MINIREVIEW

The Epidermal Growth Factor Receptor: A Link Between Inflammation and Liver Cancer

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Epidemiological studies have established that many tumours occur in association with persistent inflammation. One clear example of inflammation-related cancer is hepatocellular carcinoma (HCC). HCC slowly unfolds on a background of chronic inflammation triggered by exposure to infectious agents (hepatotropic viruses), toxic compounds (ethanol), or metabolic impairment. The molecular links that connect inflammation and cancer are not completely known, but evidence gathered over the past few years is beginning to define the precise mechanisms. A central role for cytokines such as interleukin-6 (IL-6) and IL-1 (α and β) in liver cancer has been established in experimental models. Besides these inflammatory mediators, mounting evidence points to the dysregulation of specific growth and survival-related pathways in HCC development. Among them is the pathway governed by the epidermal growth factor receptor (EGFR), which can be bound and activated by a broad family of ligands. Of special relevance is the fact that the EGFR engages in extensive crosstalk with other signaling pathways, serving as a "signaling hub" for an increasing list of growth factors, cytokines, and inflammatory mediators. In this review, we summarize the most recent evidences supporting a role for the EGFR system in inflammation-related cell signaling, with special emphasis in liver inflammation and HCC. The molecular dissection of the pathways connecting the inflammatory reaction and neoplasia will facilitate the development of novel and more effective antitumor strategies. Exp Biol Med 234:713–725, 2009

Key words: epidermal growth factor receptor; amphiregulin; heparin-binding epidermal growth factor-like growth factor; inflammation; liver; hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the most frequent type of liver cancer, accounting for almost 90% of primary malignant hepatic tumours in adults (1). Prognosis of HCC is very poor, the number of HCC-related deaths almost equals the number of cases being diagnosed each year (more than 600,000), and the 5-year survival rate is below 9% (2). HCC distribution in the world population is not homogeneous; its incidence is highest in southeast Asia and sub-Saharan Africa, areas where chronic hepatitis B (HBV) and hepatitis C (HCV) virus infection, HCCs most frequent etiological factors, are more prevalent (1–3). Other risk factors that are associated with the development of HCC include chronic alcohol abuse or genetic conditions such as hereditary hemochromatosis and α1-antitrypsin deficiency (2). Gender is another risk factor for HCC, with men being more susceptible than women, and male:female ratios between 2:1 to 4:1 (1).

Most frequently HCC unfolds slowly on a background of chronic liver injury, as evidenced by the fact that more

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than 90% of tumors are found on a chronic hepatitis or a cirrhotic background. The molecular and cellular mechanisms of HCC development are the subject of active research, and different alterations in genes controlling cell proliferation and survival have been described, however the process of liver neoplastic transformation is still incompletely understood. Differences relevant to the carcinogenic process among etiological agents have been identified. In the case of chronic HBV infection, integration of HBV-DNA in the host genome causes genomic instability and can bring about profound alterations in genes related to cell growth and survival. In addition, the expression of viral proteins, such as HBx, can also activate many pathways related to tumor promotion (4, 5). HCV is a single-stranded RNA molecule, and at least four of the HCV gene products (core, NS3, NS4B, and NS5A) have been shown to interact with numerous cellular proteins and to exhibit oncogenic activity in cellular and *in vivo* models (6). Regarding alcohol-mediated hepatocarcinogenesis, several mechanisms have been invoked, including the direct genotoxic potential of acetaldehyde, the first metabolite of ethanol, or DNA damage induced by reactive oxygen species and lipid peroxidation products elicited by Cyp2E1-mediated metabolism of ethanol (7). Besides the direct oncogenic activities of HBV, HCV, and chronic alcohol abuse, these agents are believed to cause liver tumors also *via* indirect pathways (4–7). These pathways involve the accumulation and fixation of critical mutations in the hepatocyte genome during compensatory proliferation elicited by parenchymal cell loss and chronic inflammation (8, 9).

Hepatocyte death induced by chronic oxidative stress and inflammation triggers a potent regenerative response aimed at the restoration of the lost hepatic parenchyma (10, 11). The robust regenerative reaction of the liver implicates all its different cell types and likely evolved as a defensive mechanism to preserve the viability of an organ that is essential in the detoxification of xenobiotics and noxious endobiotics. In the context of acute liver injury, hepatic regeneration is considered as a physiological wound healing process, bringing about transient and reversible changes in the extracellular matrix (ECM) of the organ and in the proliferative capacity of the hepatocytes (10–12). However, the protracted lesion inflicted to the liver under chronic viral infection, alcohol consumption, or metabolic impairment results in the perpetuation of this reparative response and the development of fibrosis and cirrhosis, which create a permissive microenvironment for HCC development. Cirrhosis is characterized by impaired liver function, the distortion of the organ's architecture caused by massive accumulation of ECM, and the presence of hyperplastic nodules of regenerating hepatocytes, encompassing initiated cells that harbour different genetic and epigenetic alterations. These lesions can progress to premalignant dysplastic nodules and eventually to frank HCC, with invasive and metastatic potential (13, 14). Therefore, regardless of the etiological factors and the molecular heterogeneity of HCC, there are common traits that characterize the early stages of liver cancer development. These include the progressive loss of liver-specific gene expression (15–17) and the persistence of an inflammatory and promitogenic milieu orchestrated by a complex network of cytokines and growth factors (9, 14, 18–21).

