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Expression of Brain Derivated Neurotrophic Factor and of its receptors: TrKB and p75NT in normal and bile duct ligated rat liver

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Abstract

Cholangiocytes are the cells lining the biliary tree from canals of Hering to larger bile ducts. By morphology, they are divided in small and large cholangiocytes, which result heterogeneous at functional and proliferative levels. Proliferating cholangiocytes acquire a neuroendocrine phenotype, modulated by several factors including neurotrophins. Brain Derivated Neurotrophic Factor (BDNF) is a neurotrophin expressed in the nervous system and also in different types of epithelial and progenitor cells. The aim of the present study is to detect the expression of BDNF and of its two receptors (TrKB and p75NT, or p75NTR) in normal and bile duct ligated (BDL) rat livers.

In normal and BDL livers, BDNF and its receptors are expressed by small and large cholangiocytes and by hepatic progenitors cells. In cholangiocytes, the expression of BDNF and of its receptors changes after different BDL timing. After one or two weeks of BDL, both BDNF and TrKB and p75NT receptors are highly expressed, while after BDL for three weeks BDNF expression is drastically reduced and p75NT receptor prevails on TrKB. The expression of BDNF and of its receptors correlates with the proliferation rate of biliary tree during BDL. Indeed, after one or two weeks of BDL, proliferation prevails on apoptosis, whereas after BDL for three weeks, apoptosis prevails on proliferation.

Our morphological results strongly suggest that BDNF plays a role in the remodeling of biliary tree during cholestasis and that it may be involved in the pathophysiology of cholestasic liver diseases.

Key words

Neurotrophins, cholangiocytes, experimental cholestasis, neuroendocrine cells, liver diseases.

List of Abbreviations

BDL: bile duct ligation
BDNF: brain derivated neurotrophic factor
CKs: cytokeratins
HPCs: hepatic progenitors cells
IP3: inositol 1,4,5-trisphosphate
NGF: nerve growth factor

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NT3, NT4: Neurotrophin 3, 4
p75NT, p75 neurotrophin: p75 neurotrophin receptor
PCNA: proliferating cells nuclear antigen
TrKB: tyrosine kinase receptor B
SR: secretin receptor

Introduction

Cholangiocytes were originally considered a "simple" epithelium lining the biliary tree. However, contrary to this classical opinion, the biliary epithelium plays important physiological functions in the modification of bile and in the detoxification of xenobiotics (Alpini et al., 1997a). Furthermore, cholangiocytes are the target cells in diseases of the biliary tree, such as primary sclerosing cholangitis, primary biliary cirrhosis and cholangiocarcinoma.

Recent studies have highlighted the heterogeneity of biliary epithelium, at morphological, functional and proliferative levels (Glaser et al., 2009). At morphological level, the biliary tree originates from the bile canaliculi and acquires a proper wall at the level of channels of Hering, extending to large extrahepatic bile ducts. The point at which the bile canaliculi continue with the canals of Hering is defined ductulo-canalicular junction. At the level of this junction, the wall of the biliary tract is partly formed by cholangiocytes and partly by hepatocytes. In this context, a compartment of partially undifferentiated cells, called hepatic progenitor cells (HPCs), has been identified. These cells constitute a stem cell compartment residing in the liver and able to differentiate towards cholangiocytes or hepatocytes (Cardinale et al., 2011). Cholangiocytes lining bile ductuli and canals of Hering are called "small cholangiocytes" because of the size and are coboidal, whereas cholangiocytes lining large bile ducts are called "large cholangiocytes" and are characterized by a columnar shape. These observations, originally made in humans, have been confirmed in several experimental studies, both in vivo and in vitro (Alpini et al., 1996; 1997a; Glaser et al., 2009). Cholangiocytes are also heterogeneous at functional level. In fact, "large cholangiocytes" possess an intense secretory activity and express the secretin receptor (SR; Alpini et al., 1997b). By contrast "small cholangiocytes" do not express SR and are involved in repairing biliary tree during chronic colangiopathies (Francis et al., 2009). The heterogeneity of biliary epithelium has been shown, finally, at proliferative level. In fact, in the course of cholestasic liver diseases, as well as in experimental cholestasis in rats, the proliferative activity is restricted to "large cholangiocytes" and is closely associated with increased expression of SR and up-regulation of intracellular cAMP (Alpini et al., 1997b; 1998). By contrast, the proliferative activity of "small cholangiocytes" occurs upon toxic injury to the bile ducts and is mediated by the over-production of inositol trisphosphate (IP3; Alpini et al., 1998).

Proliferating cholangiocytes possess the peculiar capacity of acquiring a neuroendocrine phenotype (Roskams et al., 2004). This phenotypic switch is characterized by the expression of markers typical of neuroendocrine cells (chromogranin-A and neuronal cell adhesion molecule) and by the expression of glycolipids A2-B4 - lipidic molecules typical of neuronal and glial cytoplasmic membrane - and S-100 protein, a structural cytoskeletal protein expressed by glial cells (Popper et al., 1957; Roskams

et al., 1990; 1993; 2004; Desmet et al., 1995), the over-expression and secretion of serotonin (Marzioni et al., 2005), endogenous opioid peptides and neurotrophins, and the increased cellular response to hormones and neuropeptides (Renzi et al., 2011). The acquisition of a neuroendocrine phenotype is of crucial importance in the physiopathology of cholangiocytes (reviewed in Alvaro et al., 2008). However, little is known about the factors that induce the trans-differentiation of cholangiocytes into cells with neuroendocrine phenotype. In this regard, a relevant role might be played by the action of neurotrophins, which could be also implicated in the modulation of proliferation of both small and large cholangiocytes during cholestasis.

