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Characterization of glial fibrillary acidic protein (GFAP)-expressing hepatic stellate cells and myofibroblasts in thioacetamide (TAA)-induced rat liver injury

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ABSTRACT

Hepatic stellate cells (HSCs), which can express glial fibrillary acidic protein (GFAP) in normal rat livers, play important roles in hepatic fibrogenesis through the conversion into myofibroblasts (MFs). Cellular properties and possible derivation of GFAP-expressing MFs were investigated in thioacetamide (TAA)-induced rat liver injury and subsequent fibrosis. Seven-week-old male F344 rats were injected with TAA (300 mg/kg BW, once, intraperitoneally), and were examined on post single injection (PSI) days 1–10 by the single and double immunolabeling with MF and stem cell marker antibodies. After hepatocyte injury in the perivenular areas on PSI days 1 and 2, the fibrotic lesion consisting of MF developed at a peak on PSI day 3, and then recovered gradually by PSI day 10. MFs expressed GFAP, and also showed co-expressions such cytoskeletons (MF markers) as vimentin, desmin and α-SMA in varying degrees. Besides MFs co-expressing vimentin/desmin, desmin/α-SMA or α-SMA/vimentin, some GFAP positive MFs co-expressed with nestin or A3 (both, stem cell markers), and there were also MFs co-expressing nestin/A3. However, there were no GFAP positive MFs co-expressing RECA-1 (endothelial marker) or Thy-1 (immature mesenchymal cell marker). GFAP positive MFs showed the proliferating activity, but they did not undergo apoptosis. However, α-SMA positive MFs underwent apoptosis. These findings indicate that HSCs can proliferate and then convert into MFs with co-expressing various cytoskeletons for MF markers, and that the converted MFs may be derived partly from the stem cell lineage. Additionally, well-differentiated MFs expressing α-SMA may disappear by apoptosis for healing. These findings shed some light on the pathogenesis of chemically induced hepatic fibrosis.

1. Introduction

Post-cell injury liver fibrogenesis, irrespective of the etiology, is a dynamic and highly integrated molecular and cellular process, leading to cirrhosis at the advanced stage. Activated hepatic stellate cells (HSCs) are believed to represent the principal fibroblastic cell type which plays important roles in hepatic fibrogenesis through the conversion into myofibroblasts (Forbes and Parola, 2011; Knittel et al., 1999). The myofibroblasts are the most activated cells which can produce extracellular matrices (ECMs) such as collagens (Desmouliere et al., 2003). Transformed HSC-derived myofibroblasts in rat hepatic fibrosis express such cytoskeletons as vimentin, desmin, and α-smooth muscle actin (α-SMA) in varying degrees during the development; out of them, well-differentiated myofibroblasts may express α-SMA (Desmouliere et al., 2003). Additionally, it is reported that HSC-derived myofibroblasts exhibit glial fibrillary acidic protein (GFAP), suggesting that myofibroblasts seen in hepatic fibrosis are heterogeneous in cell population, function and derivation (Forbes and Parola, 2011; Ide et al., 2004).

GFAP is a type III intermediate filament (IF) protein; its expression is specific for astroglial cells in the brain (Eng and Ghirnikar, 1994). Although myofibroblasts, characterized by expressions of vimentin, desmin and α-SMA, have been reported in cutaneous fibrosis after wound, renal fibrosis and pulmonary fibrosis (Desmouliere et al., 2003; Juniantito et al., 2012b; Willis et al., 2006), GFAP-expressing myofibroblasts are not found in the fibrotic lesions in other organs and sites. As mentioned above, interestingly, GFAP expression is seen in HSC-derived myofibroblasts (Cassiman et al., 2002); likely, GFAP expression is limited in hepatic fibrosis. The reasons why hepatic myofibroblasts can exhibit a variety of cytoskeletal proteins remains to be investigated (Guyot et al., 2006).

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Thioacetamide (TAA)-induced rat hepatic lesions are used as a useful animal model to know the pathogenesis of post-hepatocyte injury fibrosis (Ide et al., 2002, 2003). Using this animal model, to shed some light behind the chemically induced hepatic fibrogenesis, we analyzed GFAP expression patterns by means of single and double immunolabelings with antibodies for cytoskeletal marker antibodies (desmin, vimentin, and α-SMA). Furthermore, we pursued the possible derivation of GFAP-expressing myofibroblasts using double immunofluorescence with GFAP antibody and antibodies for endothelial marker (rat endothelial cell antigen-1 [RECA-1]), neurogenic stem cell marker (nestin) or stromal stem cell markers (Thy-1 and A3). This study shows that hepatic myofibroblasts may be derived partly from HSCs in the stem cell lineage.

2. Materials and methods

2.1. Experimental animals

Seven-week-old 28 male F344 rats (110–120 g body weight; Charles River Japan, Hino, Shiga, Japan) were housed in an animal room at 21 ± 3 °C and with a 12 h light–dark cycle, and fed a standard diet and tap water *ad libitum*. Twenty-four rats in TAA group were injected intraperitoneally (IP) with TAA (Wako Pure Chemical, Osaka, Japan) dissolved in physiological saline at a dose of 300 mg/kg body weight. Four rats were examined on each of post-single injection (PSI) days 1, 2, 3, 5, 7 and 10. The remaining four rats received an equivalent volume of physiological saline in the same manner and served as controls. One hour before euthanasia, all the rats received IP injection of 5-bromo-2′-deoxyuridine (BrdU: Sigma–Aldrich Co., St. Louis, MO, USA) at the dose of 50 mg/kg body weight. These experiments complied with our institutional guidelines for animal care and were approved by the local ethic committee.

