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A disintegrin and metalloproteinase 17 (ADAM17) mediates epidermal growth factor receptor transactivation by angiotensin II on hepatic stellate cells

Hiroki Oikawa [a,*], Chihaya Maesawa [a,b], Yoshinori Tatemichi [c], Yutaka Nishinari [b,d], Masao Nishiya [a], Hisata Mizugai [a,c], Aya Ikeda [a], Kanta Oikawa [c], Yasuhiro Takikawa [c], Tomoyuki Masuda [a]

[a] Department of Pathology, School of Medicine, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba-Cho, Shiwa-Gun, Iwate 028-3694, Japan
[b] Department of Tumor Biology, Institute of Biomedical Science, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba-Cho, Shiwa-Gun, Iwate 028-3694, Japan
[c] Department of Internal Medicine, School of Medicine, Iwate Medical University, Uchimaru 19-1, Morioka, Iwate 020-8505, Japan
[d] Department of Surgery, School of Medicine, Iwate Medical University, Uchimaru 19-1, Morioka, Iwate 020-8505, Japan

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ABSTRACT

Aims: Epidermal growth factor receptor (EGFR) transactivation induced by angiotensin II (Ang II) participates in the progression of various diseases. A disintegrin and metalloproteinase 17 (ADAM17) is thought to promote renal fibrosis, cardiac hypertrophy with fibrosis and atherosclerosis by activation of the EGFR through secretion of EGFR ligands. The purpose of this study was to investigate whether Ang II-induced EGFR transactivation occurs on hepatic stellate cells (HSCs) and whether the reaction is mediated via ADAM17.
Main methods: Ang II-induced EGFR transactivation and cellular proliferation of the human HSC line LI90 were investigated using Western blotting and ATP assay, respectively. Ang II-induced secretion of mature amphiregulin into the cell culture medium was evaluated by enzyme-linked immunosorbent assay (ELISA).
Key findings: An inhibitor of ADAM17, TAPI-1, as well as antagonists of EGFR and angiotensin II type-1 receptor (AT1), attenuated Ang II-induced EGFR transactivation and proliferation of LI90 cells. Furthermore, silencing of ADAM17 inhibited Ang II-induced secretion of mature amphiregulin in addition to EGFR transactivation.
Significance: These results indicate that ADAM17 mediates Ang II-induced EGFR transactivation on HSCs, and that this process may participate in the progression of liver fibrosis.

Introduction

Hepatic stellate cells (HSCs), also known as lipocytes, fat-storing cells, Ito cells or perisinusoidal cells, are thought to play a central role in liver fibrosis (Bataller and Brenner, 2005). Under normal conditions, HSCs reside in the space of Disse among endothelial cells and hepatocytes and show a vitamin A-storing quiescent phenotype. In response to inflammatory stimuli, HSCs lose their vitamin A-storing droplets and transform into myofibroblasts showing an activated phenotype with expression of α-smooth muscle actin. Activated HSCs show proliferation and migration with secretion of fibrogenic mediators and generation of extracellular matrix, thus inducing liver fibrosis (Friedman, 2008; Yang et al., 2003). Thus, activated HSCs are important as a target for the control of liver fibrosis.

The renin–angiotensin system (RAS) is activated in patients with liver cirrhosis (Ayers, 1967; Helmy et al., 2000). Furthermore, recent evidence indicates that the RAS is involved in the progression of liver fibrosis (Lubel et al., 2008; Kisseleva and Brenner, 2011). Angiotensin II (Ang II) is the principal effector peptide of this system. Ang II is produced by sequential enzymatic reactions involving renin on angiotensinogen and angiotensin converting enzyme (ACE) on angiotensin I. The effects of Ang II are promoted via two G protein-coupled receptors (GPCRs), Ang II type-1 receptor (AT1) and type-2 receptor (AT2). It is thought that the AT1 mainly mediates the progression of human diseases that involve liver fibrosis. RAS components are overexpressed in chronic liver fibrosis, and activated HSCs proliferate with production of extracellular matrix by binding of Ang II to AT1 (Bataller et al., 2000; Paizis et al., 2002; Bataller et al., 2003). In addition, prolonged infusion of Ang II has been shown to exacerbate liver fibrosis in an experimental model of chronic liver injury (Bataller et al., 2005). Some articles have reported that ACE inhibitor or AT1 antagonist attenuates experimental liver fibrosis in mice and rats (Jonsson et al., 2001; Kim et al., 2008).