Chronic Inflammation and Hepatocellular Carcinoma. The long-standing immune reaction triggered by viral infection and chronic alcohol intake is known to play a fundamental part in the induction of hepatocellular damage (5–7, 13, 18). However, it is increasingly recognized that the inflammatory reaction also participates directly in the activation and maintenance of the regenerative response of liver parenchyma as well as in the persistent ECM remodelling activity that ultimately leads to the development of hepatic fibrosis (10, 11, 22, 23). For instance, the production of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), or the engagement of interleukin-1 receptor (IL-1R) is essential to trigger hepatocyte proliferation and liver regeneration after partial hepatectomy or liver injury, as has been demonstrated in the corresponding genetically modified mice (TNF receptor type 1-TNFR1, IL-6, and IL-1R knockout animals) (10, 11, 24). Similarly, several lines of experimental evidence support the contention that the inflammatory response is also central to the development of liver fibrosis through the interaction between the immune system and ECM-producing cells (25). Activation of receptors and signal transduction mechanisms characteristic of the innate immune system, such as the pattern recognition receptor Toll-like receptor 4 (TLR4), which is expressed in liver macrophages (Kupffer cells [KC]) and activated hepatic stellate cells (HSCs) (26), have been shown to be essential for the development of experimental fibrosis. Deletion of TLR4, or different components of the TLR4 signaling system, such as CD14, the lipopolysaccharide (LPS)-binding protein (LBP), and the intracellular adaptor myeloid differentiation primary response gene 88 (MyD88), attenuates the progression of liver fibrosis in different experimental mouse models, including bile-duct ligation and chronic CCl_4 administration (26). More recently it was established that TLR4 activation on HSCs was also critical for their response to key fibrogenic signals released by KC such as transforming growth factor-β (TGF-β) (26).

TLRs like TLR4 can be bound and activated by macromolecules released by viruses and bacteria, the so-called pathogen-associated molecular patterns, or PAMPs. Activation of TLR4 triggers key intracellular pathways such as that controlled by the transcription factor nuclear factor-κB (NF-κB), leading to the production of cytokines like TNF-α, IL-6, IL-1β, and TGFβ, which mediate and amplify the inflammatory response (19, 27). Increased circulating levels of bacterial LPS and inflammatory cytokines are observed in experimental models of liver injury as well as in patients with chronic liver disease, suggesting that increased intestinal permeability allows the translocation of bacterial

products from the gut into circulation (26). A persistent endotoxemia may thus contribute to the high serum levels of inflammatory cytokines found in patients with chronic liver injury of viral or alcoholic origin (18). These findings have led researchers to propose an important role for the intestinal flora as a trigger of hepatic inflammation and as a perpetuating agent for this condition (26). Interestingly, TLR4 may be also bound and activated by endogenous ligands produced during cellular stress or released by necrotic and apoptotic cells, and mediate what has been called a "sterile" inflammatory response. These ligands are known as damage-associated molecular patterns (DAMPs) and include the chromosomal protein high-mobility group box protein 1 (HMGB1), the heat shock protein HSP-60, the ED-A domain of fibronectin and hyaluronan, and are also produced during liver injury (28–30). Altogether, these observations indicate that activation of TLR4/MyD88 signaling, be it either mediated by LPS, by endogenous ligands, or both, appears to be essential for the development of liver fibrosis.

MyD88 is a key signaling adaptor molecule used by several TLRs, but it also integrates signals generated at the IL-1R. TLR4/MyD88 and IL-1R/MyD88 signaling are important to drive the compensatory hepatocellular proliferation after liver injury. Deletion of TLR4 impairs liver regeneration after acute CCl_4-induced damage (31), and mice deficient in IL-1R or MyD88 display reduced liver injury, inflammatory response, and ultimately develop less cancer when challenged with the hepatocarcinogen diethylnitrosamine (DEN) (32, 33). Inactivation of MyD88 was accompanied by a significant reduction in the production of the inflammatory cytokine IL-6, which as demonstrated in IL-6 null mice significantly contributes to DEN-induced hepatocarcinogenesis (33). The role of inflammatory cells in hepatocarcinogenesis was further demonstrated when NF-κB activity was specifically ablated in immune cells in the liver by cell-specific deletion of IKKβ, the upstream activator of NF-κB. Impaired activation of NF-κB in KCs resulted in attenuated production of inflammatory cytokines and less cancer development (34).

Taken together, these experimental observations support the role of chronic inflammation in HCC development and highlight the important function played by inflammatory cytokines like IL-6 or cytokine receptors such as IL-1R in liver carcinogenesis. However, besides the cytokine network, inflammatory pathways also lead to the generation of additional mediators that may contribute to the progression of the disease and to the maintenance of the transformed phenotype of tumor cells. Among these, the epidermal growth factor receptor (EGFR) has emerged as a critical signaling hub capable of integrating and transducing a variety of signals from different sources that can have an impact on cancer progression (35–42). These include inflammatory signals from cytokines, TLRs, and cyclooxygenase-2 (Cox-2)-derived prostaglandins, as recently illustrated in colon carcinogenesis (40). A role for EGFR and its ligands in liver regeneration and hepatoprotection during tissue injury has been clearly established (10, 11, 43), and accumulating observations support that dysregulation of EGFR signaling participates in hepatocarcinogenesis (9, 14, 36–38). In the following sections, we briefly review the biology of the EGFR system, its crosstalk with inflammation-related pathways, and the relevance of such interaction in cancer development and potential therapeutic interventions.

The EGFR Signaling System. The EGFR, also known as ErbB1, is a 170 kDa transmembrane glycoprotein characterized by an extracellular ligand-binding domain, a single α-helical transmembrane domain, and a cytoplasmic domain that harbors a tyrosine kinase region. EGFR defines a family of four transmembrane receptors that include ErbB2, ErbB3, and ErbB4 (Fig. 1A) (42, 43). The tyrosine kinase region is followed by a carboxy-terminal tail with tyrosine autophosphorylation sites. This domain is highly conserved among the different members of the family, except in ErbB3 in which key amino acids have been substituted resulting in the ablation of the tyrosine kinase activity (43). The extracellular ligand-binding domain contains two cysteine-rich regions and is less well conserved among the different ErbB proteins, consistent with their ligand-binding specificities. With the exception of ErbB2, for which no ligand has been identified, the ErbB receptors can be bound by a family of growth factors that include EGF, transforming growth factor-α (TGF-α), amphiregulin (AR), epiregulin (EREG), β-cellulin (BTC), Epigen (EPG), and heparin-binding EGF (HB-EGF). These ligands are expressed as Type 1 transmembrane precursor proteins, characterized by the presence of an EGF-like domain, which defines receptor-binding specificity, an immunoglobulin-like domain, and additional motifs that include glycosylation sites and heparin-binding domains in AR and HB-EGF (45–47). As membrane-anchored precursor proteins, these ligands also present a hydrophobic transmembrane domain and a hydrophilic cytoplasmic tail. Figure 1B shows the overall structure of AR transmembrane precursor as an example of a typical EGFR ligand. Although the membrane-anchored peptide can be biologically active through juxtacrine signaling, in most cases the extracellular domain is proteolytically cleaved by a metalloprotease activity present in the cell membrane. This process is known as "ectodomain shedding" and leads to the release of the soluble growth factor, which may act in an endocrine, paracrine, or autocrine fashion (48). The binding of these ligands to the ErbB receptors shows specificity—all of them can interact directly with the EGFR; however, BTC, HB-EGF, and EREG can also interact with ErbB4 (Fig. 1A). Upon ligand-mediated activation, each receptor may form homo- or heterodimers and cross-phosphorylate each other. The ligandless ErbB2 and the kinase-defective ErbB3 also participate in these interactions (36). As indicated before, ligand binding triggers ErbB autophosphorylation in distinct tyrosine residues, creating docking sites for several signal-

