Original Article
Epidermal growth factor upregulates the expression of A20 in hepatic cells via the MEK1/MSK1/p-p65 (Ser276) signaling pathway

Haiyang Chen[1*], Xuan Wu[1*], Hanqiong Zhou[1*], Zhen He[1], Hongle Li[2], Qiming Wang[1]

[1]Department of Internal Medicine, Henan Cancer Hospital, Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou 450008, Henan, P. R. China; [2]Molecular Pathology Department, Affiliated Oncology Hospital, Zhengzhou University, Zhengzhou 450008, Henan, P. R. China. *Equal contributors.

Abstract: Tumor necrosis factor α-induced protein 3 (A20) suppresses inflammation by inhibiting the activation of nuclear factor kappa B (NF-κB). The aberrant expression of A20 is reportedly correlated with tumor development in human malignancies, including hepatocellular carcinoma (HCC). Proinflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin-1, and lipopolysaccharide, may induce A20 expression. The present study revealed that epidermal growth factor (EGF) significantly increased A20 mRNA and protein levels in normal hepatic and hepatoma cells via the mitogen-activated protein kinase kinase-1 (MEK1)/mitogen- and stress-activated protein kinase-1 (MSK1)/phosphorylated (p)-p65 (Ser276) signaling pathway. A significant positive correlation was observed between the expression of EGF receptor and A20 in HCC and normal healthy liver tissues. The EGF-induced A20 upregulation was NF-κB-dependent and abolished by either the overexpression of the nuclear factor of a κ light polypeptide gene enhancer in a B-cell inhibitor α or treatment with the NF-κB inhibitor BAY11-7082. However, unlike TNF-α, EGF expression did not result in the upregulation of inflammatory molecules, including intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and monocyte chemoattractant protein-1. These results indicate that EGF preferentially upregulated the protective mediator A20 over proinflammatory factors. To our knowledge, the present study is the first to demonstrate that EGF induced A20 expression by activating the MEK1/MSK1/p-p65 (Ser276) signaling pathway without causing an apparent inflammatory response. These results may further extend our understanding of liver inflammation and tumor development.

Keywords: Tumor necrosis factor α-induced protein 3 (A20), epidermal growth factor (EGF), nuclear factor kappa B (NF-κB), inflammation, liver

Introduction

Liver cancer, one of the leading causes of cancer-associated mortalities worldwide, is particularly prevalent in China. Hepatocellular carcinoma (HCC) is the major histological subtype and accounts for 70-85% of all liver cancer cases [1, 2]. The high incidence of HCC in China is largely due to the increased prevalence of chronic hepatitis B virus (HBV) infection. In fact, the inflammation induced by chronic HBV infection is observed in 60% of all liver cancer cases [3]. The nuclear factor kappa B (NF-κB) signaling pathway plays a crucial role in the inflammation process by controlling the expression of several inflammatory cytokines and chemokines, including tumor necrosis factor-α (TNF-α) and interleukin (IL)-1, IL-6, and IL-8, which are closely related to tumor development [4]. Further, a previous study revealed that activation of the NF-κB signaling pathway promotes the development of HCC by maintaining inflammatory responses in liver [5]. Therefore, suppressing chronic inflammation in the liver by targeting the NF-κB signaling pathway may prevent the development and progression of HCC.

Initially, TNF-α-induced protein 3 (A20) was identified as a TNF-α-inducible gene in human umbilical vein endothelial cells [6, 7]. Previous research demonstrated that A20 functions as a

potent regulator of inflammation due to its ubiquitin-editing activity and subsequent inhibitory effect on NF-κB activation [8-12]. Moreover, the aberrant expression of A20 is correlated with tumor development in several human malignancies, including HCC [12, 13]. In addition to TNF-α, A20 is induced by several other proinflammatory mediators, including IL-1, the Epstein-Barr virus, Latent Membrane Protein, the CD40 ligand, and lipopolysaccharide (LPS) [6, 14-16]. A mechanistic investigation revealed the induction of A20 requires two κB elements that are recognized by NF-κB transcription factors [17]. However, as an NF-κB-dependent gene, A20 participates in a negative feedback loop limiting NF-κB activation and inflammation. Furthermore, the expression of A20 is regulated by certain microRNAs, including microRNA-29b, microRNA-29c, microRNA-125a, and microRNA-125b [18-20]. A previous study revealed that hepatocyte growth factor (HGF) preferentially induces A20 but not inflammatory molecules via the p65/RelA subunit of the NF-κB pathway in renal tubular epithelial cells. This result introduced the possibility of upregulating A20 without causing inflammation, which is advantageous for preventing acute and chronic kidney diseases [21]. Based on the aforementioned study results, the present study was designed to investigate the regulation of A20 in hepatic cells.

Although the epidermal growth factor receptor (EGFR) family reportedly plays a key role in the progression of HCC, epidermal growth factor (EGF) may be one of the mitogens required for the growth of hepatoma cells [22-24]. Additionally, EGF could induce weak but notable NF-κB activation through its receptor, which belongs to a family of receptor tyrosine kinases, particularly in estrogen receptor-negative breast cancer [25-27]. However, the influence of the EGFR signaling cascade on the expression of A20 has not been investigated.

The present study demonstrates that EGF significantly upregulated A20 in normal hepatic and HCC cells via the mitogen-activated protein kinase kinase1 (MEK1)/mitogen- and stress-activated protein kinase-1 (MSK1)/p-p65 (Ser276) signaling pathway and that the EGF-induced upregulation of A20 contributed to the anti-inflammatory effect in the liver.