Ang II has been reported to induce transactivation of the epidermal growth factor receptor (EGFR). EGFR transactivation requires cleavage of a membrane-anchored EGFR ligand precursor by a disintegrin and metalloproteinase (ADAM). ADAM17 is implicated in numerous human diseases including cardiovascular disease, chronic renal disease, diabetes, rheumatoid arthritis, Alzheimer's disease and cancer (Gooz, 2010). The expression of ADAM17 has also been confirmed in chronic

* Corresponding author. Tel.: +81 19 651 5111; fax: +81 19 697 8880.
E-mail address: hioikawa@iwate-med.ac.jp (H. Oikawa).

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liver disease and activated hepatic stellate cells (Fujita et al., 2006; Perugorria et al., 2008; Bourd-Boittin et al., 2009). The available data suggest that ADAM17 may participate in liver fibrosis. However, it is still unknown whether Ang II causes EGFR transactivation in HSCs and whether the reaction is mediated through ADAM17. Among EGFR ligands, amphiregulin (AR), transforming growth factor (TGF)-α, heparin-binding EGF (HB-EGF), epiregulin, epigen and neuregulin have been shown to undergo cleavage by ADAM17 (Weber and Saftig, 2012). The expression of AR is induced during liver injury and remains elevated in cirrhosis (Berasain et al., 2005). Experimental liver fibrosis is significantly suppressed in AR-knockout mice (Perugorria et al., 2008). Thus, AR is suggested to be involved in progression of liver fibrosis.

In the present study, we examined whether Ang II-stimulation would induce EGFR transactivation via ADAM17 in HSCs, and whether ADAM17 participates in the release of AR by Ang II.

Materials and methods

Materials

Ang II, AG1478 (EGFR antagonist), PD123319 (selective AT2 antagonist), LY294002 (phosphatidylinositol 3 kinase (PI-3K) inhibitor) and monoclonal mouse β-actin antibody were purchased from Sigma (St. Louis, MO, USA). TAPI-1 (TNFα converting enzyme/ADAM17 inhibitor), U0126 (mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor) and rabbit polyclonal anti-ADAM17 antibody were obtained from Calbiochem (Darmstadt, Germany). RNH6270 (the active form of the prodrug-type AT1 antagonist) was kindly supplied by Daiichi-Sankyo (Tokyo, Japan). Rabbit polyclonal anti-human EGFR antibody was purchased from Rockland Immunochemicals (Philadelphia, PA, USA). Rabbit polyclonal anti-extracellular signal-regulated kinase (ERK) 1/2 antibody and rabbit polyclonal anti-ACTIVE® MAPK antibody were obtained from Promega Corp. (Madison, WI, USA). Rabbit polyclonal anti-phospho-epidermal growth factor receptor (pTyr1068) antibody was obtained from BioSource International Inc. (Camarillo, USA). Rabbit monoclonal anti-Akt antibody and rabbit monoclonal anti-phospho-Akt antibody (pSer473) were obtained from Cell Signaling Technology (Danvers, MA, USA). AG1478, U0126, LY294002 and TAPI-1 were dissolved in dimethyl sulfoxide (DMSO) and RNH-6270 was dissolved in methanol. PD123319 was dissolved in distilled water.

Cell culture

LI90 cells with characteristics compatible to human activated HSCs were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). Primary rat HSCs were isolated from normal Wistar rats as described previously (Senoo et al., 1993; Sato et al., 1998; Fujita et al., 2006) and used in the experiments between passages 4 and 6 for in vitro activation of HSCs. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), and then starved in FBS-free medium for 24 h before the start of all experiments. They were then subjected to preincubation for 30 min with AG1478 (10 μM), 30 min with PD-123319 (10 μM), 30 min with U0126 (10 μM), 30 min with LY294002 (20 μM), 45 min with TAPI-1 (20 μM) and 30 min with RNH-6270 (10 μM), and finally treated with Ang II (10^{-6} M).