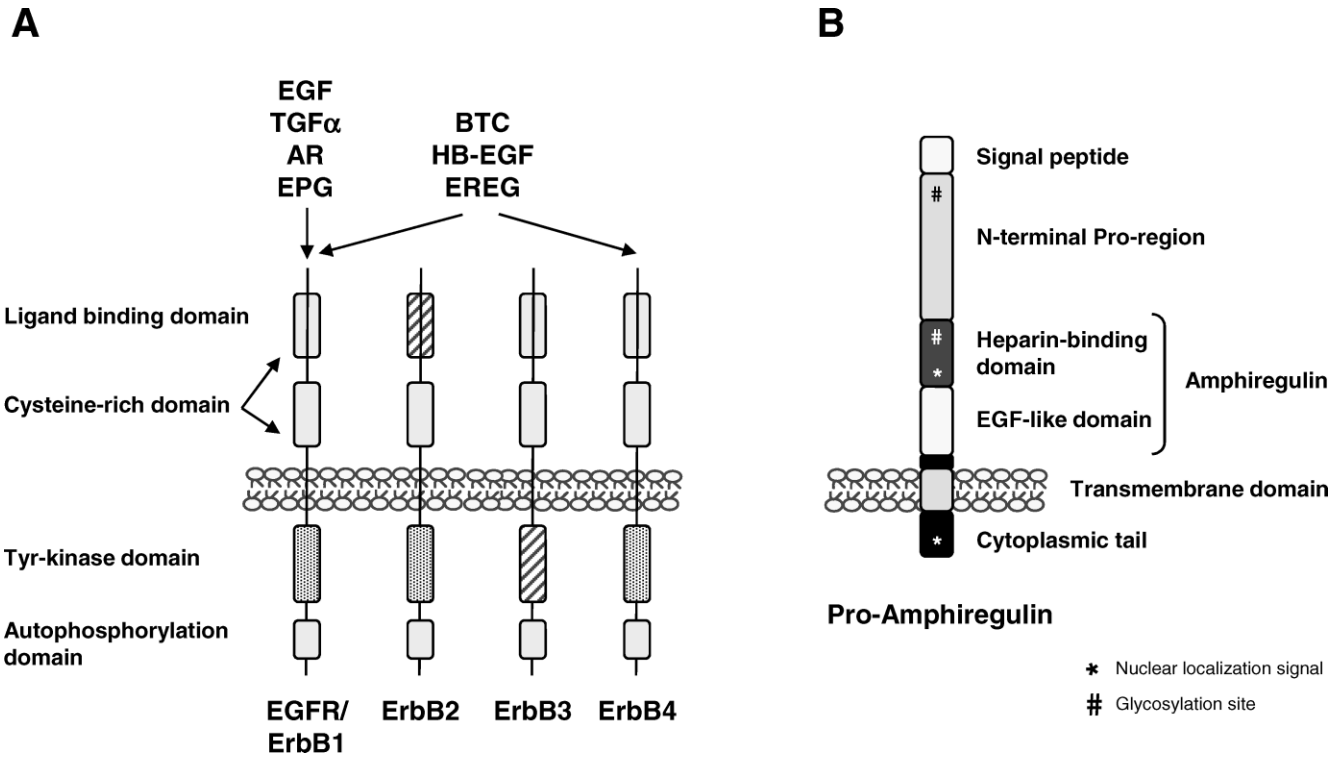

Figure 1. General structure of the EGFR family of receptors. The binding specificities of the different ligands are indicated (A). Structure of AR transmembrane precursor, a prototypical EGFR ligand (B).

ing proteins such as Shc, Grb7, Grb2, Crk, phospholipase Cγ (PLCγ), the kinases Src and PI3K, the protein phosphatases SHP1 and SHP2, and the Cbl E3 ubiquitin ligase (36, 37, 44). There are other signaling proteins, like phospholipase D (PLD) and the STAT 1, 3, and 5 proteins, that do not bind the ErbB receptors but are also activated upon ligand binding (44, 49). These interactions trigger intracellular signaling pathways such as the ras/raf/MEK/MAPK cascade, which includes the activation of ERK and c-jun NH_2-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38-MAPK), the protein kinase C (PKC) pathway, the PI3K/Akt pathway (which can lead to NF-κB activation), and the STAT pathway (37, 43, 44, 49). Intracellular pathways activated by the EGFR show a high degree of interaction and control different transcriptional programs that regulate the expression of genes involved in cell-cycle progression, survival, differentiation, and cell migration, which are dysregulated during inflammation and cancer, including HCC (9, 14, 37, 38, 43).