Materials and methods

Cell culture and human tissue specimens

The liver cancer cell line HepG2 and the human immortalized normal hepatocyte cell line QSG7701 were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The HCCLM3 cells were obtained from the cell bank of Zhongshan Hospital, Fudan University Medical College (Shanghai, China) [28]. The cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Gaithersburg, MD, USA; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen) and maintained at 37°C in a humidified incubator containing 5% CO_2. Cells were passaged every 2-3 days to maintain the logarithmic growth phase. Stable A20 knockdown QSG7701 cells (QSG7701-shA20) and their empty vector counterpart (QSG7701-shcon) were generated using a lentivirus system followed by selection in medium containing puromycin (3 μg/ml) for 2-3 weeks.

Human HCC tissues were collected from 22 patients who underwent HCC resection in the Henan Cancer Hospital from 2017 to 2018. The study was approved by the Medical Ethics Committee of Henan Cancer Hospital. Written informed consent was received from all participants in this study at the time of surgery.

Reverse transcription-quantitative real-time polymerase chain reaction

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) analysis was performed as previously described [29]. Briefly, total RNA from liver tumor and non-tumor tissues was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and reverse-transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). Gene-specific primers were obtained from Sangon Biotech Co., Ltd. (Shanghai, China) and SYBR Green PCR Master mix was purchased from Takara Biotechnology Co., Ltd. (Beijing, China). An ABI Prism 7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA; Thermo Fisher Scientific, Inc.) was used to perform qPCR. The following prim-

er pairs were used: A20 forward, 5'-CTCA-ACTGGTGTCGAGAAGTCC-3' and reverse, 5'-TT-CCTTGAGCGTGCTGAACAGC-3'; EGFR forward, 5'-CCAAGGCACGAGTAACAAGC-3' and reverse, 5'-AGGGCAATGAGGACATAACCAG-3'; intercellular adhesion molecule 1 (ICAM-1) forward, 5'-GCAAGAACCTTACCCTACGC-3' and reverse, 5'-AGTCGCTGGCAGGACAAA-3'; vascular cell adhesion molecule 1 (VCAM-1) forward, 5'-GC-AAGGTTCCTAGCGTGTA-3' and reverse, 5'-GAA-GGGCTGACCAAGACG-3'; monocyte chemoattractant protein-1 (MCP-1) forward, 5'-TGTG-CCTGCTGCTCATAG-3' and reverse, 5'-GTTTG-GGTTTGCTTGTCC-3'; and β-actin forward, 5'-AATCGTGCGTGACATTAAGGAG-3' and reverse, 5'-ACTGTGTTGGCGTACAGGTCTT-3'. The mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to endogenous control β-actin. Each sample was tested in triplicate.

Western blot analysis

Western blot was performed as previously described [29]. Briefly, the total protein was extracted from cell lines using lysis buffer [Tris-HCl (20 mM), NaCl (150 mM), glycerol (10%), Nonidet P-40 (0.2%), EDTA (1 mM), EGTA (1 mM), PMSF (1 mM), NaF (10 mM), aprotinin (5 mg/ml), leupeptin (20 mM), and sodium orthovanadate (1 mM); pH 7.4] and centrifuged at 12,000 × g for 30 min. Total protein concentration was determined using a bicinchoninic acid protein assay (BCA). Samples were prepared in 4X sodium dodecyl sulfate (SDS) sample buffer and separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. The separated proteins were transferred onto nitrocellulose membranes. Primary antibodies against A20, phosphorylated (p)-NF-κB p65 (Ser276; p-p65), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary antibodies against the nuclear factor of the κ light polypeptide gene enhancer in NF-κB inhibitor α (IkBα), p-IkBα, p65, Akt, p-Akt, Erk, and p-Erk were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Following primary antibody incubation, membranes were incubated with goat anti-rabbit (Cell Signaling Technology) or anti-mouse fluorescein-conjugated secondary antibodies (Cell Signaling Technology). Protein bands were detected using an Odyssey fluorescence scanner (LI-COR Biosciences, Lincoln, NE, USA).

Plasmids and biological reagents

The IkBα plasmid was purchased from Addgene, Inc. (Cambridge, MA, USA). The pCSII-H1-PGK-puro-WPRE-shRNA-A20 and control scramble vector were gifts from Professor Masao Seto [29]. The pEF1-A20-wt was a gift from Dr. Daniel Krappmann, Helmholtz Zentrum Munchen Gmbh, Germany. The phosphatidyl inositol 3-kinase (PI3K) inhibitor LY294002, mitogen-activated protein kinase (MAPK) kinase 1/2 inhibitor PD98059, NF-κB inhibitor BAY11-7082, EGFR inhibitor AG1478, and protein kinase A (PKA) inhibitor H89 were purchased from Selleck Chemicals (Houston, TX, USA). The cytokines TNF-α, LPS, IL-6, EGF, and HGF were purchased from PeproTech China (Suzhuo, Jiangsu Province P.R. China), and erlotinib was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

NF-κB luciferase reporter assay

Cells were plated in 24-well plates and transiently transfected with NF-κB promoter-firefly luciferase plasmid (NF-κB-luc) and internal control plasmid pRL-TK (Promega), which encodes Renilla luciferase and is used to normalize transfection efficacy. The cells were incubated for 24 h. The indicated cytokine TNFα (10 ng/ml; Peprotech) was added to the medium for 6 h and harvested with 120 μl 1 × passive lysis buffer (Promega). The firefly luciferase activity was detected with a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Each point was set in duplicate, the experiments were repeated three times, and the results are expressed as the mean ± SEM.