The Brain Derivated Neurotrophic Factor (BDNF) is a neurotrophin mainly produced in the brain and closely related to neuronal plasticity, synaptogenesis and differentiation of serotonergic neurons (Nibuya et al., 1995; Sala et al., 1998; Duman et al., 2002; Fujioka et al., 2004; Rumajogee et al., 2006; Pinnock et al., 2010; Yoshii and Constantine-Paton, 2010). BDNF is also expressed by different subtypes of epithelial cells (Peters et al., 2005; Ming et al., 2009; Prakash et al., 2010; Girard et al., 2011). During development, it promotes the proliferation of bronchial epithelial cells (Prakash et al., 2010) and the development of neuro-epithelial lung bodies (Garcia-Suarez et al., 2009). Moreover, BDNF is expressed by many types of progenitor cells of neural (review in Tonchev et al., 2011) and mesenchymal (Mousavi and Jasmin, 2006; Trzaska et al., 2009; Kompisch et al., 2010; Kan et al., 2011) origin, modulating their maturation and differentiation, and it is a trophic factor for endothelial cells (Donovan et al., 2000), promoting the proliferation, migration and differentiation of endothelial progenitor cells (Zeng et al., 2012).

BDNF binds to two classes of trans-membrane receptors: the tyrosine kinase receptor TrKB and the p75NT receptor. The activation of TrKB produces the increase of intracellular concentrations of cAMP and the recruitment of the MAPK/Erk pathway, with a consequent activation of proliferation and cellular differentiation. By contrast, activation of the p75NT can activate the transcription factor NFκB, involved in cellular growth and trophism, as well as the JNK/c-Jun pathway, responsible for the transcription of pro-apoptotic genes (reviewed in Reichardt et al., 2006). The over-production of intra-cellular cAMP and the activation of the MAPK/Erk pathway, which have been widely studied in view of neuronal plasticity, also constitute the major determinant for colangiocyte proliferation (review in Alvaro et al., 2007; 2008; DeMorrow et al., 2008). In this regard, induction of cholangiocyte proliferation has been observed in vitro, using NGF, a neurotrophin homolog of BDNF from which NGF differs for the selective binding to TrkA, instead of TrKB (Gigliozzi et al., 2004; Alvaro et al., 2006). The activation of TrKB receptor correlates also with neuronal differentiation and expression of serotoninergic markers by neurons (Ren-Patterson et al., 2005; Rumajogee et al., 2006). Since serotoninergic markers are also expressed by proliferating cholangiocytes (Marzioni et al., 2005), BDNF may be implicated in both proliferation and neuroendocrine trans-differentiation of cholangiocytes and hepatic progenitor cells (HPCs).

Therefore, in the present study we have investigated the expression of BDNF and its receptors in the rat biliary tree, both in normal conditions and upon bile duct ligation (BDL) for one, two or three weeks. BDL model is the most used model to study the proliferation of biliary tree in rodents, resulting in rapid proliferation of bile ducts with correlated increase of biliary cell mass which mimics human chronic cholestatic

liver diseases (Alpini et al., 1998). In these experimental conditions, we have correlated the expression of BDNF and its receptors with the proliferation-to-apoptosis ratio in biliary tree.

Methods

Experimental model of cholestasis

Twenty-four Wistar rats, males, 8 weeks aged and weighing about 225 g, were divided into four experimental groups consisting of 6 rats each:

Controls: not subjected to BDL;

BDL 1 week: subjected to BDL for one week;

BDL 2 weeks: subjected to BDL for two weeks;

BDL 3 weeks: subjected to BDL for three weeks.

The ligation procedure of the bile duct was carried out as previously described (Glaser et al., 2007). Before each experimental procedure the animals were anesthetized with pentobarbital (50 mg/kg ip) and every effort was carried out to minimize any suffering and discomfort, in accordance with the recommendations for the use of laboratory animals of "Sapienza University of Rome" and in compliance with guidance for use of laboratory animals of the NIH (NIH Publication nos. 85-23, revisited 1996).

Processing of samples, Immunohistochemistry and immunofluorescence

Rat livers were fixed in 2% paraformaldehyde for 24 hours. Each liver was divided into blocks of 2-4 mm^3, which were processed for embedding in paraffin and sectioning into 3.5 μm thick sections with a rotary microtome.

After deparaffinization and rehydration, the sections were incubated with the primary polyclonal antibody in the following experimental conditions, specific for each antibody:

- sheep anti-BDNF (ab 75040 Abcam, Cambridge, UK), 1: 400, for 2 days at + 4 ° C;
- rabbit anti p75NTR (AB1554, Millipore, Temecula, CA), 1: 500, for 2 days at + 4 ° C;
- rabbit anti-TrKB (ab 51190 Abcam, Cambridge, UK), 1: 200, for 2 days at + 4 ° C;
- rabbit anti-pan cytokeratin (CK; cholangiocytes specific, comprising CKs 7-8 and 7-20; Dako, Glostrup, Denmark), 1: 50, for one hour at room temperature;
- rabbit anti proliferating cells nuclear antigen (PCNA; Santa Cruz Biotechnology, Dallas, TX), 1: 50, for 60 minutes at room temperature.