As indicated above, to allow paracrine or autocrine interaction of the EGFR ligands with the receptor, the membrane-tethered ligand precursors need to be released by a proteolytic reaction. This important step is mediated mainly by membrane-anchored metalloproteases of the ADAM family (a disintegrin and metalloprotease) (50, 51). Various members of the ADAM family have been implicated in EGFR ligand cleavage, including ADAM 9, 10, 12, 15, 17, and 19 (50). However, ADAM17, which is also known as tumour necrosis factor-α (TNF-α)-converting enzyme, or TACE, together with ADAM10, are thought to play a central role (51). ADAM17 can cleave the AR, EREG, TGF-α, and HB-EGF membrane anchored precursors, while ADAM 10 is a key sheddase for EGF, BTC, and can also cleave the HB-HGF transmembrane precursor (50–54). The proteolytic activity of ADAMs is therefore crucial for the generation of soluble EGFR ligands and receptor activation. Importantly, the proteolytic activity of ADAMs is in turn subject to regulation by multiple upstream signals, which adds another layer of complexity to the system. In fact, there is a growing list of physiological stimuli that can trigger EGFR signaling through the stimulation of ligand shedding, a process known as EGFR transactivation (50, 55) (Fig. 2). This process has important biological implications, because it places the EGFR system at the center of converging signals for cell proliferation, survival, migration, and, as will be discussed later, also for the integration of inflammatory signals. Transactivation of the EGFR by ligands of G protein-coupled receptors (GPCRs) is perhaps the best characterized example of EGFR activation by heterologous ligands (50). These include angiotensin II (ANG II), lysophosphatidic acid (LPA), endothelin-I, thrombin, IL-8, and prostaglandins such as PGE_2 (40, 50, 54, 56, 57). Different mechanisms have been proposed to mediate ADAM activation by GPCRs. Elevation of the intracellular levels of Ca^{2+} or reactive oxygen species (ROS) are likely to be involved as well as phosphorylation reactions involving protein kinase C (PKC), ERK, or c-Src (50). However, the existence of specific ADAM kinases

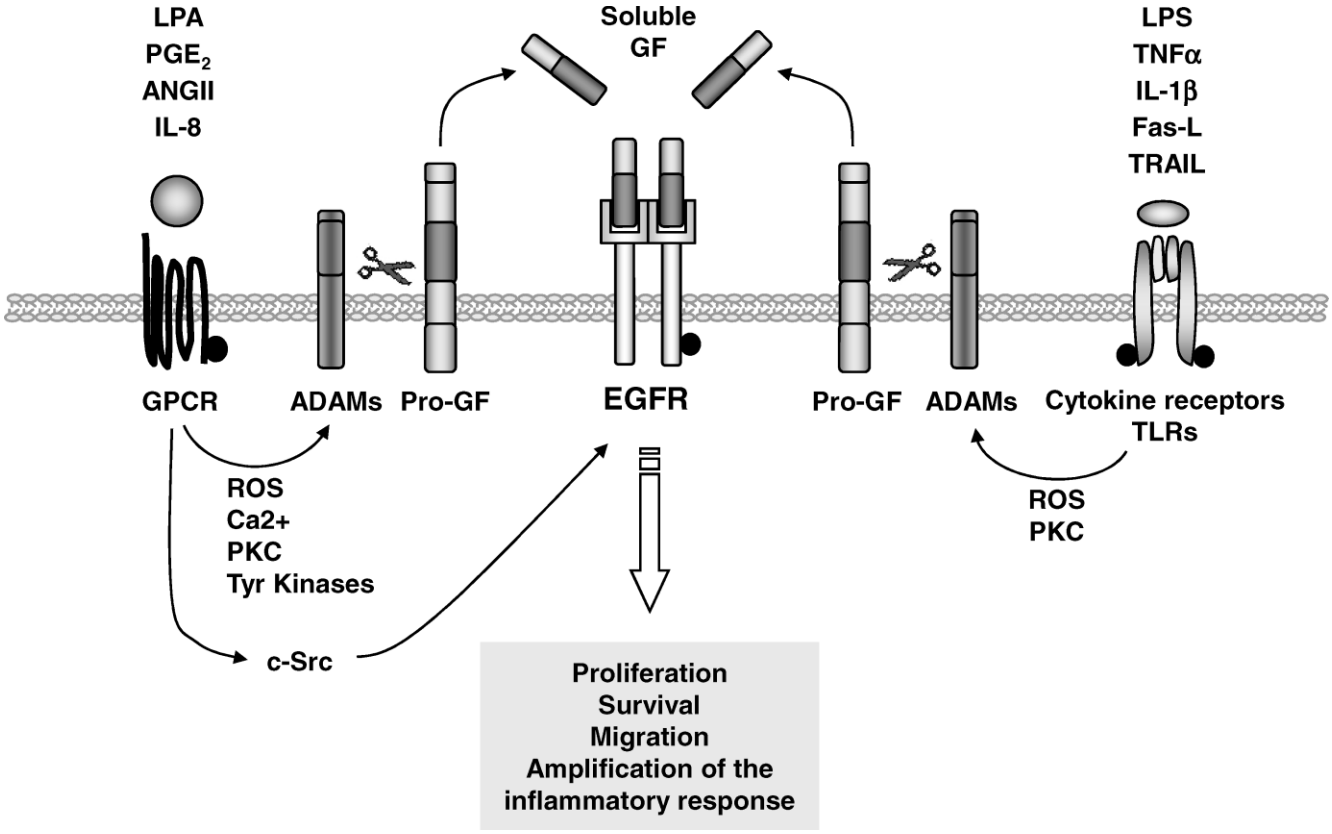

Figure 2. EGFR transactivation. Crosstalk between the EGFR and other signaling systems involved in inflammatory pathways. Pro-GF: pro-growth factor. See text for details.

activated by GPCRs cannot be excluded, and therefore the precise mechanisms involved in ADAM activation remain to be established.

The EGFR Signaling System in Inflammation. The expression of EGFR ligands is known to be increased during injury and inflammation in different organs and tissues. Perhaps one of the best studied cases is the inflammatory reaction of the skin, where the expression of ligands such as TGF-α and HB-EGF is markedly increased during wound healing and the targeted disruption of the EGFR has been shown to impair re-epithelialization after injury (58). An interesting interaction between the EGFR system and innate immunity in epithelial cells is currently being elucidated. In this context, signaling through the EGFR seems essential to induce the expression of TLRs, such as TLR5 and 9, and it synergizes with these receptors to upregulate the production of inflammatory cytokines like IL-8 and antimicrobial peptides (58). The expression of AR and TGF-α is also known to be induced in chronic skin lesions such as psoriasis, where they are thought to promote IL-8 expression. Interestingly, IL-8 is in turn able to stimulate EGFR signaling through the metalloprotease-mediated release of EGFR ligands, leading to a self-perpetuating loop (58).