Statistical analysis

All data are expressed as the mean ± SEM. The Student's t-test was used to determine statistical significance among variables. Pearson's correlation test was used to assess the relationship between the mRNA expression of A20 and EGFR. All statistical analyses were carried out using SPSS PASW Statistics 18.0 software (SPSS, Inc., Chicago, IL, USA). A statistically significant difference was defined as P < 0.05.
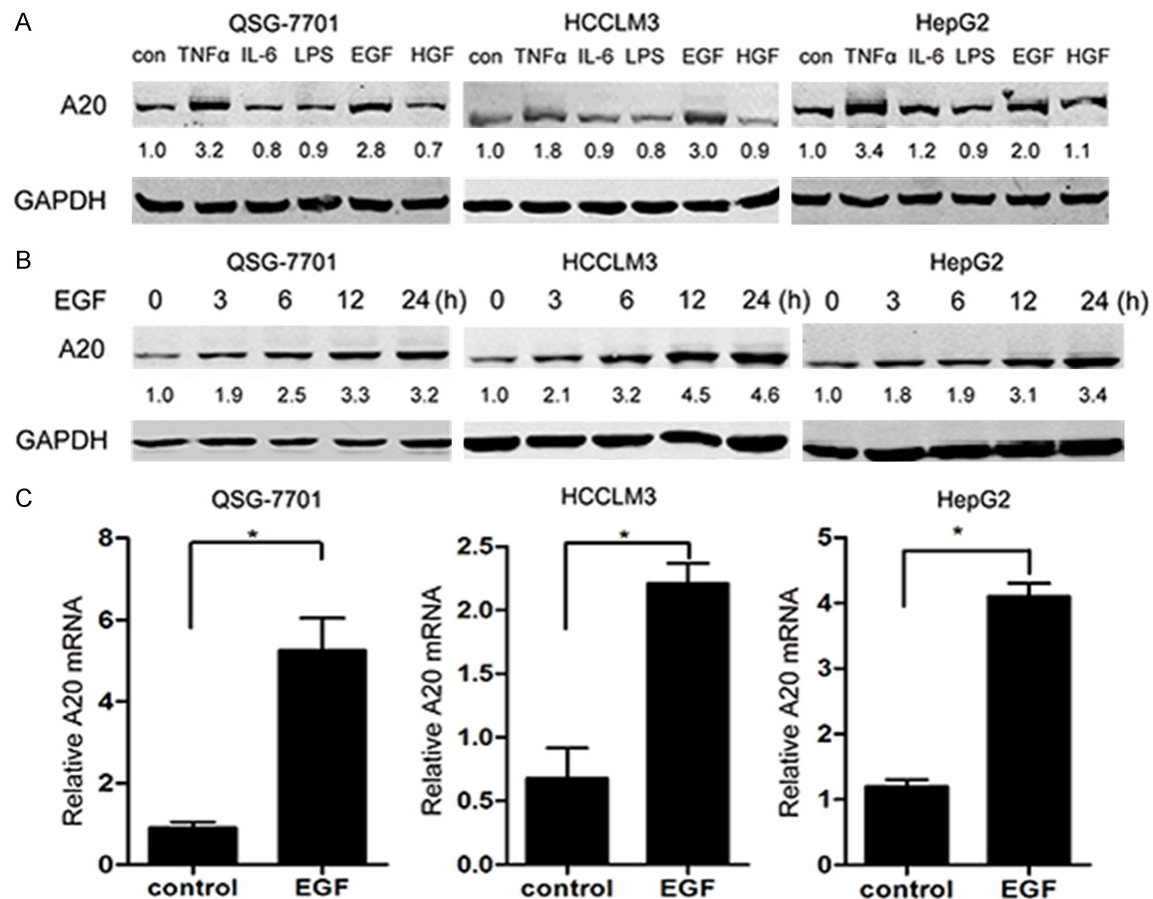
Figure 1. EGF upregulates A20 expression in QSG7701, HepG2, and HCCLM3 cell lines. A. Western blot of A20 in QSG7701, HCCLM3, and HepG2 cells stimulated with TNF-α (10 ng/ml), IL-6 (100 ng/ml), LPS (100 ng/ml), EGF (50 ng/ml) or HGF (50 ng/ml) for 6 hours. B. Western blot of A20 in QSG7701, HCCLM3, and HepG2 cells treated with EGF for different periods. C. The reverse transcription-quantitative PCR of A20 mRNA in QSG7701, HCCLM3, and HepG2 cells treated with EGF. Key: EGF, epidermal growth factor; A20, tumor necrosis factor α-induced protein 3; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; LPS, lipopolysaccharide; HGF, hepatocyte growth factor.

Results

EGF upregulated the expression of A20 in normal hepatocytes and HCC cell lines

Evaluation of whether EGF regulates the expression of A20 in liver cells was determined by western blot analysis following treatment of liver cells with EGF, TNF-α, IL-6, LPS, or HGF. The results reveal that TNF-α and EGF significantly increased A20 protein (**Figure 1A**). In contrast, IL-6, LPS, and HGF did not significantly affect the expression of A20. Further analysis revealed that EGF significantly upregulated A20 expression in a time-dependent manner, with the maximum protein level (over threefold) observed approximately 12 h after the addition of EGF (50 ng/ml; **Figure 1B**). Correspondingly, RT-qPCR analysis results show that A20 mRNA was upregulated following EGF treatment for 12 h in QSG7701, HCCLM3, and HepG2 cells

(**Figure 1C**). Cells were also treated with EGF inhibitors AG1478 (2 µM) and erlotinib (2 µM) for 3 h, and western blot analysis revealed that the expression of A20 was significantly reduced in the EGFR inhibitor group compared with the blank group (Figure S1).

