by a number of pro-inflammatory stimuli (12). Here we have observed the consistent upregulation of AR, Btc and Ereg gene expression in the livers of young EPP mice, and in normal mice after short term griseofulvin exposure. These observations, together with the finding of a significant direct correlation between hepatic levels of protoporphyrin and AR gene expression in the genetic model of EPP, led us to examine the potential direct contribution of protoporphyrin and/or its metabolic precursors to the upregulation of these genes in liver cells. We observed that treatment of AML-12 hepatocytes with ALA induced a rapid protoporphyrin accumulation, and resulted in a significant induction in the expression of EGFR ligands. These results suggest that the enhanced expression of EGFR ligands in the *in vivo* models could be attributed at least in part to the protoporphyrin accumulation.

Expression of high levels of the EGFR is a characteristic of the adult and differentiated hepatocyte (11). Chronic liver injury is characterized by the loss of liver-specific functions, which is attributed in part to the downregulation of the expression of genes highly expressed in the mature hepatocyte (6, 11). Compared to wild-type animals, hepatic EGFR expression decreased in young C57BL/6J EPP mice, and more significantly in the SJL/J background, as well as in mice fed with the griseofulvin supplemented diet. Interestingly, EGFR mRNA levels were maintained in young $Fech^{m1Pas}/Fech^{m1Pas}$ mice bred into BALB/cJ background, however at an older age, when disease has progressed (1, 30), these mice also showed a reduction in EGFR expression. Although at the present time we do not have an explanation for these findings, changes in EGFR mRNA levels might be attributed to the progression of the disease and the loss of the differentiated features of the normal hepatocyte.

Taken together these observations suggest that the induction of the EGFR signaling system represents an endogenous protective and pro-regenerative response elicited by tissue injury and a high protoporphyrin accumulation in hepatocytes. However, when liver damage persists and the expression of EGFR ligands such as AR is sustained, this protective response may participate in the progression of the disease, contributing to liver fibrosis and promoting the development of a neoplasic phenotype (20). According to our findings in BALB/cJ EPP mice, high AR, Btc and Ereg mRNA levels were maintained up to one year of age in the liver of mutant mice (Figure 1D and data not shown). Moreover, AR mRNA levels correlated with liver protoporphyrin concentration in EPP mice at different ages. In this context, the expression of genes associated with hepatocellular proliferation, extracellular matrix accumulation, preneoplastic lesions and hepatocellular carcinoma foci that progressively develop with age in $Fech^{m1pas}/Fech^{m1pas}$ mice bred into the BALB/cJ background previously described (18, 30), might be related in part to the chronic upregulation of these EGFR ligands. Nevertheless, further experiments, including studies carried out in the corresponding knockout mice, are needed to fully elucidate the role of these growth factors in the pathogenesis of liver disease in EPP.

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