As previously indicated, transactivation of the EGFR is not exclusive of GPCR-triggered signaling. Studies carried out in keratinocytes have established that the expression and release of EGFR ligands can be elicited by the cytokines TNF-α and interferon-γ (INF-γ) (58). This has been recently observed also for the pro-apoptotic factor Fas ligand (FasL). Interestingly, it was shown that transactivation of the EGFR through the secretion of ligands such as AR contributed to mediate part of the inflammatory responses to FasL in human epidermis (59). Similarly, in the airway epithelium the activation of different TLRs has been reported to promote wound repair and to trigger inflammatory signaling through EGFR transactivation (60, 61). In this case, ROS generated by the NADPH oxidase dual oxidase 1 were implicated in the activation of ADAM17, and in the initiation of innate immune responses in airway epithelial cells (61). The expression and release of EGFR ligands in airway epithelial cells is also stimulated by infectious agents and their PAMPs. This has been recently reported in a model of rhinovirus infection in bronchial epithelial cells, where it was also observed that EGFR activation contributed to the upregulation of TLR2 and TLR4 gene expression and to the amplification of the inflammatory response (62, 63). Of interest is also the interaction between the EGFR system and TGF-β1 signaling in bronchial epithelial cells. As described in a recent report, activation of the EGFR by AR is required for the upregulation of COX-2 expression and PGE_2 production elicited by TGF-β1 (64).

These findings are of particular relevance, given the implications of COX-2 in chronic airway inflammation and the development of lung cancer (64). A close interaction between TGF-β1 and the EGFR system has been also exposed in normal and transformed hepatocytes as will be discussed later.

The gastrointestinal tract is another organ system where important crosstalk between inflammatory signals and the EGFR system is being elucidated. It has been observed that IL-1β and IL-8 can induce the shedding of EGFR ligands in gastric cancer cells (65) and that the TNF-related apoptosis-inducing ligand (TRAIL) promotes ADAM17-mediated TGF-α shedding in a c-Src-dependent fashion in colorectal cancer cells (66). Importantly, the transactivation of EGFR by TRAIL constitutes a mechanism of resistance towards TRAIL-induced apoptosis in these tumoral cells (66).

Further links between inflammation-related TLR activation and the EGFR system also have been established in an experimental model of chemically induced colitis-associated cancer (67). The authors of this study first observed that TLR4 was overexpressed in human colon cancers arising in chronic ulcerative cholitis. The relevance of TLR4 for tumor development was then demonstrated by the lower incidence of tumors in TLR4 knockout mice subjected to an inflammation-related colon carcinoma model. Interestingly, while in colonic tissues obtained from wild type mice undergoing chronic inflammation there was a significant activation of EGFR phosphorylation, this modification was almost undetectable in samples obtained from TLR4 null mice (67). Consistent evidence was then provided demonstrating that TLR4 activation by LPS in colon cells leads to AR-mediated EGFR transactivation. The increase in AR production upon LPS treatment in colon cells is shown to be mediated through the upregulation of COX-2 expression and PGE_2 synthesis (67). This is in agreement with the previously reported activation of AR gene transcription by PGE_2 in colon cancer cells, in which the production of AR mediates the growth-promoting effects of this prostanoid (68). On the other hand, TLR4 is also highly expressed in inflammatory cells like macrophages; therefore, its activation by LPS can also elicit the production of EGFR ligands and further support epithelial proliferation and colon carcinogenesis in a paracrine manner (41). Moreover, transactivation of the EGFR by ADAM17-mediated AR release is responsible for the recently discovered growth-promoting effect of the chemokine MIP-3α on colon cancer cells (69), an observation that further expands the list of inflammation-related mediators that use the EGFR system to convey their cellular signals. Furthermore, in a recent report it was shown that ADAM17 is overexpressed in colorectal cancer and that the simultaneous inhibition of ADAM17 and EGFR activity resulted in cooperative growth inhibition (70). Together these studies provide consistent evidence of the links between inflammatory signaling and the EGFR system in chronic inflammation-related carcinogenesis and suggest the therapeutic potential of interfering with the EGFR axis.

The EGFR Signaling System in Chronic Liver Injury and Cancer. Mounting evidence indicate that the EGFR system plays an important role in liver regeneration and hepatocyte protection in acute and chronic liver injury (36). In fact, expression of EGFR in the hepatocyte is very high as compared with other cell types, and when isolated hepatocytes are treated with EGFR ligands such as EGF, TGF-α, AR, HB-EGF, and EREG, a potent mitogenic and antiapoptotic effect is observed (36). The hepatoprotective and pro-regenerative potential of the EGFR axis has been demonstrated in transgenic mice overexpressing TGF-α or HB-EGF, or by the direct intraperitoneal administration of these ligands in models of acute injury and regeneration (36). More recently, the *in vivo* relevance of EGFR activation during hepatic regeneration has been directly addressed in mice with conditional deletion of this receptor in the liver. In agreement with previous *in vitro* and *in vivo* observations, it was found that mice with targeted ablation of the EGFR show enhanced mortality, hepatocellular injury, and delayed regeneration after partial hepatectomy (71). The expression of different EGFR ligands is known to be upregulated in the hepatic parenchyma during surgically induced regeneration and experimental tissue injury (36). Interestingly, it has been published recently that the expression of ADAM17 is also upregulated during liver regeneration after partial hepatectomy in the rat (72). The relative contribution to hepatic regeneration of most of the EGFR ligands has been established in their respective knockout mice. Although lack of TGF-α or EREG did not seem to affect the course of liver regeneration after partial hepatectomy (73, 74), lack of HB-EGF or AR resulted in a delayed proliferative response of the hepatocytes (75, 76). These observations indicate that there is a certain degree of redundancy in the effects of the different EGFR ligands during liver injury and regeneration. Nevertheless, AR knockout mice showed a more prominent phenotype, and in addition to having a delayed regenerative response after liver tissue resection, these mice also manifested an enhanced death rate when challenged with a lethal dose of a Fas agonistic antibody, which induces mouse death through massive liver failure (77). These findings underscore the fundamental role of the EGFR as a defensive and pro-regenerative signaling system in acute liver injury.