EGF-induced upregulation of A20 was dependent on NF-κB activation

According to previous reports, NF-κB activation is required to induce A20 transcription in response to proinflammatory stimuli [14, 17, 30]. Therefore, the effect of NF-κB inhibition on the EGF-mediated A20 upregulation in QSG7701 cells was investigated by overexpressing IkBα or inhibiting NF-κB signaling using BAY11-7082. The results show that overexpression of IkBα significantly suppressed the EGF-induced upregulation of A20 expression compared with transfection with the control
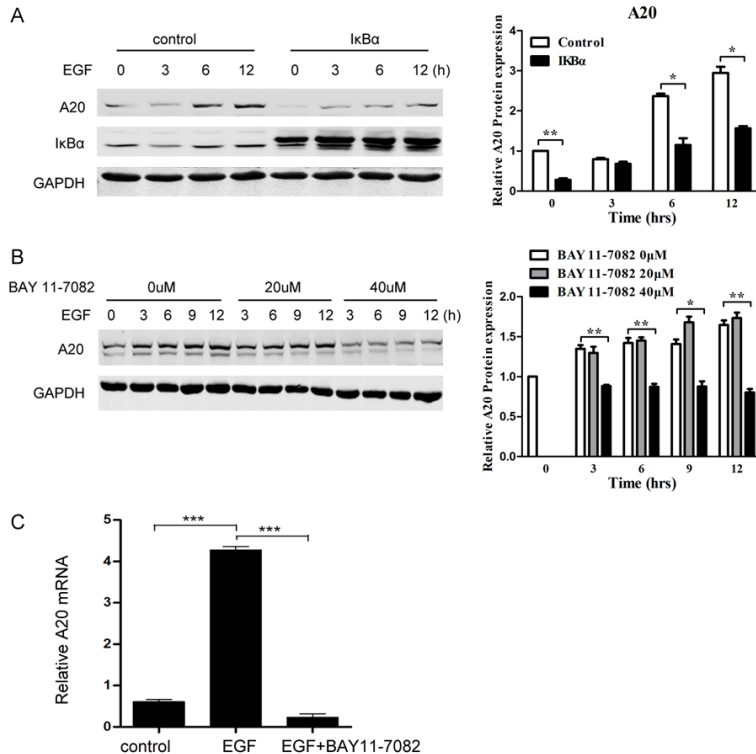
Figure 2. EGF-induced A20 upregulation is dependent on NF-κB activation. A. Western blot of A20 protein in QSG7701 cells with pCDNA3.1-IkBα or control plasmids treated with EGF for the indicated time. B. Western blot of A20 protein in QSG7701 cells pretreated with the NF-κB inhibitor BAY11-7082 at the indicated concentrations and followed by EGF treatment. C. Reverse transcription-quantitative PCR of A20 mRNA in QSG7701 cells pretreated with BAY 11-7082 or DMSO and after treatment with EGF. Key: EGF, epidermal growth factor; A20, tumor necrosis factor α-induced protein 3; IkBα, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α; DMSO, dimethyl sulfoxide. All data are expressed as the mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

vector (Figure 2A). Furthermore, the cytoplasmic and nuclear protein separation assay results reveal that the transfer of p65 from the cytoplasm to the nucleus was inhibited when IkBα was upregulated. Similarly, blocking NF-κB signaling with BAY11-7082 inhibited the EGF-induced upregulation of A20 protein and mRNA levels (Figure 2B, 2C). Collectively, the results indicate that EGF-induced upregulation of A20 required NF-κB activation.

Activation of the MEK1/MSK1/p-p65 (Ser276) pathway is required for EGF-induced A20 upregulation

EGF binding to the EGFR leads to receptor dimerization, autophosphorylation, and the activation of downstream pathways involving PI3K-Akt and MEK1-ERK [31]. Previous studies have shown that NF-κB is activated by either the PI3K-Akt or MEK1-ERK signaling pathway [32-

34]. To elucidate the molecular mechanism underlying EGF-induced A20 upregulation, PI3K-Akt and MEK1-ERK signaling in QSG7701 cells was blocked with the PI3K inhibitor LY294002 (20 μM) or the MEK1 inhibitor PD-98059 (50 μM), respectively. The results reveal that EGF-induced upregulation of A20 was not suppressed by inhibiting PI3K-Akt activation (Figure 3A). By contrast, when ERK phosphorylation was inhibited using PD98059, upregulation of A20 by EGF was significantly decreased (Figure 3B). These findings suggest that MEK1-ERK rather than PI3K-Akt activation was required for EGF-induced upregulation of A20.