In order to inactivate endogenous peroxidase, the incubation with primary antibody was preceded in all cases by preincubation with 3% H_2O_2 for 20 minutes at room temperature. On the sections treated for anti-pan CK, in order to expose the antigenic sites, the incubation was preceded also by enzymatic digestion with proteinase K (Dako, Glostrup, Danmark), for 15 minutes at room temperature.

After incubation with the primary antibody the sections were incubated with the specific biotinylated secondary antibody, diluted 1: 1000, for 2 hours at room temperature, followed by incubation with peroxidase-coupled streptavidin (Dako, Glostrup, Danmark), for 20 minutes at room temperature. The immunoreaction was

revealed with by diaminobenzidine with a commercial kit (Dako), according to the instructions of the producer, controlling the yield under the microscope. The sections were then dehydrated in alcohol, cleared in xylene and mounted in an hydrophobic medium for light microscopy examination.

For double immunofluorescence labeling (BDNF + pan-CK), the sections were incubated simultaneously with primary antibodies diluted to the following concentrations: 1:100 (anti-BDNF) and 1:100 (anti pan-CK), overnight at + 4 ° C. Subsequently, the sections were incubated with byotinilated donkey anti sheep (Jackson Laboratories, Berlingame, USA), diluted 1:500, for 2 hours, followed by incubation with Cy3--conjugated donkey anti rabbit IgG, diluted 1:400, and with Cy2-conjugated streptavidin (both from Jackson Laboratories, Berlingame USA), diluted 1:500. Finally, the sections were mounted in a specific mounting medium containing DAPI (Santa Cruz Biotechnology) and observed in a fluorescence microscope (Leica, Solms, Germany).

TUNEL method for visualization of apoptotic cells

The TUNEL method was used for visualization of apoptotic cells following the instructions of the appropriate kit (Apoptag, Millipore, Temecula, California, USA).

Results quantification and data analysis

The percentage of cholangiocytes immune positive for BDNF and its receptors (TrKB, p75NT) among the total number of cholangiocytes was quantified by counting the cells positive to each antibody in different microscopic fields from each experimental group.

Quantification of immunostaining for BDNF and its receptors (TrKB, p75NTR) was performed also by densitometry analysis of different microscopic fields from the four different experimental groups. A specific software (IAS, Delta Sistemi, Rome, Italy) was used as previously described (Casini et al., 2011). In brief, the optical density of the reaction product was measured by light transmittance, assuming 0 value as maximum darkness, thus the maximal staining signal, and 100 as maximum brightness, therefore the absence of signal.

The percentage of cells positive to PCNA (assumed as proliferating) or to TUNEL (assumed as apoptotic) on the total number of cholangiocytes were evaluated by counting positive cells in different microscopic fields and computing, for each experimental group, the ratio between proliferation and apoptosis.

The mean percentage values of PCNA and TUNEL positive cells from different microscopic fields, +/- standard error, were analyzed by ANOVA and t-test. Differences were considered significant for $p<0.005$.

Results

In normal rat liver BDNF appeared expressed by small and large cholangiocytes and by HPC (Fig 1). This finding was substantiated by double immunofluorescence labeling (Fig. 2, A-C) for BDNF (A, red) and biliary cytokeratins (CKs) (B, green),