The expression and activity of the EGFR axis has been also assessed in chronic liver injury. Upregulation of AR, TGFα, and HB-EGF gene expression was reported in models of chronic liver injury as well as in liver tissue samples obtained from cirrhotic patients (36, 76, 78). Importantly, as occurs during experimental liver regeneration, the expression of ADAM17 is also increased in human liver cirrhotic tissues, suggesting that the availability of soluble EGFR ligands is further enhanced during chronic liver injury (79). As introduced before, the sustained wound healing response triggered during chronic damage leads to

the accumulation of ECM and the development of liver fibrosis (22). The ECM produced in chronic liver injury originates from myofibroblastic cells that derive from distinct cell populations, including the previously mentioned HSCs and portal fibroblasts. The activation of these ECM-producing cells occurs through a complex interplay among different cell types and results in their proliferation, enhanced survival, and the synthesis of collagen. Profibrogenic mediators can be produced by hepatocytes, KCs, endothelial cells, and infiltrating inflammatory cells and can act on fibrogenic cells in a paracrine fashion (22, 80, 81). In addition, ECM-producing cells are capable of autocrine stimulation through the concomitant expression of activating factors and their receptors (81). As occurs in the mitogenic stimulation of hepatocytes during liver regeneration, the factors involved in the activation of fibrogenic cells include inflammatory cytokines such as IL-1β, IL-6, or TNF-α, and growth factors like platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), and TGF-β (22, 80, 81). The participation of EGFR ligands in this process has also been suggested. *In vitro* treatment of fibrogenic cells with EGFR ligands has been shown to contribute to their phenotypic transformation, mainly through the stimulation of the proliferation and migratory properties of these cells (82–85). In fact, ECM-producing cells such as HSCs and liver myofibroblasts are highly responsive to EGFR ligands, which is in agreement with their elevated expression of the EGFR (Fig. 3A). Within the hepatic parenchyma, EGFR ligands can be produced by different cell types. For example the expression of TGF-α and AR has been detected in hepatocytes and can be increased by inflammatory cytokines and mediators like TNF-α, IL-1β, and PGE_2 (76, 86). Similarly, upon activation ECM-producing cells also release TGF-α and AR, which can engage in autocrine stimulation through binding to the EGFR present in these cells (83, 85). It was also known that when activated with phorbol esters, zymosan or LPS resident liver macrophages also express EGFR ligands like TGF-α (83). Once released, TGF-α can act on hepatocytes or fibrogenic cells in a paracrine fashion (83), but not on KCs, since in these cells, as occurs in bone marrow–derived macrophages, the EGFR mRNA is barely detectable (Fig. 3A). More recently, other EGFR ligands have been identified to be produced by KCs and their expression found to be upregulated by pro-inflammatory stimuli related to the fibrogenic process. For instance, the expression of AR and HB-EGF can be induced by bacterial LPS in murine KCs (85, 87), and as shown in Figure 3B these two ligands show a more prominent response to bacterial endotoxin when compared with other members of the family. The expression of EGF-related growth factors can be also elicited by pro-inflammatory cytokines such as IL-1β and TNF-α in murine KCs (Fig. 3C) (85).

In spite of this abundant information on the expression and activity of EGFR ligands during chronic liver injury, their *in vivo* contribution to hepatic fibrogenesis remains largely unexplored. However, in a recent report it was demonstrated that AR knockout mice develop attenuated liver fibrosis when chronically challenged with CCl_4 than do wild-type animals (85). Furthermore, the expression of important pro-fibrogenic mediators such as TGF-β and CTGF was reduced in the liver of AR null mice after chronic CCl_4 administration (85). Interestingly, it was also shown that AR directly induced the expression of CTGF when added to cultured human and murine fibrogenic cells, including HSCs, and that AR also was a proliferative and survival factor for these cells (85).

The existence of EGFR transactivation mechanisms in liver fibrogenic cells has been recently shown. In an attempt to characterize the mechanisms behind the resistance of HSCs to the pro-apoptotic effects of death receptor agonists such as FasL, TNF-α, or TRAIL, a ligand-dependent activation of the EGFR was uncovered (88). It was found that these death receptor agonists were able to stimulate EGFR signaling and HSC proliferation through the protease-mediated release of EGF, therefore identifying novel cellular mechanisms potentially related to liver fibrogenesis that involve the EGFR system.

Persistent activation of the EGFR has been demonstrated to participate in the pathogenesis of tissue fibrosis in different organs such as the kidney (89). Interestingly, treatment with the EGFR inhibitor gefitinib proved to be an effective strategy to prevent experimental renal vascular and glomerular fibrosis (90), suggesting that the pharmacological targeting of the EGFR system may be also effective in the prevention of liver fibrosis.

As described above, chronic tissue damage and inflammation in the liver results in the sustained overexpression and overstimulation of the EGFR pathway. On the other hand, the implication of a dysregulated EGFR signaling system in the development of HCC is gaining considerable support. Already from the early stages of experimental carcinogenesis it has been observed that the production of EGFR ligands may influence the growth of premalignant cells (91). Pro-inflammatory stimuli elicit the release of EGFR ligands such as HB-EGF from liver KCs and endothelial cells, which in turn stimulate the proliferation of initiated hepatocytes in a paracrine fashion (87, 91). Furthermore, activation of the EGFR in liver cancer cells by ligands released from inflammatory cells seems to further potentiate their aggressive behaviour (92).