Some researchers have reported that the MEK1 inhibitor blocks phosphorylation of p65/RelA (Ser276) but not Ser536, suggesting that this phosphorylation is dependent on the activation of the MEK1/2-ERK1/2 pathway [35]. They also show that inhibition of MSK1, a kinase acting downstream of MEK1/2-ERK1/2, also inhibits phosphorylation of p65/RelA (Ser276). These findings suggest that phosphorylation is dependent on MSK1 (Figure 4A). To detect whether phosphorylation of p65 at Ser276 was involved in EGF-induced A20 upregulation, QSG7701 cells were pretreated with an inhibitor of MSK1 (10 μM H89) or dimethyl sulfoxide (DMSO) prior to treatment with EGF. As shown in Figure 4B, phosphorylation of p65 at Ser276 was inhibited in cells pretreated with H89, and A20 protein levels were significantly decreased compared with control cells. These results indicate that EGF promoted A20 expression via the MEK1-MSK1-p-p65 (Ser276) signaling pathway.

Relationship between the expression of A20 and EGFR in normal liver and HCC tissues

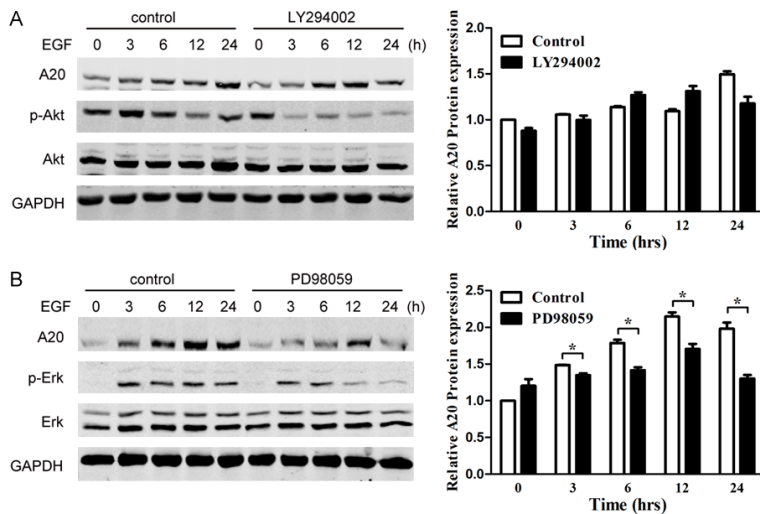The mRNA expression of EGFR and A20 was detected in 22 pairs of human hepatocarcino-

Figure 3. EGF induced the expression of A20 through the MEK1/MSK1/p-p65 (Ser276) signaling pathway. Western blot of A20 protein in QSG7701 cells pretreated with (A) the PI3K inhibitor LY294002 or (B) the MEK1 inhibitor PD98059 followed by treatment with EGF for different periods. Key: EGF, epidermal growth factor; A20, tumor necrosis factor α-induced protein 3; MEK1, mitogen-activated protein kinase; MSK1, ribosomal protein S6 kinase A5; p, phosphorylated. All data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
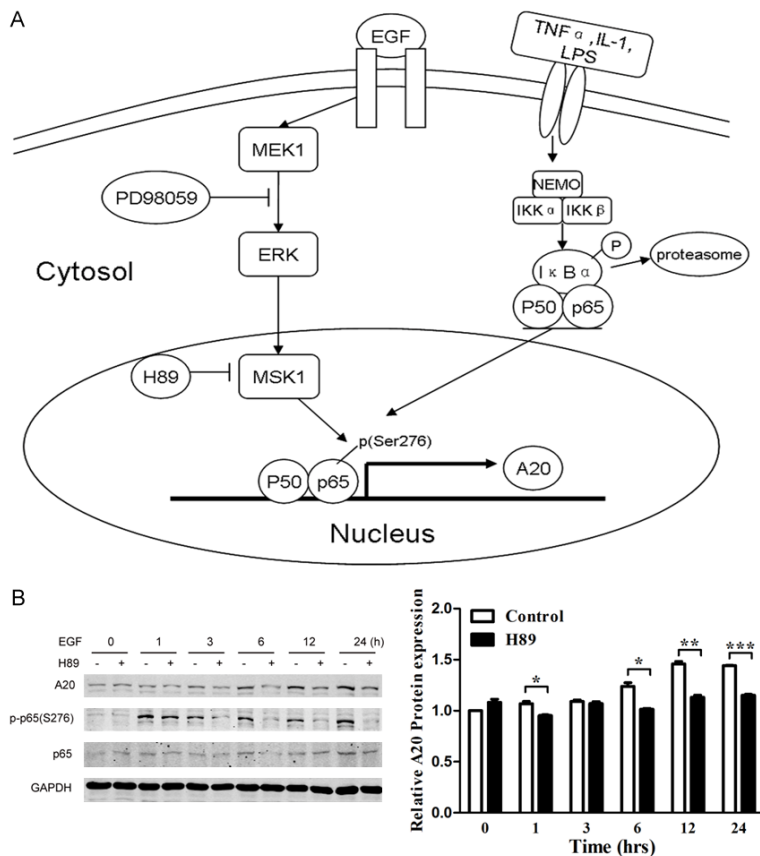

Figure 4. EGF induced A20 expression via the MEK1/MSK1/p-p65 (Ser276) signaling pathway. A. EGF activated the MEK1/ERK signaling pathway, resulting in the activation of MSK1 and the subsequent phosphorylation of

p65 at Ser276, increased p65 activity, and increased expression of A20. The inhibition of MEK1 by PD98059 resulted in reduced MSK1 phosphorylation, whereas the inhibition of MSK1 by H89 repressed the phosphorylation of p65 and the induction of A20. B. Western blot of A20 protein in QSG7701 cells pretreated with the MSK1 inhibitor H89 to inhibit the phosphorylation of p65 at Ser276 and then treated with EGF (50 ng/ml) for the indicated time. Key: EGF, epidermal growth factor; A20, tumor necrosis factor α-induced protein 3; MEK1; mitogen-activated protein kinase kinase; MSK1, ribosomal protein S6 kinase A5; p, phosphorylated. All data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

ma and adjacent normal tissues. These data were analyzed using the Pearson's correlation test and indicate a significant positive correlation between the expression of EGFR and A20 mRNA in both HCC and normal liver tissues (**Figure 5A**, **5B**).