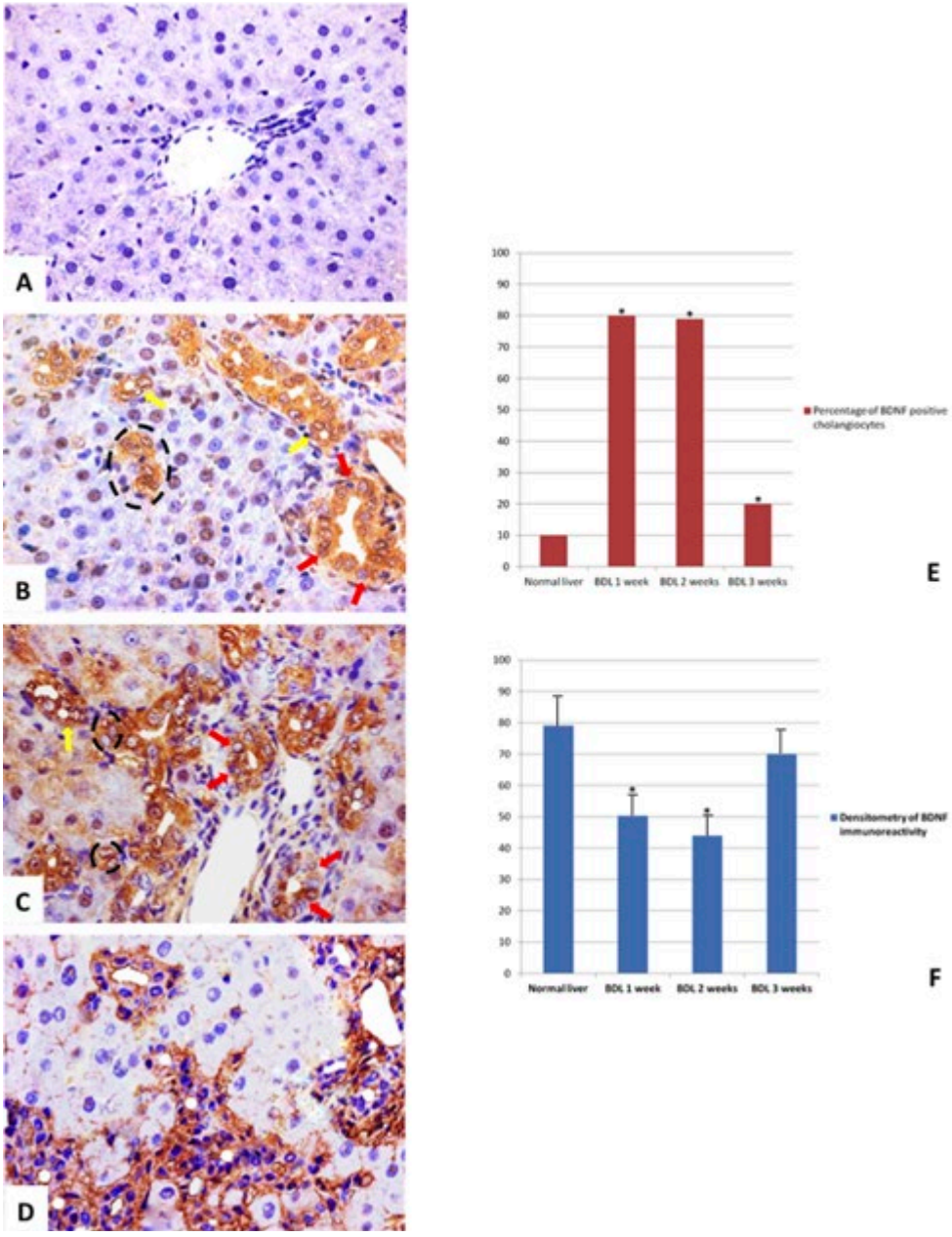

Figure 1 – Immunolocalization of BDNF in normal rat liver (A), and in liver of rats subjected to one week (B), two weeks (C) or three weeks (D) BDL. Red arrows indicate large cholangiocytes, whereas yellow arrows indicate small cholangiocytes. HPCs are evidenced in the squared areas. Quantification of BDNF immunostaining in the different experimental groups, by counting BDNF positive cholangiocytes or by densitometry is reported in E and F, respectively. The differences among experimental groups were significant ($p<0.005$; ANOVA). Magnification: 40x.

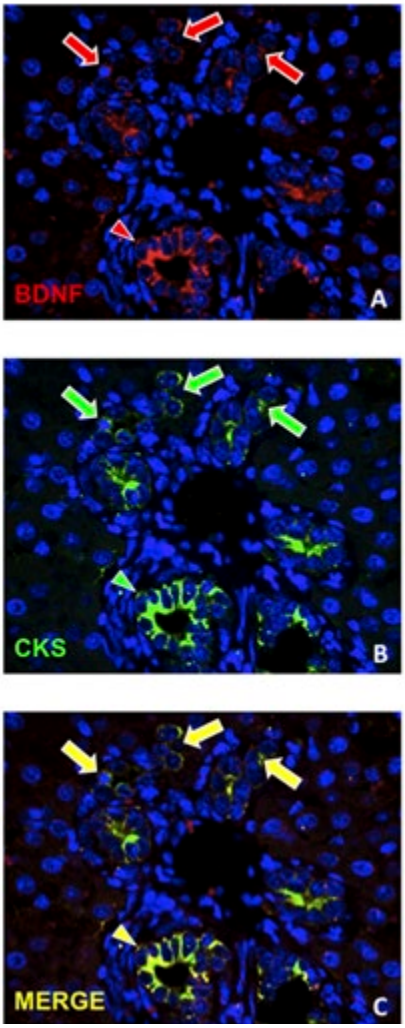

Figure 2 – Double immunolocalization for BDNF (A, red) and CKs (B, green). The merge figure (C) shows that BDNF and CKs are colocalized in cholangiocytes (arrowheads) and in some oval cells around the bile ducts, which are likely to be HPCs (arrows). Magnification: 40x.

which are a validated marker of both cholangiocytes and HPC. Merged images (C, yellow) showed the colocalization of BDNF and CKs. There were no significative differences in the immunostaining for BDNF among the different cellular subtypes of biliary tree (HPCs, small cholangiocytes and large cholangiocytes).

The intensity of BDNF immunoreactivity was different among experimental conditons (Fig 1E,F). In detail: in normal rat liver (Fig 1A), a slight BDNF immunoreactivity was detectable in cholangiocytes. After BDL for 1 week (Fig 1B), BDNF immunoreactivity was drastically increased both in small and in large cholangiocytes. After