Dysregulation of the EGFR system in human HCC tissues includes the overexpression of EGFR and ErbB3, as well as their ligands HB-EGF, TGF-α, BTC, and AR, and ADAM17 (9, 14, 36, 79, 93). Experimental models using genetically modified mice also attest to the relevance of this system in the development of liver cancer. For example, mice transgenic for TGF-α or EGF show a high tendency to develop HCC (94, 95), while hepatocarcinogen treatment of TGF-α null mice results in smaller tumors than in wild-type animals (73). Experiments carried out in human HCC cell lines indicate that autocrine signaling through the EGFR is

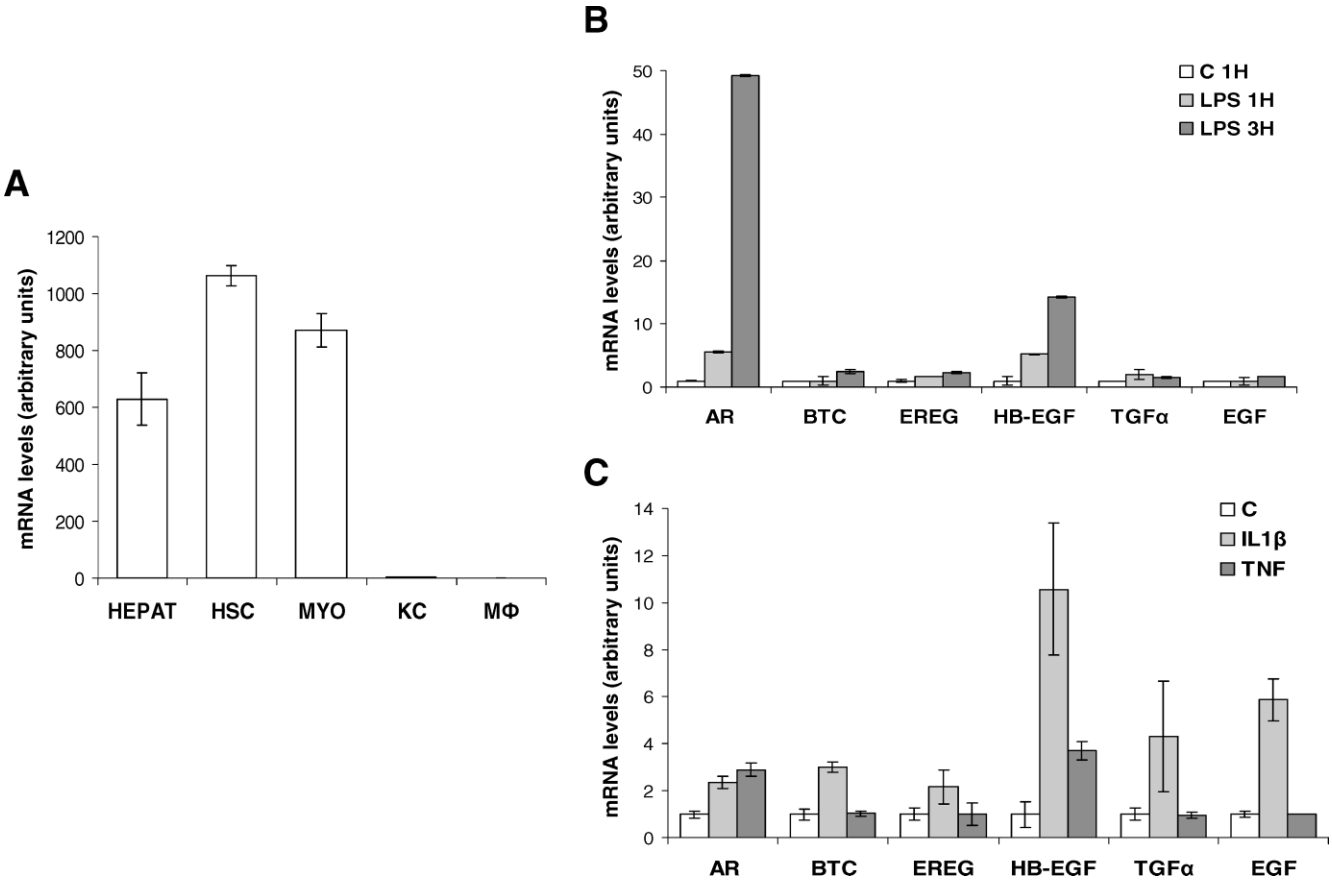

Figure 3. (A) Quantitative real-time PCR analysis of the expression of the EGFR in freshly isolated and cultured mouse hepatocytes (HEPAT), hepatic stellate cells (HSC), hepatic myofibroblasts (MYO), Kupffer cells (KC), and bone marrow macrophages (MΦ). (B) Effect of *E. coli* LPS (100 ng/mL) treatment on the expression of EGFR ligands in isolated murine KCs as determined by quantitative real-time PCR. (C) Effect of IL-1β (2 ng/mL) or TNF-α (20 ng/mL) treatment for 1 h on the expression of EGFR ligands in isolated murine KCs as determined by quantitative real-time PCR. Cells were isolated and cultured, and gene expression was analyzed as described in refs. 77 and 85.

important for the survival and proliferation of liver cancer cells. Treatment of HCC cells with pharmacological inhibitors of the EGFR significantly impairs their viability. This has been shown with monoclonal antibodies that compete with the binding of activating ligands, or small-molecule inhibitors of the receptor tyrosine kinase activity, which induce growth inhibition, cell-cycle arrest, and apoptosis (see below) (36, 96). Interestingly, the specific silencing of AR gene expression in HCC cells results in reduced constitutive EGFR signaling, inhibition of cell proliferation, anchorage-independent growth, and increased apoptosis in response to cytotoxic drugs (79). These findings suggest that this particular EGFR ligand plays a nonredundant role in the malignant phenotype of human HCC cells.

The interaction of the EGFR system with the inflammatory and pro-tumorigenic cytokine TGF-β may be of special relevance in liver carcinogenesis. TGF-β has been demonstrated to induce the expression of EGFR ligands such as HB-EGF and TGF-α in isolated fetal rat hepatocytes through the activation of the inflammatory transcription factor NF-κB (97). Moreover, TGF-β also stimulates ADAM17 activity promoting the release of EGFR ligands, which in turn contribute to mediate resistance toward the pro-apoptotic effects of TGF-β in normal hepatic cells (98). Activation of ADAM17 and EGFR ligand shedding by TGF-β appears to be a more general mechanism involved in tumorigenesis. In support of this notion is the recent demonstration of the activation of AR and TGF-α shedding in breast cancer cells upon TGF-β treatment and the importance of this mechanism in the aggressive behaviour of these tumor cells (99). Upon malignant transformation, HCC cells also seem to rely on the EGFR system to resist apoptosis induced by TGF-β. For instance, it has been demonstrated that interference with AR gene expression in human HCC cells results in the disruption of an autocrine loop that protects tumor cells from apoptosis induced by TGF-β (79).

Other inflammatory and tumor environmental stimuli that have been demonstrated to engage the EGFR system in liver cancer cells include TNF-α (100), fibronectin (101), and ANGII (102). TNF-α was initially shown to mediate its proliferative effects in nontransformed hepatocytes through the shedding of TGF-α (103). Subsequently, the trans-

activation of the EGFR receptor proved to be relevant for the pro-metastatic activity of TNF-α in HCC cells, although the identity of the ligand(s) involved was not established (100). A recent report on the crosstalk between ANGII and the EGFR demonstrated the potential influence of this cytokine on HCC proliferation and invasion (102). ANGII levels are frequently elevated in patients with chronic liver injury as well as in experimental models of chronic liver damage and fibrogenesis (80). ANGII has been shown to activate human HSCs in culture and to play a critical role in the development of hepatic fibrosis (104). Together, these observations suggest that a cross-talk between ANGII and the EGFR system in the liver may exist well before neoplastic transformation of the hepatocytes and therefore can contribute to this process from early stages. Moreover, although it has not been established yet, interaction between these two pathways might also be involved in the proliferation of ECM-producing cells in the liver and the development of fibrosis.

Targeting of the EGFR-Signaling System in HCC. Anti-EGFR agents were initially tested for the treatment of epithelial cancers such as non-small-cell lung cancer (NSCLC), in which the expression and function of the EGFR signaling system is dysregulated (38, 105). Two classes of EGFR antagonists are currently available: anti-EGFR monoclonal antibodies and small-molecule EGFR tyrosine kinase inhibitors (105). Monoclonal antibodies, such as cetuximab, bind to the extracellular domain of the EGFR, blocking ligand binding and receptor activation (105, 106). Tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, compete with ATP to bind to the intracellular catalytic domain of EGFR tyrosine kinase and inhibit downstream signaling from the receptor (105, 106). Although gefitinib, the first anti-EGFR agent tested, showed clinically relevant antitumor activity in phase 2 studies carried out in patients with NSCLC, a subsequent phase 3 trial failed to improve survival (105, 106). Erlotinib, in contrast to gefitinib, yielded a significant survival benefit in a phase 3 study also performed on patients with this type of lung cancer (107). Cetuximab administration to patients with advanced NSCLC was only marginally active; however, the combination of cetuximab with platinum-based compounds was of clinical benefit (105). A number of trials have also combined chemotherapy with the administration of EGFR-TKIs; however, no clear-cut benefits were observed (105, 108). A possible explanation to the limited efficacy of EGFR-TKIs was that these compounds were tested in unselected NSCLC patients. It was found that lung cancer patients responsive to EGFR-TKIs harbored activating mutations in the tyrosine kinase domain of the *EGFR* gene (106, 109). These mutations concentrated in exons 18 to 21, near the ATP cleft of the TK domain, where gefitinib and erlotinib compete with ATP for binding (106, 109). In addition to these *EGFR* somatic mutations, the overexpression of EGFR ligands such as TGF-α and AR has been demonstrated as independent prognostic factors in the response to gefitinib therapy in NSCLC patients (110, 111).

Regarding HCC, encouraging observations using anti-EGFR agents in cultured HCC cells (96, 112, 113) and in *in vivo* models of liver cancer (78) led to the clinical evaluation of these compounds. However, when tested as single agents, gefitinib and cetuximab showed no objective responses in HCC patients (114–116), while erlotinib had a modest disease-control benefit, manifested by modestly prolonged progression-free survival and overall survival in a phase 2 study (117). A recent multicenter phase 2 study showed a relatively good disease control rate and progression-free survival in patients with advanced HCC treated with a combination of gemcitabine plus oxaliplatin with cetuximab (118).

The reasons for these modest responses are not completely known. Sequencing of exons 18–21 of the *EGFR* gene in a significant number of HCC samples found no activating mutations (119). As previously mentioned, these activating mutations predict a good response to gefitinb, and their absence in liver cancer may be related to the observed limited efficacy of EGFR-TKIs. In addition, as occurs in NSCLC, the overexpression of EGFR ligands commonly observed in HCC may also participate in gefitinib resistance. Crosstalk with other signaling systems that are also dysregulated in HCC, such as the insulin-like growth factor-2 (IGF-2)/IGF-1 receptor (IGF-1R), and the constitutive activation of downstream signaling effectors, have been invoked. It was demonstrated that while IGF-2 exerted its mitogenic effect on HCC cells through AR-mediated transactivation of the EGFR, its pro-survival activity was mediated through the PI3K pathway and was completely resistant to EGFR inhibitors (120). Interestingly, combination of EGFR and IGF1-R inhibitors overcomes resistance to EGFR blockade and results in enhanced HCC cell killing in preclinical studies (112, 120). Moreover, simultaneous targeting of downstream signaling effectors of the EGFR and IGF-1R pathways such as mTOR enhanced therapeutic efficacy in experimental models (112, 121). These findings highlight the molecular complexity of HCC and provide a rationale to test combination of targeted therapies in HCC patients. These combinations may also include inhibitors of the COX-2 system, which as previously stated extensively crosstalks with the EGFR axis (40) or newly developed inhibitors of ADAM17, which have shown promising results in the inhibition of colorectal cancer cell growth (122). Additionally, although ErbB2 overexpression is not common in HCC, given the capacity of ErbB receptors to form heterodimers and the potent intracellular signaling generated from heterodimeric complexes (123), a dual EGFR and ErbB2 inhibitor, lapatinib, is currently being tested in experimental HCC and early clinical trials (116).

In view of the findings summarized in this review, the EGFR signaling system is situated at a critical junction between inflammation-related signals and potent cell-

regulating machineries. These experimental evidences connecting liver cancer development and inflammation provide novel strategies for the prevention and treatment of this deadly disease that warrant clinical testing.

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