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EXPERIMENTALLY INDUCED DISEASE

Expression of Nestin in Remodelling of α-Naphthylisothiocyanate-induced Acute Bile Duct Injury in Rats

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Summary

The function of the intermediate filament protein nestin is poorly understood. The significance of nestin expression was assessed in α-naphthylisothiocyanate (ANIT)-induced cholangiocyte injury lesions in F344 rats. Liver samples obtained from rats injected intraperitoneally with ANIT (75 mg/kg) on post-injection days 0 (control) and 1–12 were labelled immunohistochemically for expression of nestin and markers specific for mesenchymal cells (vimentin), hepatic stellate cells (HSCs) (desmin and glial fibrillary acidic protein [GFAP]), endothelial cells (rat endothelial cell antigen [RECA]-1), cholangiocytes (cytokeratin [CK] 19) and cellular proliferation (Ki67). Cholangiocyte injury led to infiltration of neutrophils and macrophages followed by aggregation of mesenchymal cells and regeneration of bile ducts. Nestin expression was detected in mesenchymal cells (vimentin positive) on days 1–7 with a peak on days 3–5 and in newly-formed RECA-1-positive endothelial cells on day 3. Nestin expression was also observed in regenerating CK19-positive cholangiocytes on days 2–5, with a peak on day 3. Labelling for Ki67 showed proliferation of cholangiocytes, mesenchymal cells and HSCs. Real-time reverse transcriptase polymerase chain reaction with microdissected samples showed significantly elevated nestin mRNA on day 3. The findings suggest an association between nestin expression and cellular proliferation. Based on these findings, it was considered that nestin-expressing mesenchymal cells, HSCs and endothelial cells may be possible progenitors of repopulating cholangiocytes. Nestin expression may serve as an indicator for cellular remodelling and behaviour of injured and repopulating bile ducts.

Keywords: bile duct; nestin; rat; remodelling

Introduction

The enhanced capacity of stem/progenitor cells for proliferation, survival and motility contributes to tissue modelling and remodelling. Cholangiocytes are the lining epithelial cells of bile ducts in the Glisson's sheath of the liver and have a low replicative ability (LeSage *et al.*, 1996). Transient injury to cholangiocytes is followed by successful regeneration, while repeated injury results in permanent damage evidenced by bile duct proliferation, often with ductopenia (Degott *et al.*, 1992). Little is known about why regeneration of cholangiocytes fails to keep pace with injury. Cytoskeletal intermediate filaments are critical and dynamic structural elements that control a variety of cell processes, including proliferation, migration and survival in different pathobiological conditions (Coulombe and Wong, 2004). Characterization of the expression dynamics of intermediate filaments may help improve the understanding of the mechanisms underlying cholangiocyte injury and may suggest new therapeutic interventions.

Nestin is a class VI intermediate filament protein with a molecular weight of 240 kDa. Initially, nestin was identified as a marker of neuroepithelial stem cells and progenitor cells in the nervous system (Lendahl *et al.*, 1990). Since then, nestin has been shown to be expressed by a wide range of cell types including non-

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neural progenitors and stem cells of pancreatic islets, haemopoietic cells and hair follicle sheath cells (Hunziker and Stein, 2000; Zulewski *et al.*, 2001; Lechner *et al.*, 2002; Li *et al.*, 2003; Abraham *et al.*, 2004; Walczak *et al.*, 2004; Hoffman, 2006). However, the detailed function of nestin and the factors that regulate its expression are currently unclear.

The aim of the present study was to investigate nestin expression in α-naphthylisothiocyanate (ANIT)-induced acute cholangiocyte injury in the rat liver.

Materials and Methods

Animals and Experimental Induction of Pathology

Five-week-old, male F344 rats (100–120 g body weight) purchased from Charles River Japan (CRJ, Hino, Shiga, Japan) were used. These animals were maintained at a controlled temperature (21 ± 3°C) and humidity with a 12 h light–dark cycle; they were provided with commercial rodent chow (DC-8; CLEA Japan, Inc., Tokyo, Japan) and tap water *ad libitum*. After a 1-week acclimatization period, they were divided into control ($n = 3$) and ANIT injection groups ($n = 21$). The animals in the ANIT group were given a single intraperitoneal injection of ANIT (75 mg/kg) dissolved in olive oil (Golbar *et al.*, 2011). Three rats were killed by exsanguination under deep isoflurane anaesthesia on each of post-injection days 1, 2, 3, 5, 7, 9 and 12. Control rats received an equal volume of olive oil and were killed immediately after the injection (day 0).

The animal experiments were conducted in compliance with the institutional guidelines and protocol approved by the Ethical Committee of Osaka Prefecture University.

Histopathology and Immunohistochemistry

Tissues from the left lateral lobe of the livers were fixed in 10% neutral buffered formalin and Zamboni's fixative (0.21% picric acid and 2% paraformaldehyde in 130 mM phosphate buffer, pH 7.4). These tissues were dehydrated and embedded in paraffin wax. Formalin-fixed dewaxed sections (4 μm) were stained with haematoxylin and eosin (HE). Sections fixed in Zamboni's solution were subjected to immunohistochemistry (IHC) with mouse monoclonal antibodies specific for cytokeratin (CK) 19 and nestin (Table 1). The immunohistochemical methodology was as described by Golbar *et al.* (2013). Briefly, after retrieval of epitope with trypsin (for CK19) or microwaving (for nestin), endogenous peroxidase activity and non-specific protein binding were blocked by treating sections with 3% H_2O_2 and 5% skimmed milk, respectively. The sections were incubated with each primary antibody overnight at 4°C, followed by reaction with horseradish peroxidase-conjugated secondary antibody (Histofine® Simple Stain MAX-PO, Nichirei, Tokyo, Japan) for 30 min. Positive reactions were 'visualized' with 3, 3′-diaminobenzidine (DAB Substrate Kit, Vector Laboratories, Burlingame, California, USA). Sections incubated with non-immune mouse IgG (N-universal negative control; Dako, Carpinteria, California, USA) instead of primary antibody produced no signal and served as control. Sections were counterstained lightly with haematoxylin. Cells showing distinct expression of nestin in the area of the Glisson's sheath were evaluated semi-quantitatively (Table 2).

Immunofluorescence Labelling

Double immunofluorescence labelling was carried out using fresh frozen liver sections obtained on day 3 after ANIT injection. The combinations in the dual immunofluorescence labelling were nestin with CK19, vimentin, desmin, rat endothelial cell antigen (RECA)-1 and glial fibrillary acidic protein (GFAP); GFAP with vimentin and desmin; as well

Table 1
Primary antibodies used for immunolabelling

Antibody specificity	*Clone, dilution*	*Antibody sources*	*Labelling specificity*
CK19	b170, 1 in 500	Novocastra Laboratories Ltd., Newcastle, UK	Cholangiocytes
Nestin	Rat-401, 1 in 500	Millipore, Temecula, CA, USA	Cholangiocytes, mesenchymal and endothelial cells
Vimentin	V9, 1 in 500	Dako, Glostrup, Denmark	Mesenchymal cells
Desmin	D33, 1 in 500	Dako	Hepatic stellate cells
Ki67*	SP6, prediluted	Nichirei, Tokyo, Japan	Proliferating cells
RECA-1	HIS52, 1 in 100	AbD Serotec, Oxford, UK	Endothelial cells
GFAP†	Polyclonal, 1 in 300	Dako	Neuronal cells

*rabbit monoclonal antibody.
†rabbit polyclonal antibody; the rest are murine monoclonal antibodies. CK19, cytokeratin 19; GFAP, glial fibrillary acidic protein; RECA-1, rat endothelial cell antigen-1.