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Succinate induces hepatic fibrogenesis by promoting activation, proliferation, and migration, and inhibiting apoptosis of hepatic stellate cells

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ABSTRACT

Liver fibrosis is a progressive pathological process that accompanies wound healing; however, therapeutics for reversing hepatic fibrosis are unavailable. Activation of hepatic stellate cells (HSCs) play a critical role in liver fibrosis. Recent reports showed that succinate and its receptor, G-protein coupled receptor 91 (GPR91), act as signaling molecules during the activation of HSCs. However, the role of succinate in proliferation, apoptosis, and migration of HSCs has not been studied. In this study, we determined whether succinate regulates proliferation, apoptosis, and migration of HSCs and induces liver fibrosis in a mouse model.

Succinate treatment not only induced activation of HSCs, but also increased the proliferation and migration of LX-2 HSCs and inhibited apoptosis. To investigate whether succinate causes hepatic fibrosis, 100 mg/kg succinate or control PBS was administered by intraperitoneal injection to mice once a day for four weeks. There were significant molecular changes such as increased α-SMA and collagen type 1 production and increased production of inflammatory cytokines such as IL-6 and TNF-α, but not TGF-β, in the succinate-treated group compared to the control group. However, no morphological changes were observed in Masson's trichrome staining. In conclusion, the present study demonstrated that succinate induces activation, proliferation, and migration of HSCs and attenuates apoptosis in LX-2 HSCs. Therefore, inhibition of succinate accumulation may be an effective method for reversing liver fibrosis by controlling HSC survival and growth.

1. Introduction

The incidence of nonalcoholic fatty liver disease (NAFLD) is increasing rapidly, which represents significant challenges in terms of prevention and treatment. Despite its high prevalence, most patients exhibit only simple steatosis without subsequent clinical consequences, and only 5–10% of the affected patients develop steatohepatitis with consequent higher risk of developing liver fibrosis, cirrhosis, and hepatocellular carcinoma [1]. Hepatic stellate cells (HSCs) are the principal hepatic fibrogenic cells that produce extracellular matrix (ECM) components such as collagen type 1 and α-smooth muscle actin (α-SMA) in response to persistent liver injury, and HSC activation is a central mechanism underlying liver fibrogenesis [2,3].

Recent reports showed that succinate and its receptor, G-protein coupled receptor 91 (GPR91), act as signaling molecules during the activation of HSCs [4,5], renin release in macula densa cells [6], VEGF release in retina [7,8], cardiac hypertrophy [9], and osteoclastogenesis [10]. However, the role of succinate in proliferation, apoptosis, and migration of HSCs has not been studied so far. In this study, we assessed whether succinate regulates the proliferation, apoptosis, and migration of HSCs and induces liver fibrosis in a mouse model.

2. Materials and methods

2.1. Materials

The reagents used in this study were obtained from the

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indicated suppliers: succinate from Sigma (St. Louis, MO, USA); antibodies against cleaved PARP, PARP, caspae-3, and cleaved caspae-3 from Cell Signaling Technology (Richmond, CA, USA); antibodies against GAPDH from GeneTex (Irvine, CA, USA); antibodies against GPR91 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against α-SMA from Abcam (Cambridge, England); Transwell filters from Costar (Corning, NY, USA). All other materials were obtained from Sigma (St. Louis, MO, USA).

2.2. Cell culture

LX-2 cells are immortalized human HSCs and were kindly provided by Prof. Ja June Jang of Seoul National University, Korea. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 mL/L fetal bovine serum (FBS) with 100,000 U/L penicillin and 100 mg/L streptomycin.

2.3. Cell viability assay and apoptosis assay

To assess the effects of succinate on growth, LX-2 cells were plated in 24-well plates with DMEM containing 100 mL/L FBS at 37 °C. After 24 h, the medium was replaced with 0, 800, or 1600 μmol/L succinate in fresh DMEM containing 100 mL/L FBS. Viable cell numbers were estimated using the CCK-8 assay (Dojindo Molecular Technologies, Inc., Rockville, USA) in accordance with the manufacturer's instructions. Succinate was dissolved in phosphate buffered saline (PBS).

Cell-proliferation was also measured using the BrdU cell proliferation assay kit from Millipore/Chemicon (Temecula, CA, USA).