EGF inhibits the proinflammatory response induced by TNF-α in hepatic cells via an A20-dependent mechanism

A previous study shows that the expression of the adhesion molecules ICAM-1, VCAM-1, and the chemokine MCP-1 are NF-κB-dependent [21], so we examined the effects of EGF on the expression of ICAM-1, VCAM-1, and MCP-1 in QSG7701 cells. The cells were treated with EGF or TNF-α for 6 h at the concentrations indicated in **Figure 6**, and ICAM-1, VCAM-1, and MCP-1 mRNA levels were detected using RT-qPCR analysis. As shown in **Figure 6A**, TNF-α increased ICAM-1, VCAM-1, and MCP-1 in a concentration-dependent manner. By contrast, EGF did not
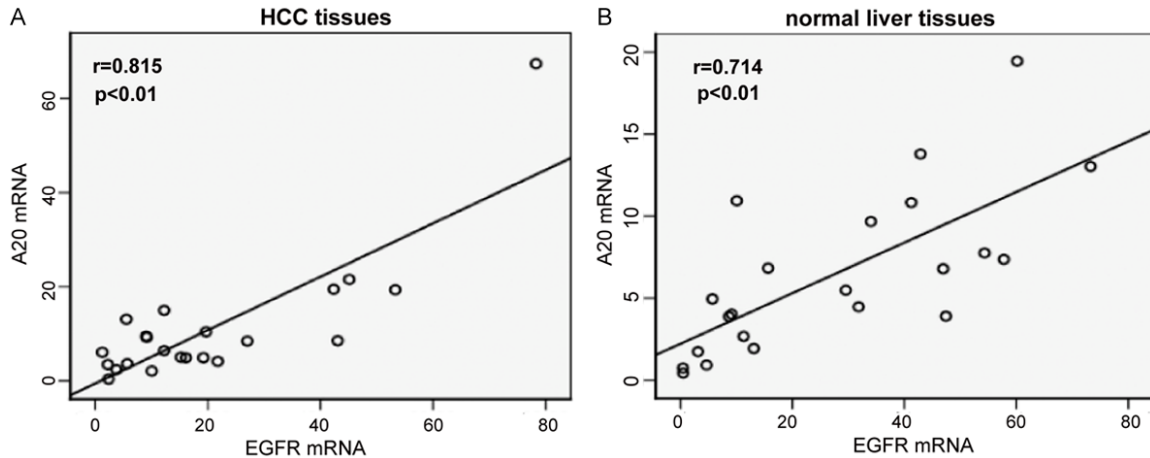
Figure 5. Relationship between the expression of A20 and EGFR in HCC and liver tissues. mRNA was extracted from 22 pairs of (A) HCC and (B) adjacent normal tissues. The levels of A20 and EGFR were measured by reverse transcription-quantitative PCR. The correlation of A20 and EGFR expression in tumor (T) or peri-tumor (N) tissues was analyzed by Pearson's correlation test. Key: A20, tumor necrosis factor α-induced protein 3; EGFR, epidermal growth factor receptor; HCC, hepatocellular carcinoma.
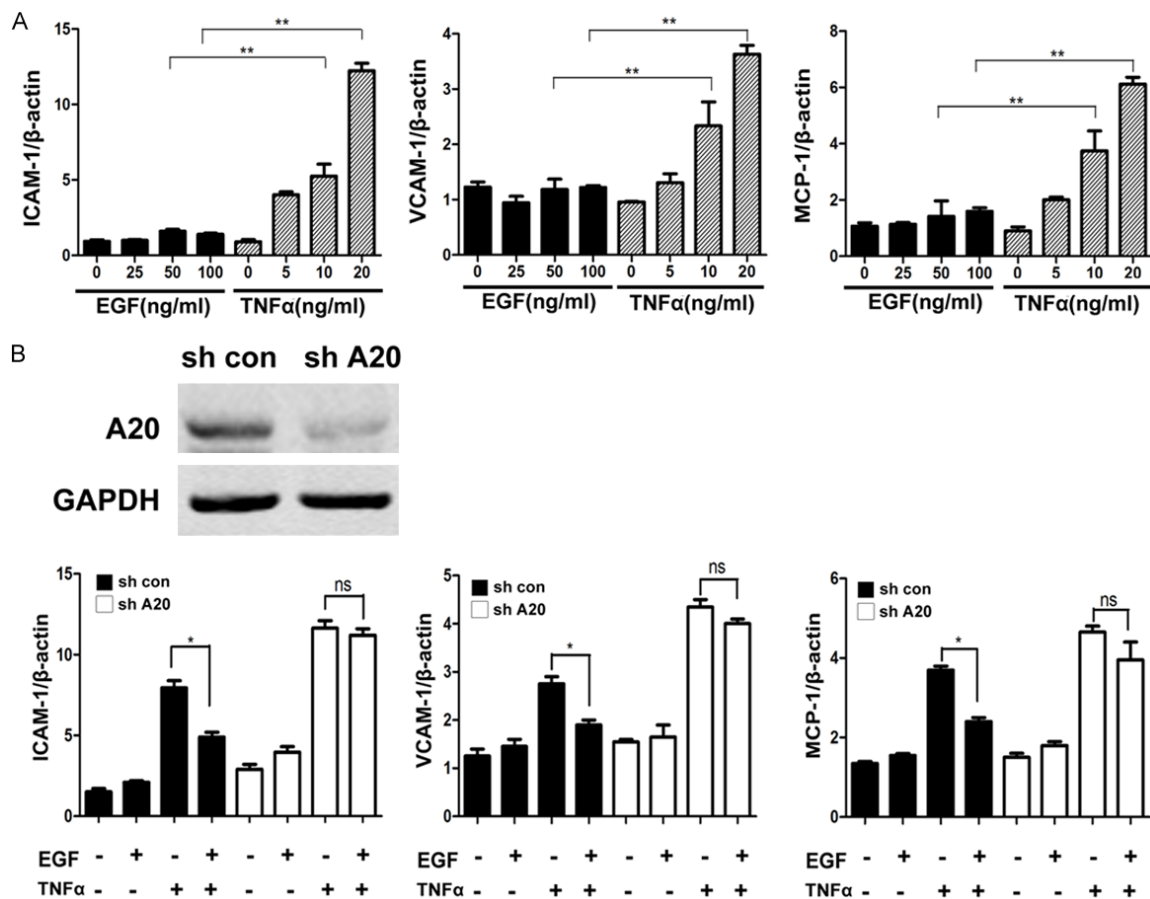