2.2. Histopathology

All rats were euthanized under isoflurane anesthesia. Liver samples were immediately fixed in 10% neutral buffered formalin, PLP-AMeX (Suzuki et al., 2000) or in Zamboni's solution (0.21% picric acid and 2% paraformaldehyde in 130 mM phosphate buffer, pH 7.4). The fixed tissues were embedded in paraffin and sectioned at 3–5 μm in thickness. Formalin-fixed, deparaffinized sections were stained with hematoxylin and eosin (HE) for histopathological observations and with the Azan–Mallory stain for collagen deposition.

2.3. Single immunohistochemical labeling

Livers fixed in Zamboni's solution or PLP-AMeX was analyzed immunohistochemically by the indirect immunoperoxidase method (Histofine Simple Stain Kit: Nichirei Corp., Tokyo, Japan). Primary antibodies, pre-treatment and dilution for each antibody are listed in Table 1. After pre-treatment, sections were treated with 3% H_2O_2 in phosphate buffered saline (PBS) for 30 min to quench endogenous peroxidase and then with 5% skimmed milk in PBS for 30 min at room temperature (RT) to inhibit non-specific reactions, the sections were incubated with the primary antibody overnight at 4 °C. The sections were then incubated for 30 min with peroxidase-conjugated secondary antibody, which consisted of goat anti-mouse IgG Fab fragment antibody (Histofine simplestain MAX PO®; Nichirei, Tokyo, Japan) or goat anti-rabbit IgG Fab fragment (Histofine simplestain MAX PO®; Nichirei, Tokyo, Japan). Positive reactions were visualized with 3,3′-diaminobenzidine (DAB; Vector Laboratories Inc., Burlingame, CA, USA) and the sections were lightly counterstained with hematoxylin. As negative controls, tissue sections were treated with mouse or rabbit non-immunized serum instead of the primary antibody.

2.4. Double immunohistochemical labeling

To investigate co-localization between cell-specific antigens, the double immunofluorescence labeling was carried out using cryostat tissue sections (10 μm thick) from the controls and TAA group on PSI days 2 and 3. The combinations in the dual immunofluorescence labeling were GFAP/vimentin, GFAP/desmin, GFAP/α-SMA, vimentin/desmin, vimentin/α-SMA, desmin/α-SMA, GFAP/nestin, GFAP/A3, GFAP/RECA-1 and GFAP/BrdU. The sections except for GFAP/BrdU were fixed in methanol/acetone (1:1) for 10 min at −4 °C and washed in PBS. For GFAP/BrdU staining, sections were fixed in 4% paraformaldehyde for 10 min. Blocking was performed with 10% normal goat serum for 1 h. Subsequently, appropriate primary antibodies were applied (Table 1). After overnight incubation at 4 °C, the sections were washed and exposed for 45 min to appropriate flourochrome-conjugated secondary antibodies. Goat anti-rabbit-Alexa 488 (Invitrogen, Carlsbad, CA, USA) was used for rabbit antibodies. Mouse monoclonal antibodies were visualized with goat anti-mouse IgG-Cy3 (Jackson Immunoresearch, West Grove, PA, USA) or goat anti-mouse IgG2a-Alexa 568 (Invitrogen, Carlsbad, CA, USA). Additionally, isotype specific antibody, goat anti-mouse IgG2a-Alexa 488 (Invitrogen, Carlsbad, CA, USA) was used for the α-SMA antibody. Vimentin and RECA-1 directly labeled with HyLyte flour 555 labeling Kit-NH2 (Dojindo Laboratories, Kumamoto, Japan) were used for desmin/vimentin and GFAP/RECA-1 combinations. Slides were mounted with mounting medium including 4′,6-diamino-2-phenylindole (DAPI; VECTASHIELD®; Vector Laboratories, Burlingame, CA, USA). Fluorescence signal and co-localization were examined by a laser scanning confocal imaging microscope (Nikon C1Si; Nikon, Tokyo, Japan) and processed with EZ-C1 3.20 Free Viewer (Nikon). Negative control immunostainings were performed, either by omission of the primary antibody or by treatment with non-immune rabbit or mouse IgG.

To identify the co-expressions of antigens recognized by nestin and A3, the modified double immunohistochemistry was performed according to the method described by Hasui et al. (2003). Firstly, the sections were immune-labeled with nestin antibody and visualized red by the Fuchsin substrate–chromogen system (Dako, Carpinteria, CA, USA). Secondly, sections used for nestin were reacted with A3. The positive reactions at the second labeling were visualized brown with DAB as mentioned above.

2.5. Terminal deoxyribonucleotide transferase (TdT)-mediated deoxyuridine triphosphate nick end labeling (TUNEL)

A standard *in situ* TUNEL (Appop Tag® Peroxidase *In situ* Apoptosis Detection Kit, Millipore, Bedford, USA) method was used for detection of DNA fragmentation in apoptotic cells according to manufacturer's instructions. The formalin-fixed and deparaffinized tissue sections were retrieved with proteinase K (100 μg/ml) for 20 min, and then, with 3% H_2O_2 for 20 min to inactivate endogenous peroxidase. Afterwards, sections were incubated with TdT enzyme and digoxigenin DNA labeling mixture in the TdT reaction buffer for 60 min at 37 °C, and then, treated with horseradish peroxidase-conjugated anti-digoxigenin for 60 min. The sections were visualized with DAB (Vector Laboratories). Negative control sections were incubated with distilled water instead of TdT enzyme.

2.6. TUNEL and GFAP or α-SMA double labeling

Double labeling was carried out with the TUNEL method for apoptosis and GFAP or α-SMA using cryostat tissue sections (10 μm