2.4. Migration assays

For the wound migration assay, LX-2 cells were plated in six-well plates at 5×10^5 cells/well in DMEM containing 100 ml/L of FBS. Cells at 90% confluence were incubated for 1 h with 1 mg/L mitomycin C. After mitomycin C treatment, an injury line was made using a yellow tip, and the cell monolayers were rinsed with PBS. The cells were then incubated for 0 or 16 h with 0, 800, or 1600 μmol/L succinate in DMEM containing 100 mL/L FBS. Cell migration was measured by microscopy at the indicated time points, and the measured widths of the injury lines were plotted as moving distance.

For the Transwell migration assays, we used a 8-μm pore size Transwell system (Corning, NY, USA). Briefly, LX-2 cells (3×10^4 cells/filter) were plated onto Transwell filters in a 24-well plate; the Transwell filters were precoated with 10 μg type IV collagen. The lower chambers of the wells were filled with DMEM containing 15% FBS as a chemoattractant. Cells were incubated with 0, 800, 1600 μmol/L succinate. After 6 h incubation, the migrated cells were fixed and stained with hematoxylin and eosin (H & E). Migrated cell numbers in eight separate fields were counted using light microscopy at 200 × magnification. The assay was independently repeated thrice.

2.5. Western blot analyses

Cells were lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) with phosphatase and protease inhibitors. The protein contents of total cell lysates were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The proteins of the cell lysates were separated by sodium

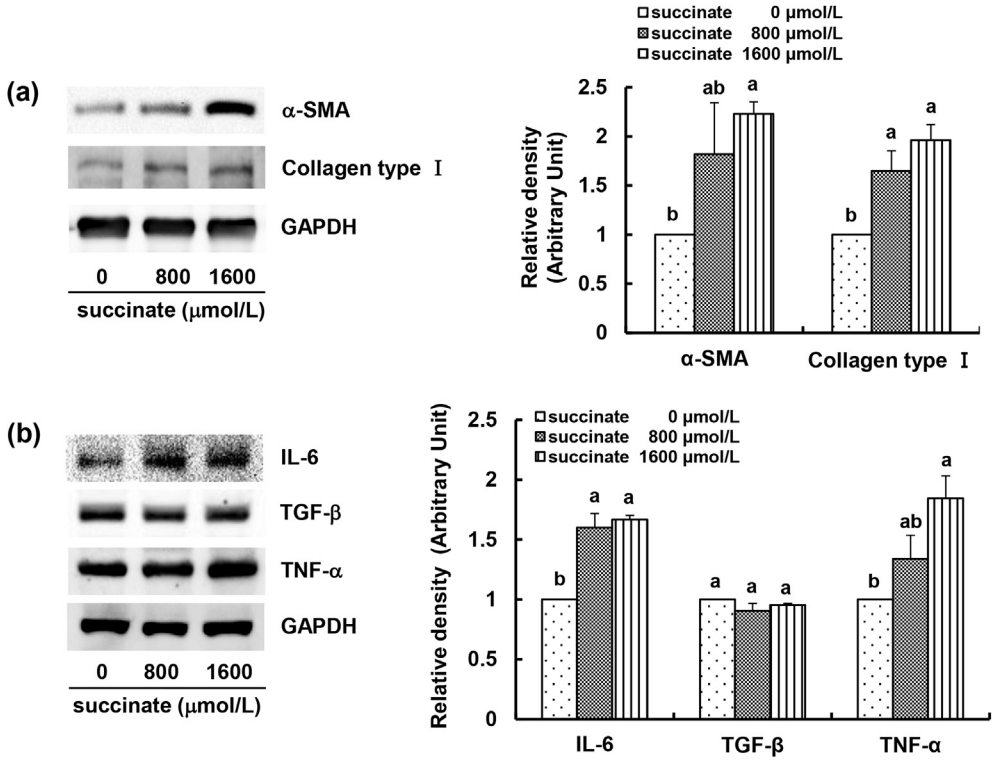

Fig. 1. Succinate induces activation of HSC and inflammatory cytokine production in LX-2 cells. Cells were treated with 0, 800, or 1600 μmol/L of succinate for 48 h. Total cell lysates were subjected to immunoblotting with α-SMA, collagen type 1 **(a)**, IL-6, TGF-β, and TNF-α **(b)**. [**Left panel]** Representative photographs of chemiluminescent detection of blots from three independent experiments. The relative abundance of each band was estimated by densitometric scanning, and the expression levels were normalized to that of GAPDH. [**Right panel]** Each bar represents the mean ± SEM (n = 3). Means with different letters differ significantly, $P < .05$.