Construction of miR RNAi vector and infection of adenoviral stock

We used a BlOCK-iT Pol II miR RNAi expression vector kit (Life Technologies), which facilitates the expression of microRNA (miRNA) to silence human ADAM17. The targeting mRNA sequence (AGCATG GATTCTGCATCGGTT) corresponded to nucleotides 2335–2355 in the coding region of human ADAM17. The miRNA double-strands containing the targeting sequence were cloned into the pcDNA6.2-GW/EmGFP-miR vector in accordance with the manufacturer's protocol. As a control, pcDNA6.2-GW/EmGFP-miR-Neg was prepared. The insert of the control vector encoded an mRNA not targeting any known vertebrate gene. To obtain highly efficient delivery of miRNA to LI90 cells, these inserts containing EmGFP were additionally transferred to the pAd/CMV/V5-DEST vector (Ad-miR-ADAM17 for ADAM17 knockdown and Ad-miR-Neg for control, Life Technologies) by a BP/LR recombination reaction in accordance with the manufacturer's protocol. These vectors were then digested with PacI and transfected into 293A cells to generate the adenoviral stocks. The titers of adenoviral stock were determined using a plaque-forming assay and cultured 293A cells. LI90 cells were infected at a MOI (multiplicity of infection) of 20 on Ad-miR-ADAM17 or Ad-miR-Neg for 2 h and incubated in fresh growth medium for 48 h. The efficiency of adenoviral infection to LI90 cells was evaluated by observation of EmGFP fluorescence and Western blotting.

Western blotting

After the treatment, cells were lysed with ice-cold buffer (10 mM Tris–HCl, pH 7.4/100 mM NaCl/1% Triton X-100/0.1% SDS/10% glycerol/50 mM NaF/1 mM sodium orthovanadate/2 mM PMSF/2% protease inhibitor cocktail Set 1 (Calbiochem, Darmstadt, Germany). The lysate was mixed with 6-fold concentrated loading dye, heated for 5 min at 95 °C, and subjected to SDS-PAGE on a 7.5% polyacrylamide gel (Bio-Rad Laboratories, Mississauga, Canada). The proteins were then transferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK) by electroblotting. The membrane was blocked in 5% casein buffer in Tris-buffered saline with 0.1% Tween 20 for 1 h at room temperature and incubated with each primary antibody (1:500 for anti-ADAM17, 1:1000 for anti-EGFR, 1:5000 for anti-ERK 1/2, 1:5000 for anti-ACTIVE® MAPK, 1:5000 for anti-phospho- EGFR, 1:5000 for anti-β-actin) overnight at 4 °C. The membrane was then incubated with the second antibody, anti-mouse IgG (GE Healthcare) or anti-rabbit IgG (GE Healthcare), for 60 min at room temperature. The signals were detected with ECL Plus (GE Healthcare) and ChemiDoc XRS (Bio-Rad Laboratories).

Proliferation assay

Five thousand cells were seeded into each well of 96-well plates and their viability was assessed by CellTiter-Glo Luminescent Cell Viability assay (Promega). Upon serum deprivation for 24 h, the cells were treated with Ang II (10^{-6} M) for 60 h with and without pretreatment with each inhibitor and antagonist. For examining the effects of adenovirus infection, infected cells were similarly cultured in serum-free medium and treated with Ang II (10^{-6} M) for 60 h. The assay substrates were then added to each well on the plate and the samples were evaluated using a luminometer (Centro LB960; Berthold Tech, Bad Wildbad, Germany). For examining the blocking of AR released by Ang II, a goat polyclonal AR antibody and a goat control IgG were obtained from R&D Systems (Minneapolis, MN, USA) and used at a concentration of 5 μg/ml.

Quantitative real-time RT-PCR

Total RNA was isolated with TRIzol reagent (Life technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Superscript™ III First-Strand Synthesis SuperMix (Life technologies) was used for the synthesis of single-stranded complementary DNA from 1 μg of total RNA. Quantitative real-time PCR analysis was performed with TaqMan Gene Expression Assays (Applied Biosystems; ABI, Foster City, CA, USA) and an ABI 7500 real time PCR thermocycler (ABI). The assays employed were for amphiregulin (AR) (Hs00950669_m1) and glyceraldehyde-3-