Figure 6. A20 induced by EGF contributed to the anti-inflammatory effect of this growth factor in hepatic cells. A. RT-qPCR analysis of ICAM-1, VCAM-1, and MCP-1 mRNA in QSG7701 cells treated with EGF or TNF-α at the indicated concentrations. B. RT-qPCR analysis of ICAM-1, VCAM-1, and MCP-1 mRNA in QSG7701-shcon and QSG7701-shA20 cells pretreated with EGF and then stimulated with TNF-α. Key: A20, tumor necrosis factor α-induced protein 3; EGFR, epidermal growth factor receptor; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; MCP-1, monocyte chemoattractant protein-1; RT-qPCR, reverse transcription-quantitative PCR.

significantly affect the expression of these proinflammatory molecules. Furthermore, to detect the influence of EGF on TNF-α-induced inflammatory responses, A20 knockdown QSG7701 cells (QSG7701-shA20) and control cells (QSG7701-shcon) were pretreated with EGF for 6 h and then stimulated with TNF-α for an additional 6 h. The mRNA expression of ICAM-1, VCAM-1, and MCP-1 was subsequently determined by RT-qPCR. The results show that pretreatment with EGF significantly inhibited the TNF-α-induced upregulation of ICAM-1, VCAM-1, and MCP-1 in QSG7701-shcon cells. However, in A20 knockdown cells (QSG7701-shA20), the inhibitory effect of EGF on the TNF-α-mediated upregulation of these inflammatory molecules was reduced (**Figure 6B**). This result indicates that EGF-induced A20 expression was responsible for the anti-inflammatory effect of EGF in hepatic cells.

Discussion

HCC frequently develops in patients who are chronically infected with HBV or hepatitis C virus (HCV). Chronic HBV/HCV infection in the liver results in hepatocyte death and inflammatory cell infiltration, which trigger long-lasting compensatory liver repair and regeneration that eventually lead to severe liver fibrosis or cirrhosis [36]. Although the mechanisms underlying hepatitis virus infection-induced HCC have not been fully elucidated, inflammation and compensatory regeneration are thought to play a crucial role [37]. Multiple signaling pathways are involved in the injury-inflammation-regeneration response leading to human HCC development [38, 39]. IKK-dependent NF-κB signaling is one of the most important pathways that are activated during liver injury and inflammation [40]. In addition, hepatocyte IKK/NF-κB signaling promotes HCC development by maintaining liver inflammatory responses in the MDR2$^{-/-}$ mouse model [41]. Persistent NF-κB activity in human tumors is correlated with a higher incidence of metastasis, faster disease progression, increased tumor recurrence, poorer survival time, and therapeutic resistance [42]. Therefore, preventing the excessive activation of NF-κB signaling is essential for maintaining homeostasis, and the identification of NF-κB pathway inhibitors has become the focus of numerous studies.

In several studies, A20 is recognized as a central regulator of NF-κB signaling cascades. As a

dual function ubiquitin-editing enzyme, A20 is thought to attenuate NF-κB signaling by editing the ubiquitylation status of proximal signaling proteins, including receptor-interacting serine/threonine kinase 1, TNF receptor-associated factor 6, inhibitor of nuclear factor κ B kinase regulatory subunit γ, and ubiquitin-conjugating enzyme E2 N. Therefore, A20 serves as a central inhibitor of inflammation downstream of the TNF receptor superfamily member 1A, an MYD88 innate immune signal transduction adaptor and nucleotide binding oligomerization domain containing two signaling pathways [12, 43]. Given the link between chronic inflammation and tumorigenesis, A20 may serve as a tumor suppressor gene, as has been demonstrated in lymphoma [44]. A20 has been shown to protect against acute toxic lethal hepatitis [45] and to attenuate free fatty acid-induced lipid accumulation in non-alcoholic steatohepatitis [46]. Our previous study [13] showed that A20 plays a negative role in the development and progression of HCC by inhibiting twist 1 expression, which is regulated by the A20-induced attenuation of NF-κB activity. We also analyzed the relationship between A20 expression and clinicopathologic features in HCC patients using a tissue microarray containing 143 pairs of HCC specimens. The results of the Kaplan-Meier analysis indicate that patients in the high-A20 expression group have a significantly prolonged disease-free survival and overall survival than those in the low-A20 expression group [13]. These results emphasize that A20 expression is an important factor for maintaining liver homeostasis.