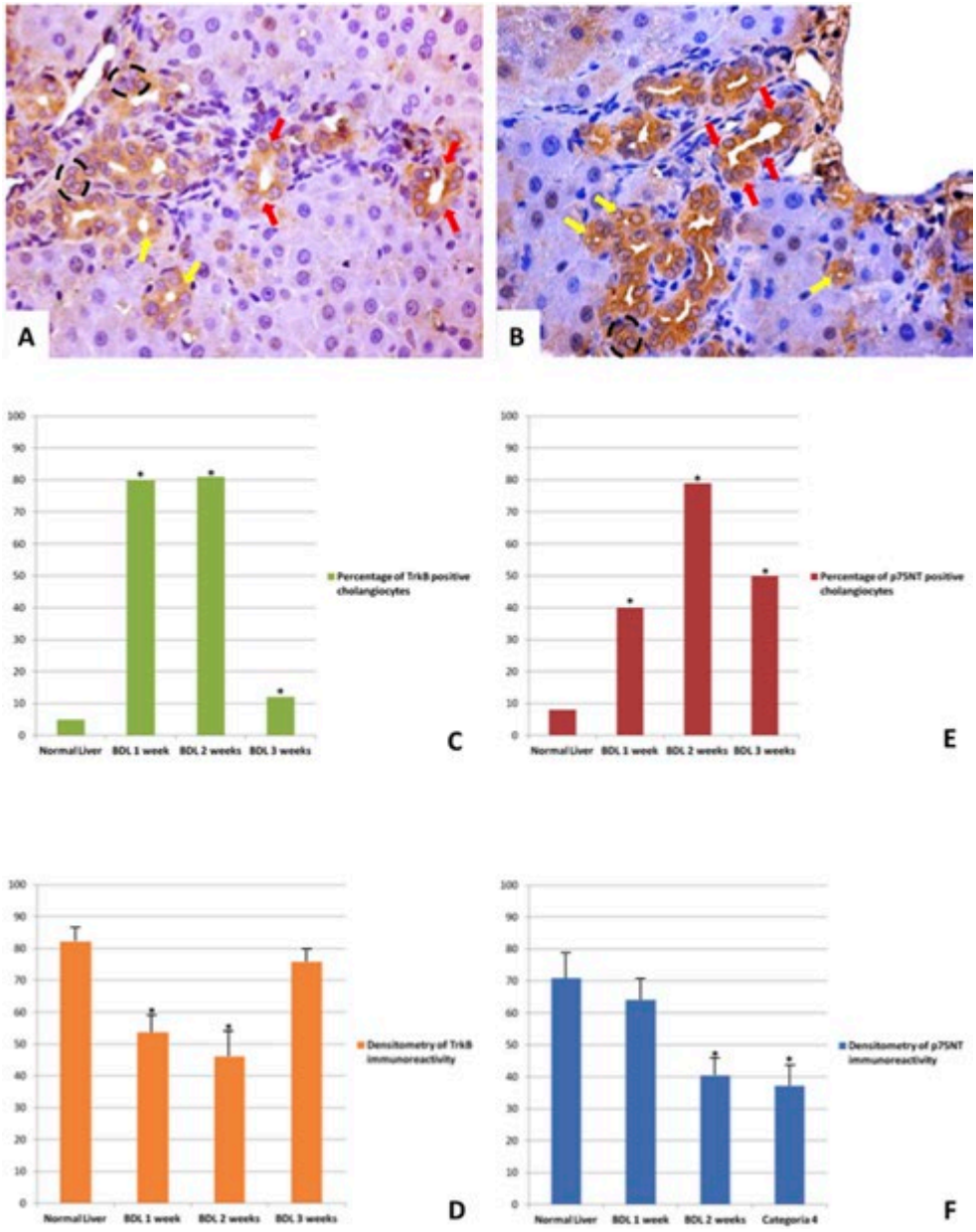

Figure 3 – Immunolocalization of TrKB (A) and p75NT (B) upon BDL. Red arrows indicate large cholangiocytes and yellow arrows indicate small cholangiocytes. HPCs are visible in the squared areas. Quantification of receptors immunostaining in the different experimental group, by counting positive cholangiocytes or by densitometry, is reported in C and D, respectively. The differences among experimental groups were significant for both receptors ($p<0.005$; ANOVA). Magnification: 40x.

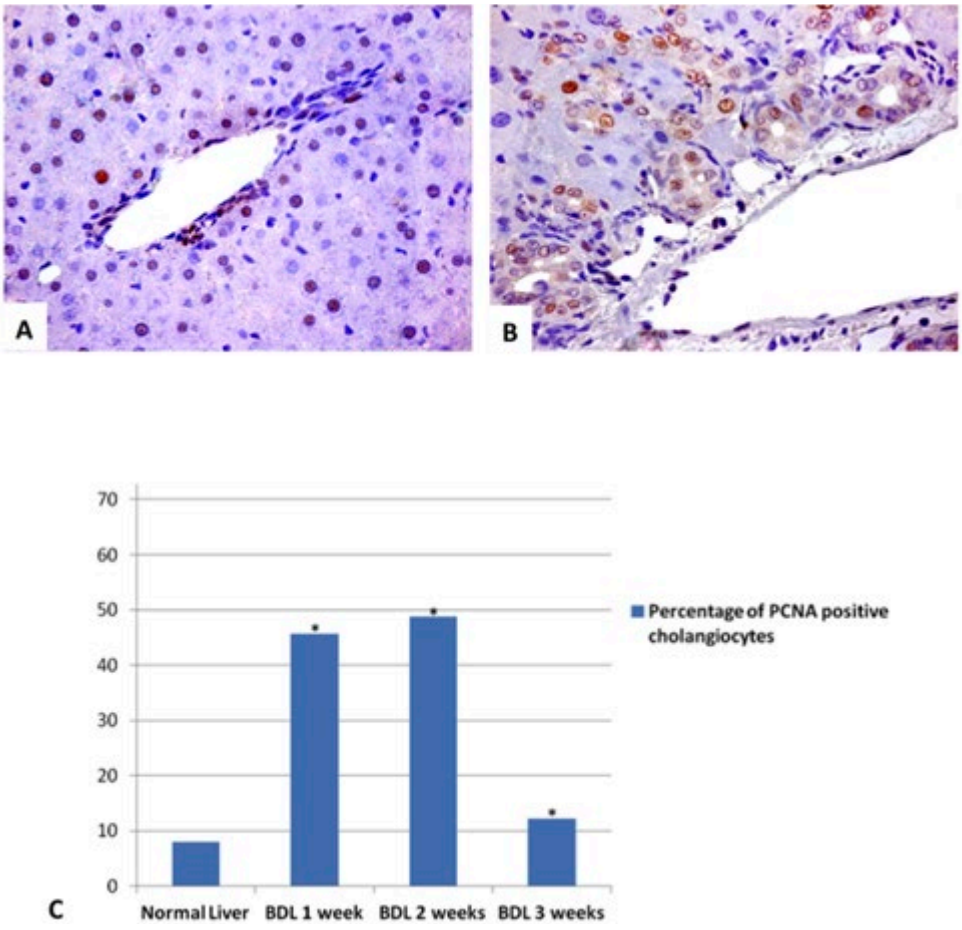

Figure 4 – PCNA immunostaining in a control rat liver (A) and in the liver of a rat subjected to BDL (B). The percentage of cells positive to PCNA (*i.e.* proliferating) among the total number of cholangiocytes (C) was quantified by counting positive cells in different microscopic fields and obtaining a percent value for each experimental group (normal, BDL 1 week, BDL 2 weeks, BDL 3 weeks). The differences among experimental groups were significant ($p<0.005$; ANOVA). Magnification: 40x.

two weeks BDL (Fig 1C) the results were similar to those upon one week BDL. Conversely, after three weeks BDL (Fig 1D), BDNF immunoreactivity was significantly reduced from that after one or two weeks BDL. In both normal and 3 weeks BDL rats liver, BDNF immunoreactivity was mostly confined to the basolateral membrane with only slight reactivity into the cytoplasm.

Similar to BDNF, BDNF receptors (TrKB, p75NT) immunoreactivity was detected in small and large cholangiocytes and in HPCs (Fig 3A,B). Even for these molecules, no substantial difference in immunoreactivity was appreciated among the different cellular subtypes of biliary tree (HPCs, small and large cholangiocytes). As well as BDNF,

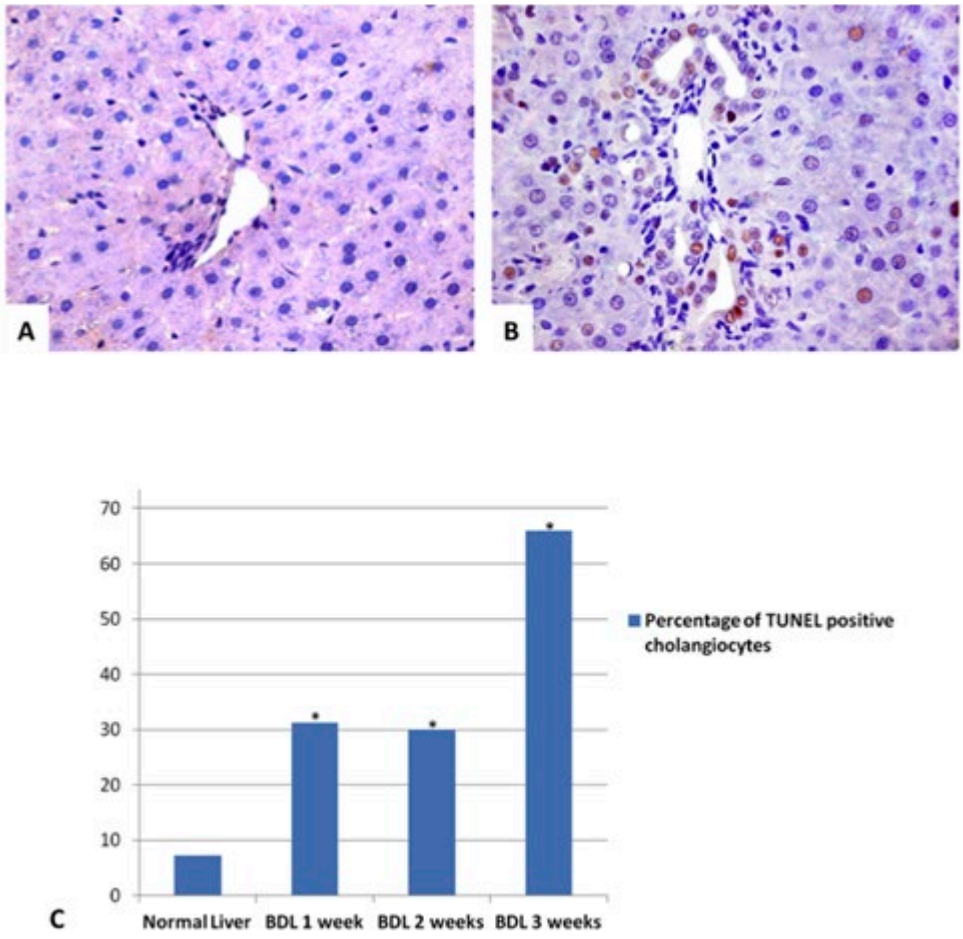

Figure 5 – TUNEL staining in a control rat liver (A) and in the liver of a rat subjected to BDL (B). The percentage of cells(positive to TUNEL (*i.e.* apoptotic) among the total number of cholangiocytes (C) was quantified by counting positive cells in different microscopic fields and obtaining a percent value for each experimental group (normal, BDL 1 week, BDL 2 weeks, BDL 3 weeks). The differences among experimental groups were significant for both receptors ($p<0.005$; ANOVA). Magnification: 40x.

both receptors were differentially expressed among experimental conditions. TrKB and p75NT immunoreactivities were drastically increased after one or two weeks of BDL (Fig 2C,D) as compared with normal rat liver. However, after one week of BDL, TrKB immunoreactivity resulted higher than that of p75NT, whereas after two weeks of BDL the immunoreactivity was almost the same for the two receptors. After three weeks of BDL, instead, the immunoreactivity for p75NT prevailed on that for TrKB; the latter was confined to the basolateral cell membrane. Therefore, the ratio between the immunopositive cells for each BDNF receptor (TrKB/p75NT) yielded the following values: normal liver: 1; BDL 1 week: 2; BDL 2 weeks: 1; BDL 3 weeks: 0,2.

To search for possible relationships between the expression of BDNF and its receptors and the balance between proliferation and apoptosis of cholangiocytes, we labelled proliferating cells by PCNA immunostaining (Fig 4) and apoptotic ones by TUNEL (Fig 5). In normal rat liver, proliferation and apoptosis of cholangiocytes were substantially balanced, whereas prevalence of proliferation was detected in the rats submitted to BDL for one or two weeks. Conversely, after three weeks BDL we found prevalence of apoptosis. Quantitative data are summarized in Figs. 4C and 5C. The ratio between proliferation and apoptosis was obtained from that of PCNA positive to TUNEL positive cells, with the following results: normal liver: 1,1; BDL 1 week: 1,46; BDL 2 weeks: 1,61; BDL 3 weeks: 0,18.

An inflammatory infiltrate was detected in the liver of rats upon BDL, more marked after three weeks than after one or two weeks BDL.

Discussion

The main findings of the present study are that: 1) BDNF and its receptors, TrKB and p75NT, are expressed by small and large rat cholangiocytes and by HPCs; 2) the expression of BDNF and its receptors changes with time upon BDL; 3) during BDL, the balance between the expression of BDNF receptors (TrKB and p75NT) correlates with that between proliferation and apoptosis of biliary epithelium.

The results were appreciated both in terms of positive cells and of intensity of immunostaining and suggest that neurotrophins, in particular BDNF, are involved in the pathophysiology of biliary epithelium during BDL.

The expression of neurotrophins in human and rat liver had been investigated in previous studies, with different results. Shibayama et al. (1996) failed to show any reactivity to TrK antibodies in human liver. Other studies, by RT-PCR or immunohistochemistry on human or rat liver sections, found only trace levels of TrKB transcript or protein (Zhou et al., 1993; Yamamoto et al., 1996). Lomen-Hoerth and Shooter (1995) showed a low level of p75NT receptor in rat liver, using ribonuclease protection assay, but they were not able to show any TrKA or TrKB transcription. Finally, Gigliozzi et al. (2004) reported that the neurotrophin NGF, as well as its receptors TrKA and p75NT are expressed by normal rat cholangiocytes and overexpressed after one week of BDL. They also found that NGF stimulates the proliferation of cholangiocytes activating both Erk and IP3 intracellular pathways.

Among the different animal models mimicking cholestasic liver diseases and utilized to investigate the mechanisms underlying cholangiocytes proliferation and bile duct damage, BDL is the most commonly used (Alpini et al., 1988; Glaser et al., 1997; Alpini et al., 1998). BDL induces proliferation of cholangiocytes, however prolonged BDL makes cholangiocytes also more susceptible to injury (Lesage et al., 2001; Alpini et al., 2003).

In the present study, we found that the expression of BDNF and of its receptors correlates with the remodeling of biliary epithelium during BDL. One week upon BDL we detected that PCNA expression prevailed on TUNEL reactivity, indicating that proliferation prevailed on apoptosis. Upon two weeks of BDL the expression of PCNA was increased in respect to one-week BDL, whereas only irrelevant differences were detectable for TUNEL staining. On the contrary, after three weeks of

BDL, TUNEL reactivity was drastically increased, whereas only few cholangiocytes expressed PCNA. Therefore, one to two weeks after BDL cholangiocytes proliferation was prevalent and a low level of apoptosis occurs, whereas when BDL was prolonged to three weeks apoptosis became prevalent on proliferation. Three weeks after BDL we found an increased expression of p75NT and a reduced expression of TrKB. At previous time points, instead, the expression of TrKB prevailed (after one week), or was balanced (after two weeks) with that of p75NT.

Both TrKB and p75NT are receptors for BDNF (Rodriguez-Tebar et al., 1990; Frade and Barde, 1998; Reichardt et al., 2006). TrKB is a high affinity, tyrosine kinase receptor, specific for BDNF (Urfer et al., 1998; Wiesmann and De Vos, 2001), whereas p75NT is a low affinity receptor shared by different neurotrophins, including NGF, BDNF, NT3 and NT4. The molecular intracellular pathways activated by these two receptors are also different. TrKB is a tyrosine kinase transmembrane receptor which activates different pro-proliferative and growth pathways, including the Akt and Erk pathways, involved in the proliferation of large cholangiocytes (English et al., 1999), as well as the IP3/PKC pathway, responsible of the proliferation of small cholangiocytes (Huang and Reichardt, 2003). P75NT receptor, instead, belongs to the family of TNF receptors (Liepinsh et al., 1997; He and Garcia, 2004). It activates two distinct intracellular pathways one of which includes the activation of the cytoplasmic factor JNK and of the transcription factor c-JUN, with a pro-apoptotic effect (Chi et al., 2005), whereas the other is characterized by the activation of the transcription factor NFκB and has pro-proliferative and differentiative properties (Hamanoue et al., 1999; Middleton et al., 2000). Activation of NFκB has been reported in cholestasis, possibly to reduce the effect of bile duct injury (Miyoshi et al., 2001). The activation of the pro-proliferative pathway, related to p75NT requires the coactivation of TrKB or of another neurotrophin tyrosine kinase receptor, such as TrKA or TrKC (Yoon et al., 1998). Therefore, it is possible that, after one or two weeks of BDL, the coactivation of TrKB and p75NT neurotrophin receptors has a positive effect on proliferation of both small and large cholangiocytes and prevents apoptosis. Indeed, after two weeks of BDL, when the expression of these two receptors was balanced, we found the maximal proliferative activity, as detected by PCNA immunostaining. In contrast, after three weeks of BDL a prevalent expression of p75NT occurs and the activation of this receptor by BDNF may lead to the activation of a pro-apoptotic intracellular pathway, because of lack of coactivation of TrKB. Therefore, in the early stages of BDL, BDNF, which is directly produced by small and large cholangiocytes, can stimulate proliferation of biliary epithelium and exert an anti-apoptotic effect, whereas at later stages, its production by cholangiocytes decreases drastically and the small amount secreted can induce apoptosis through activation of p75NT without coactivation of TrKB, which results less expressed. These results, obtained in the liver, are in line with those obtained in the nervous system, where survival, apoptosis, differentiation and response to injury are all controlled through the modulation of the above described signaling pathways activated by neurotrophins (Patel et al., 2000; Teng et al., 2005).

The expression of TrKB in the cytoplasm of proliferating cholangiocytes likely represents the intracellular trafficking of the receptor. Binding of neurotrophins to their TrK receptors, in fact, induces the formation of signaling endosomes, containing both the neurotrophin and the receptor, the latter trafficking between the cell surface and the cytoplasm (Makkerh et al., 2005; Arevalo et al., 2006). The fact that, after three

weeks of BDL, TrKB seems localized mainly at the basolateral cell surface, suggests that this receptor may not be activated by BDNF, further supporting the hypothesis that upon prolonged BDL the activation of p75NT occurs without coactivation of TrK receptors, exerting a pro-apoptotic effect.

Proliferating cholangiocytes acquire a neuroendocrine phenotype and secrete and respond to a number of hormones, neurotransmitters and neuropeptides, including neurotrophins (Roskmas et al., 2004; Renzi et al., 2011). The formation of this neuroendocrine compartment predominated by cholangiocytes represents a unique opportunity for these cells to regulate their own proliferation via autocrine pathways and to influence other nearby cell types, such as vascular endothelial cells, portal fibroblasts, stellate cells and HPCs (Alvaro et al., 2007). In our study we found that BDNF and BDNF receptors are expressed by HPCs, which were identified according with Forbes et al. (2002). The expression of BDNF and BDNF receptors by HPCs during BDL may be part of this neuroendocrine-paracrine crosstalk. On the other hand, HPCs expressing BDNF and BDNF receptors might represent a subpopulation of these cells committed to a neuroendocrine phenotype.

Besides HPCs, proliferating cholangiocytes crosstalk with other cellular subtypes, including lymphocytes, macrophages and other inflammatory cells (Alvaro et al., 2007). In human pathology, as well as in experimental models of cholestasis, in fact, proliferation of cholangiocytes occurs always associated with marked changes in surrounding mesenchymal cells and extracellular matrix (Lazaridis et al., 2004). In chronic cholestatic liver diseases (primary biliary cirrhosis, primary sclerosing cholangitis), it occurs in a milieu of an intense portal inflammation (Fava et al., 2005). Inflammatory cells produce interleukins (IL-1, IL-6, IL-8, INF-γ), TGF and TNF which are involved in the remodeling of biliary tree, as well as in the genesis of peribiliary fibrosis (Alvaro et al., 2007). In particular TNF can induce apoptosis of cholangiocytes, by activating its transmembrane receptor which activates, in turn, a pro-apoptotic intracellular signal (Loffreda et al., 1997; Rosen et al., 1997; Guicciardi and Gores, 2009). p75NT is member of the TNF receptor superfamily (Liepinsh et al., 1997; He and Garcia, 2004). After three weeks of BDL, we have found high expression of p75NT, together with decreased expression of TrKB and BDNF. The activation of the p75NT may contribute to the apoptosis of cholangiocytes, for which BDNF may cooperate with inflammatory cytokines and TNF. Moreover, the decreased production of BDNF by cholangiocytes might exacerbate this process because BDNF has an anti-inflammatory effect (Gigliozzi et al., 2004). All these mechanisms could contribute to ductopenia occurring in cholestasic liver diseases as well as in late stages of BDL models.

In conclusion, we should like to stress the original aspects of this immunohistochemical study: 1) it is the first study focused on the expression of the neurotrophin BDNF and of its receptors (TrKB, p75NT) in rat liver; 2) it shows, for the first time, that the expression of BDNF and of related neurotrophins receptors is closely associated with the proliferation as well as with the apoptosis of biliary epithelium, being a possible determinant for remodeling of biliary tree during BDL; 3) it reports that BDNF and its receptors are expressed by HPCs during proliferation of cholangiocytes, suggesting that, as well as in the brain, neurotrophins, including BDNF, could modulate the HSCs compartment and intervene in the restoration of liver damage.

These findings call for more detailed investigations on neurotrophic signal in the liver, and lay the bases for new insights on the application of neurotrophins in the

management of chronic cholestasic liver diseases, as well as in the modulation of HPCs compartment.

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