To the best of our knowledge, the present study was the first to investigate the influence of EGF on A20 expression and to reveal that EGF enhanced A20 transcription and protein expression in normal hepatocytes and HCC cells. A20 had been reported to be an NF-κB-dependent gene activated by NF-κB signaling in response to proinflammatory cytokines [17]. In our study, when NF-κB signaling was blocked with IkBα or the inhibitor of BAY11-7082, the induction of A20 by EGF stimulation was inhibited. These findings suggest that this process was also NF-κB dependent, which is consistent with the findings of previous studies [13]. Several studies have shown the interaction between NF-κB signaling and the PI3K/Akt pathway or MEK1/ERK pathway [32-34]. To elucidate which pathway was involved in the present study, LY294002 and PD98059 were used to

block activation of the PI3K/Akt and MEK1/ERK pathways, respectively. The results show that only PD98059 suppressed the influence of EGF on A20 expression. Furthermore, inhibiting phosphorylation of p65 at Ser276 with H89 blocked the induction of A20 by EGF, suggesting that EGF promotes A20 expression through the MEK1/MSK1/p-p65 (Ser276) pathway.

The present study results also show a significant positive correlation between the mRNA levels of A20 and EGFR in HCC and normal liver tissues. These results suggest that the EGF pathway affects the expression of A20 in the liver.

Stimulation with TNF-α activates the NF-κB signaling pathway and further triggers proinflammatory and anti-inflammatory responses, including the expression of A20, which subsequently inhibits NF-κB activation [13]. TNF-α stimulation could promote the expression of inflammatory molecules, including ICAM-1, VCAM-1, and MCP-1. However, EGF did not significantly affect these molecules. Moreover, pretreatment with EGF suppressed TNF-α-induced upregulation of these inflammatory molecules, and the inhibitory effect of EGF was dependent on the expression of A20. Similarly, dose-dependent activation of NF-κB signaling and increased expression of cytokines involved in immunoregulatory responses, such as IL-6, IL-7, TNFSF4, and IRF-5, have been observed in human cervical carcinoma cells treated with the EGFR inhibitor PD153035 [47].

In summary, EGF upregulated the expression of A20 via the MEK1/MSK1/p-p65 (Ser276) signaling pathway without increasing expression of inflammatory molecules. Pretreatment with EGF suppressed TNF-α-induced inflammatory responses. These results unveil the possibility of inducing A20 without causing liver inflammation; however, the potent carcinogenic effects of EGF should also be considered. Thus, further investigation of the relationship between EGF and liver inflammation and tumorigenesis are needed.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Qiming Wang, Department of Internal Medicine, Henan Cancer Hospital, Affiliated Cancer Hospital of Zhengzhou University, 127 Dong Ming Road, Zhengzhou 450008, Henan, China. E-mail: qimingwang1006@126.com; Hongle Li, Molecular Pathology Department, Affiliated Oncology Hospital, Zhengzhou University, 127 Dong Ming Road, Zhengzhou 450008, Henan, China. E-mail: llhl73@163.com

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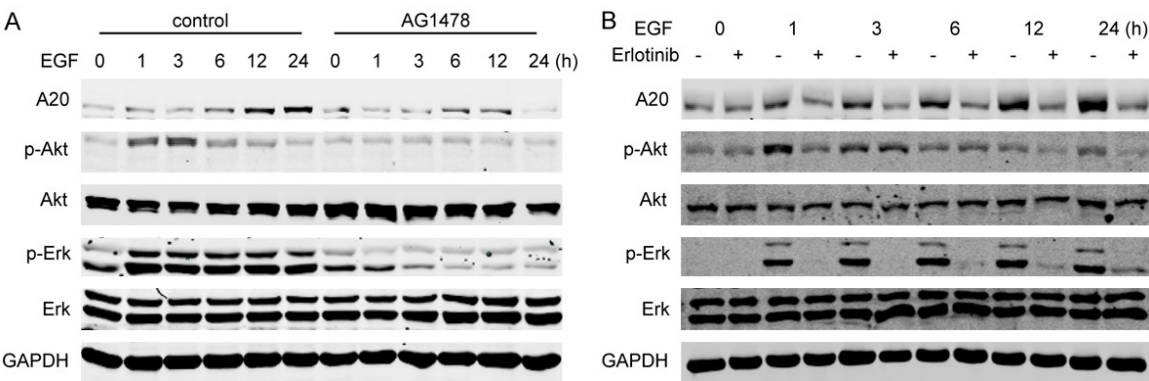
Figure S1. EGF-induced A20 upregulation was blocked by EGFR inhibitors. Western blot of A20 protein in QSG7701 cells pretreated with (A) the EGFR inhibitor AG1478 or (B) the EGFR-tyrosine kinase inhibitor erlotinib, followed by EGF treatment for the indicated time. Key: A20, tumor necrosis factor α-induced protein 3; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor.