Molecular mechanisms of cholangiopathy in primary biliary cirrhosis

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Abstract

Primary biliary cirrhosis (PBC) is histologically characterized by chronic nonsuppurative destructive cholangitis (CNSDC) and the progressive loss of intrahepatic small bile ducts. Cellular immune mechanisms involving T cell-reaction are thought to be significantly involved in the formation of CNSDC and bile duct loss. In inflammed portal tracts of PBC, $CD4^+$ T cells of Th1-type expressing IFN-γ or CXCR3 are aggregated and more commonly detected around injured bile ducts than Th2-type $CD4^+$ T cells expressing IL-4 or CCR4, indicating that Th1-dominant cellular immunity play a more prominent role in recruitment of memory T cell subsets in PBC and may be responsible for the progressive bile duct damage. Biliary epithelial apoptosis is demonstrated to be a major pathogenic process of bile duct loss in PBC. In CNSDC, several biliary apoptotic cells, an aberrant expression of Fas antigen (pro-apoptotic molecule), and decreased expression of bcl-2 and mcl-1 (anti-apoptotic molecules) are found, though interlobular bile ducts express bcl-2 and mcl-2, but lack Fas. In addition, the up-regulation of WAF1 and p53 related with biliary apoptosis is found in biliary epithelial cells of PBC, which may be due to cell senescence in response to genotoxic damages such as oxidative stress. Several steps and mechanisms during induction and progression of cholangitis and biliary apoptosis followed by bile duct loss are now being proposed in PBC, but future analysis of an etiopathogenesis explainable for these characteristic histopathogenesis of PBC will be required.

Introduction

Necroinflammatory and fibrous changes and these combinations in parenchyma and portal tracts characterize the histology of most hepatobiliary diseases, and the characteristic cholangiopathy also take a part in biliary diseases such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). PBC primarily affects middle-aged women and is characterized by the presence of antimitochondrial antibodies (AMA).[1] The initial histology of PBC is a distinctive pattern of bile duct damage referred to as chronic nonsuppurative destructive cholangitis (CNSDC) and bile duct loss and chronic cholestasis are prominent from the early stage.[2,3] Non-biliary diseases such as HCV-related chronic hepatitis (CH-C) and autoimmune hepatitis (AIH) also often show mild bile duct damage, but the occurrence of bile duct loss or cholestasis is very rare. This cholangiopathy found in non-biliary diseases, therefore, is denominated as hepatitic duct lesion or hepatitis-associated bile duct injury to entirely differ from the cholangiopathy in PBC.[4-6] In this review, we describe the molecular mechanisms of cholangitis and bile duct loss in PBC.

Histopathology of cholangiopathy in PBC

The intrahepatic biliary tree is dividable anatomically into large bile ducts, septal bile ducts, interlobular bile ducts and bile ductules.[7] The morphology and functions of biliary epithelial cells are different along the intrahepatic biliary tree. CNSDC usually distribute in intrahepatic small bile ducts (interlobular bile ducts and septal bile ducts), but bile duct loss progresses mainly in the level of interlobular bile ducts. Biliary epithelial cells consisting CNSDC show the proliferative change such as papillary and stratified ingrowths with high

cell kinetic activity, and simultaneously the destructive change such as eosinophilic and swollen degeneration.[2,8,9] Chronic inflammatory cells including lymphocytes and plasma cells infiltrate around the injured bile ducts in PBC, and eosinophils and macrophages are also noted in several portal tracts. A focal aggregate of epithelioid cells, sometimes forming well-developed granuloma, is present and surrounds the CNSDC (granulomatous cholangitis) (Fig.1). These injured bile ducts finally disappear by the mechanism of enhanced biliary apoptosis from the early stage of PBC (see below). Significant bile duct loss refers to the condition in which more than 50% of the portal tracts which lack these bile ducts in liver specimens, though peripheral portal tracts constantly contain well-formed interlobular or septal bile ducts in addition to portal vein and hepatic arterial branches in normal livers.[2,10] Hepatitis-associated changes in parenchyma such as necroinflammatory changes, Kupffer cell hyperplasia, and intrasinusoidal lymphocytic infiltration are generally mild in PBC. However, the characteristic findings of copper deposition and liver cell dysplasia suggesting chronic cholestatic conditions are prominent from the early stages and very useful for pathological diagnosis of PBC. Especially, copper deposition is a sensitive finding suggesting the presence of chronic cholestasis and orcein staining (Shikata's orcein) is the most useful staining for evaluating copper-associated proteins.

Cytokines and chemokines around bile ducts in PBC

Cell population within and around bile ducts in PBC

Cytokines produced by lymphocytes infiltrating around CNSDC, are closely associated with the progression of bile duct injury in PBC, because biliary epithelial cells bear several

cytokine receptors against at least IL-4, IL-6, IFN-γ, and TNF-α (type2-TNF receptor).[11] In addition, biliary epithelial cells themselves also produce TNF-α and IL-6, so these cytokines affect themselves in autocrine and paracrine matters.[12] In PBC, it is generally believed that T cell-related cellular immunity is involved in the pathogenesis of CNSDC. Indeed, it has been demonstrated that $CD8^+$ and $CD4^+$ lymphocytes are the predominant cell type in the inflammatory cells within portal tracts in PBC.[13,14] $CD8^+$ lymphocytes are mostly cytotoxic T cells and affect the targets via the perforin/granzyme exocytosis pathway.[15,16] $CD4^+$ lymphocytes, especially pathogenic autoreactive T cells, regulate the autoimmunity around bile ducts in PBC. Moreover, in the development of cholangiopathy, the infiltration of immune cells within the biliary epithelial layer and the direct adhesion between biliary epithelial cells and immune cells are key events leading to cell-mediated cytotoxicity and apoptosis of biliary epithelial cells.[15,16] The finding of lymphocytic recruitment around the bile ducts and of penetration into the biliary layer is termed as an ‘epitheliotropism’, which is associated with the increased cellular permeability of bile ducts caused by significant reduction of tight junction molecules in PBC.[17-19] This epitheliotropism is found in normal livers as well as PBC livers. In normal livers, intra-epithelial lymphocytes existing in interlobular bile ducts are mainly $CD8^+$ lymphocytes and though to be involved in the immune homeostasis of intrahepatic bile ducts.[20]

Cytokine milieu around bile ducts in PBC

A number of (pro)inflammatory cytokines are known to be elevated in the local portal tract microenvironment in PBC, contributing to development of chronic inflammatory reaction

around bile ducts and biliary epithelial cells as well as immune cells actively participate in this inflammatory process. Immunoreactivity and autoimmunity are regulated by two different types of $CD4^+$ helper T cells, Th1 and Th2 subsets, principally subdivided by distinctive cytokine production and effector functions. Th1 cells involved in the cell mediated response, provide help to cytotoxic $CD8^+$ T lymphocytes, activate natural killer cells, and produce delayed hypersensitivity reactions. Th1 clones secrete IL-2, IFN-γ and TNF-β. In contrast, Th2 cells involved in the humoral response is characterized by the differentiation and activation of B cells into antibody secreting plasma cells. Th2 clones secrete IL-4, IL-5, IL-6, IL-10 and IL-13. A polarized cytokine profile plays a pivotal role as a pathophysiological factor in autoimmunity; organ-specific autoimmune diseases are mainly mediated by Th1 cells, while in systemic autoimmune disorders Th2 subset predominates. In PBC, the presence of a predominant Th1 cytokine profile is demonstrated.[21] Cytokine profiles determined primarily from stimulated peripheral blood and liver-derived T lymphocytes may be misleading for defining a Th1/Th2 cytokine profile in PBC.[22,23] *In situ* hybridization study reveals that IFN-γ mRNA-expressing mononuclear cells are more commonly detected primarily around damaged bile ducts in PBC livers than IL-4 mRNA-expressing cells and that levels of IFN-γ mRNA expression are highly correlated with the degree of portal inflammatory activity (Fig.2).[24] Cytokine levels in sera and whole liver may not accurately reflect local hepatic tissue levels, because cytokines primarily have a local or paracrine mode of action. However, the analyses of cytokine synthesis using peripheral blood mononuclear cells and whole livers also reveal that a slight elevation of Th1 prevalence, as well as a significant decline of Th2 prevalence, is observed in PBC.[25,26]

Recent study has reported that $CD8^+$ and $CD4^+$ (in particular $CD4^+$ $CD28^-$) are markedly increased as intra-epithelial lymphocytes within damaged bile ducts in PBC.[20] Because these unique $CD4^+CD28^-$ T cells proliferate in target tissue of autoimmune diseases and are associated with Th1/Th2 balance in the regulation of spontaneous autoimmune diseases by possessing the high expression of IFN-γ and autoreactive and cytolytic function, $CD4^+CD28^-$ T cells may be involved in the pathogenesis of autoimmune-mediated bile duct damage of PBC.[27,28]

Chemokine milieu around bile ducts in PBC

Continued recruitment of pathogenic lymphocytes including autoreactive T cells contributes to the progressive bile duct destruction in PBC. Chemokines (chemotactic lymphokines) have well-defined roles in the regulation of leukocyte recruitment and retention, and positioning in tissues. Fractalkine (CX3CL1) consisting of a membrane-bound form and a soluble chemotactic form, is produced by several epithelial cells and associated with the cell-adhesion and the chemoattractant for its receptor (CX3CR1)-expressing cells such as $CD8^+$ and $CD4^+$ T cells.[29,30] The production of fractalkine is demonstrated in biliary epithelial cells and upregulated by several cytokines including IFN-γ and TNF-α. In PBC, the expression of fractalkine is upregulated in injured bile ducts of PBC and the $CD4^+$ and $CD8^+$ lymphocytes expressing CX3CR1 are found in portal tracts and within biliary epithelial layer of injured bile ducts.

The preferential association of some chemokine receptors with Th1 or Th2 cells has been reported, providing a basis for tissue-specific recruitment of memory T cell subsets. CXCR3

and CCR5 are expressed at higher levels on Th1 cells.[31,32] In contrast, CCR3 and CCR4 are found on Th2 cells.[31] At early stage of PBC, the mononuclear cells positive for CXCR3 (Th1) are dense around the damaged bile ducts and the ratio of CXCR3-/CCR4-positive mononuclear cells (Th1/Th2) in portal tracts of early PBC is significantly higher than that of advanced PBC.[33] In addition, the expression of IFN-γ-inducible protein-10 (IP-10, CXCL10) and monokine induced by IFN-γ (MIG, CXCL9) is increased in plasma and portal tracts in PBC, which chemokines are synthesized predominantly by macrophages following exposure to IFN-γ and involved in the selective recruitment of Th1 cells via CXCR3.[34] These data also suggest that the enhanced shift toward Th1 occurs in portal tracts of PBC

Mechanism of bile duct loss in PBC

Apoptosis of biliary epithelial cells

Apoptosis (programmed cell death) conventionally has been defined as nuclear condensation and fragmentation by electron microscopy (Fig.3). Recent molecular analysis revealed that apoptosis is biochemically characterized by DNA fragmentation caused by the activation of endonuclease as a final event and constitutes a strictly regulated mechanism for the removal of unnecessary, aged or damaged cells. In addition, apoptosis plays an essential role in the pathogenesis and the development of many human diseases including autoimmune diseases as well as in physiological condition. Light-microscopically, apoptotic cells of biliary epithelial cells are characterized by shrinkage of cytoplasm and condensation of nucleus and scatter in intrahepatic biliary tree in physiological conditions (Fig.3). Approximately 4% apoptotic cells are found in the large bile ducts and septal bile ducts, whereas very rare in

interlobular bile ducts (below 1%).[35] This suggests that the cell kinetics of biliary epithelial cells with respect to apoptosis differs according to the anatomical level of the biliary tree. That is, the loss and renewal of biliary epithelial cells occur relatively rapidly in the large and septal bile ducts compared with the interlobular bile ducts. The type and/or amounts of physiologic stimuli responsible for the induction of apoptosis may vary along the biliary tree and be a cause of the different homeostasis (cell death-renewal mechanism) of intrahepatic bile ducts. Morphological recognition of biliary epithelial apoptosis may underestimate the real rate of apoptosis, because apoptotic biliary epithelial cells are probably rapidly shed into bile and eliminated from the biliary tree without histologic recognition. In recent studies, terminal deoxynucleotidly transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-nick end labeling (TUNEL) is used in practice as a method to identify apoptotic cells *in situ*. Conventional and morphological apoptotic biliary cells certainly show the TUNEL-positivity, and some biliary cells lacking morphological apoptosis are also positive for TUNEL staining. In PBC, TUNEL-positive apoptotic cells are frequently found on biliary epithelial cells, particularly more cholangitic bile ducts.

Apoptosis-related molecules in biliary epithelial cells

The process of apoptosis can be subdivided into three phases: initiation, effector and degradation. The initiation stage of apoptosis depends on the type of apoptosis-inducing stimulus, but the effector and degradation stages are common to all apoptotic processes. In biliary epithelial cells, several apoptosis-related molecules including Bcl-2-family, Fas-Fas ligand, and WAF-1, act in the initiation stage.[9,35-40] The regulatory mechanisms of biliary

apoptosis are different according to the anatomical location of the intrahepatic biliary tree depending the expression of these apoptosis-related molecules.[35]

Bcl-2-family proteins are resident proteins of the mitochondrial membrane and share several highly conserved regions and interact with one another through the formation of homo- and heterodimers. The susceptibility of cells to apoptotic stimuli is thought to be controlled by the relative ratios of these different Bcl-2 family proteins.[41] Bcl-2 and Mcl-1 (both anti-apoptotic) prevent cells from undergoing apoptosis when overexpressed, whereas Bax (pro-apoptotic) functions to promote apoptosis. Bcl-X is subdivided to Bcl-XL (long) and Bcl-XS (short) through the alternative RNA splicing of Bcl-X gene, which are anti-apoptotic and pro-apoptotic, respectively.[42] Overexpression of Bax functions to promote cell death via apoptosis through the formation of Bax homodimers[41] and to release cytochrome C (an apoptosis-inducing factor) from mitochondria to cytosol. This release is known to lead to an activation of caspase 9 and subsequently caspase 3. However, the formation of heterodimers between Bax and Bcl-2 homologues with death repressor function (Bcl-2, Mcl-1 and Bcl-XL, especially Bcl-2) leads to inhibition of the death promoting effects of Bax.[41] In liver, the expression of Bcl-2 is restricted in the interlobular bile ducts and bile ductules, but not detectable in the large and septal bile ducts and hepatocytes.[43] In contrast, Bcl-XL and Mcl-1 (anti-apoptotic) and Bax (pro-apoptotic) are diffusely expressed in the intrahepatic biliary tree.[35] Therefore, the ratio of Bax vs Bcl-2 is higher in the large and septal bile ducts than in the interlobular bile ducts and bile ductules, and is responsible for the unique distribution of conventional apoptotic biliary cells along the intrahepatic biliary tree in normal conditions as described above.[35]

Fas receptor, also known as APO-1 or CD95, belongs to TNF superfamily and has the intracellular death domain. Fas activation causes a complex sequence of events, which finally leads to Fas membrane targeting and formation of the death-inducing signalings complexes (DISC), that is, the recruitment of the Fas-associated death domain (FADD) and procaspase 8 to the Fas. Fas ligand is also a cell surface molecule on the effector cells including cytotoxic T cells, natural killer cells and the Th1 subset of T helper cells; Fas ligand induces apoptosis in target cells by triggering Fas in susceptible target cells. In liver, Fas antigen plays an important role in liver homeostasis, because hepatocytes constantly express Fas on their cellular membrane and are exquisitely sensitive to apoptosis mediated by Fas-Fas ligand signaling. Biliary epithelial cells also express Fas antigen, but the distribution of Fas shows heterogeneity in the intrahepatic biliary tree. Fas expression is observed in the large bile ducts, but rarely in the interlobular bile ducts and bile ductules. In the septal bile ducts, its expression is intermediate, and positive and negative biliary cells often mingled in the same ducts.[35] This suggests that biliary epithelial cells consisting of large bile ducts are also sensitive to Fas-mediated apoptosis and supports that the number of conventional apoptosis is relatively larger in biliary epithelial cells of the large bile ducts than those of interlobular bile ducts as mentioned above. Among caspase cascades, caspase 3, caspase 8, and caspase 9 play a pivotal role in the intracellular signaling of Fas-mediated apoptosis; Fas activation induces the cleavage of caspase 8 followed by directly or indirectly the cleavage of caspase 3. Biliary epithelial cells constantly express caspase 3, caspase 8, and caspase 9, but the intracellular signaling pathway leading apoptosis in biliary epithelial cells is entirely unknown.

Cell senescence of biliary epithelial cells is also noted as a process of apoptosis.[40]

Replicative cell senescence caused by replication and/or by diverse agents such as oxidative stress and bile acids, and other detergent substances in the bile ("bad bile"), may be related to the induction of DNA damage and apoptosis of biliary epithelial cells.[19,44-47] WAF1 ($p21^{WAF1/Cip1}$) is a potent and reversible inhibitor of cell-cycle progression at both the G1 and G2 checkpoint and an important molecule of cell-cycle regulation and homeostasis. p53-dependent WAF1 up-regulation followed by irreversible G1 arrest is one of major mechanism to delete the genetical damaged cells in carcinogenesis and cellular senescence of biliary epithelial cells. [40,48,49]

Enhanced biliary apoptosis in PBC

TUNEL-positive apoptotic cells are frequently found on biliary epithelial cells, particularly more cholangitic bile ducts in PBC, but very a few in control livers (Fig.4). These biliary epithelial cells showing cholangitis aberrantly express Fas antigen in their luminal surface and/or basolateral membrane accompanying the infiltration of Fas ligand-expressing inflammatory cells.[16,35,37] Because TNF-α and IFN-γ have been shown to induce an increase in Fas receptor expression and enhance anti-Fas-induced apoptosis, these cytokines may involved in the occurrence of apoptotic cell death on damaged bile ducts in PBC through this system. The bcl-2 family reportedly is also associated with the biliary epithelial apoptosis in PBC.[38,50] Among bcl-2 family, the downregulation of bcl-2, mcl-1, and bcl-XL leads to a decrease in the threshold of apoptosis and increases in the vulnerability to apoptotic stimuli in the damaged bile ducts, followed by the progressive bile duct loss in PBC.[9,36,37,51] Celli *et al*., have demonstrated that using a human biliary epithelial cell line the reduction in the cellular

levels of an antioxidant cytoprotective molecules such as glutathione results in increased degradation of bcl-2 protein and an increase in biliary epithelial apoptosis.[52] By the close examination of Fas and bcl-2 expression in interlobular bile ducts, we have confirmed that Fas-positive and bcl-2-negative bile ducts are very specific for PBC livers, suggesting the increased susceptibility for apoptotic signals in biliary epithelial cells of PBC (Fig.5). In addition, the up-regulation of WAF1 and p53 is found in biliary epithelial cells and relates to the biliary apoptosis in cholangitis of PBC, which may be due to cell senescence of biliary epithelial cells in response to genotoxic damages such as oxidative stress.[40]

In addition, infectious agents including bacteria and virus are suspected of being involved in the etiopathogenesis of PBC and/or the pathogenesis of bile duct damage in PBC. *Mycobacterium*, *Escherichia coli* (*E.coli*), *Propionibacterium acnes* (*P.acnes*), *Lactobacillus*, and retrovirus have been reported as possible candidates based on serological examinations or on molecular analysis.[53-59] Although it is a matter of controversy whether these microorganisms are directly or indirectly causative pathogens of PBC or not, infectious agents may increase the susceptibility for apoptosis in biliary epithelial cells.

Conclusions

Th1 cytokines including IFN-γ predominate over Th2 in PBC livers and enhanced biliary apoptosis is responsible for the progressive bile duct loss in PBC. However, the mechanisms for such dysregulation of cytokine milieu and apoptosis-related molecules in biliary epithelial cells are unknown. In view of recent reports of possible microorganisms involvement in the multi-factorial process that leads to the development of PBC, further studies are needed to

address this issue.

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Figures and figure legends

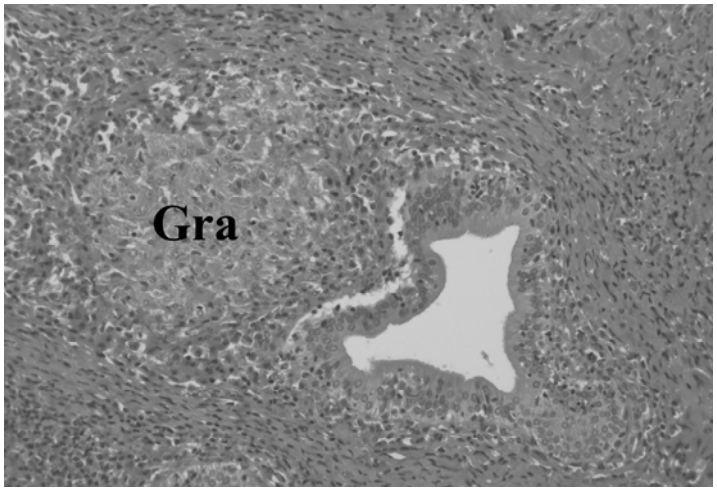


Fig 1 Granulomatous cholangitis in primary biliary cirrhosis. Chronic non-suppurative destruction cholangitis accompanying well-developed epithelioid cell granuloma (Gra) is referred as granulomatous cholangitis. (HE staining, Original magnification, x100)

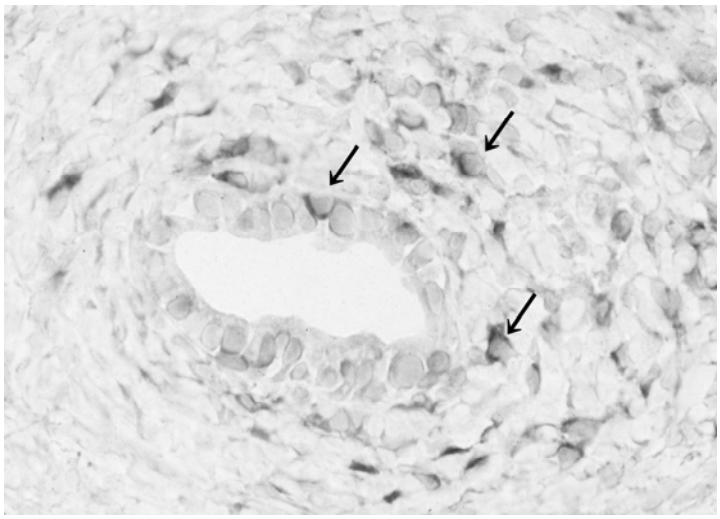


Fig 2 IFN-γ-mRNA expressing mononuclear cells around and within injured bile ducts in primary biliary cirrhosis (arrows). (*In situ* hybridization for IFN-γ mRNA, Original magnification, x200)

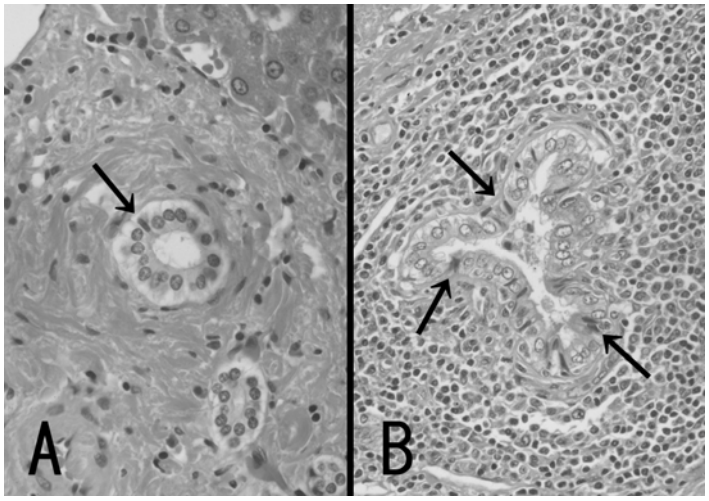


Fig 3 Interlobular bile ducts in normal liver (A) and injured bile ducts in PBC (B). Apoptosis of biliary epithelial cells are characterized morphologically by cytoplasmic shrinkage and nuclear condensation (arrows). (HE staining, original magnification x200 (A) and x400 (B))

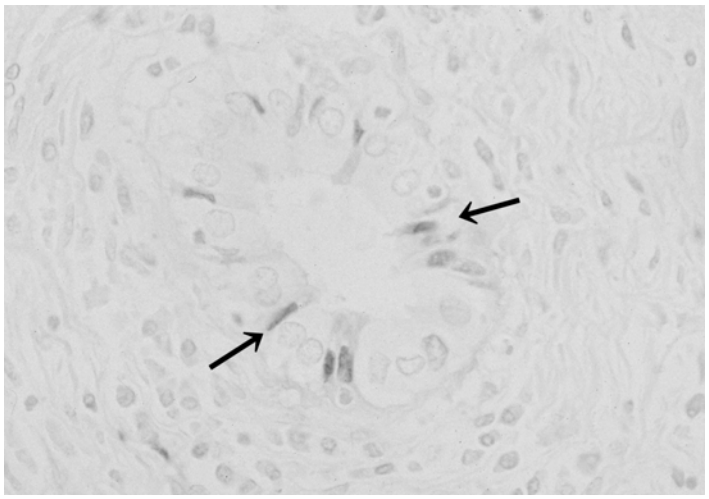


Fig 4 Primary biliary cirrhosis. Conventional apoptotic cells (arrows) certainly show the TUNEL-positivity. (Terminal deoxynucleotidly transferase-mediated deoxyuridine triphosphate-biotin nick end labeling staining, Original magnification, x200).

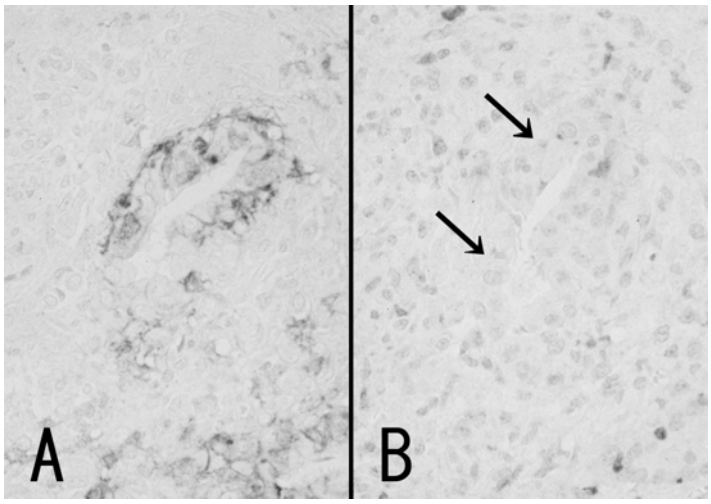
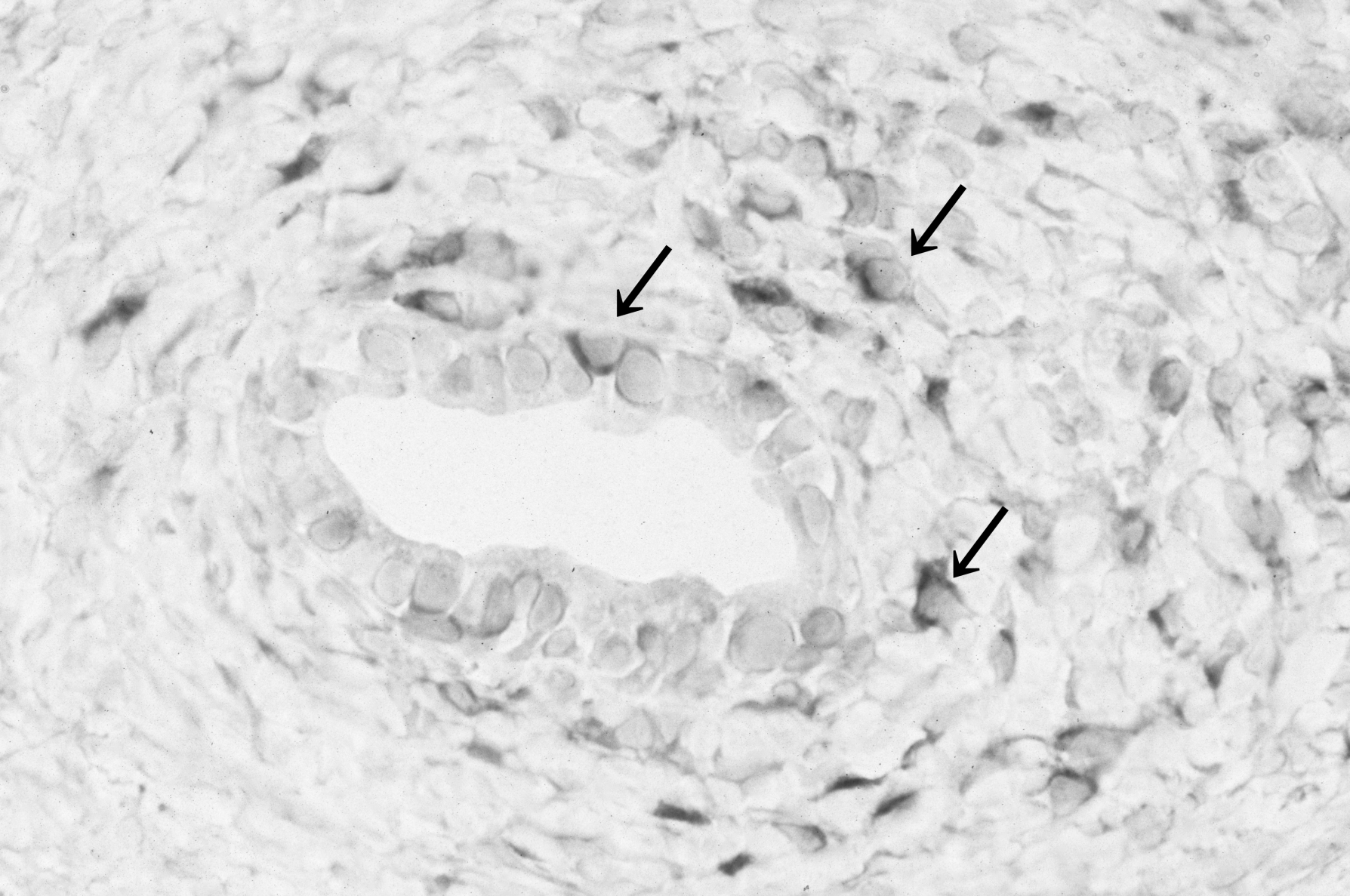
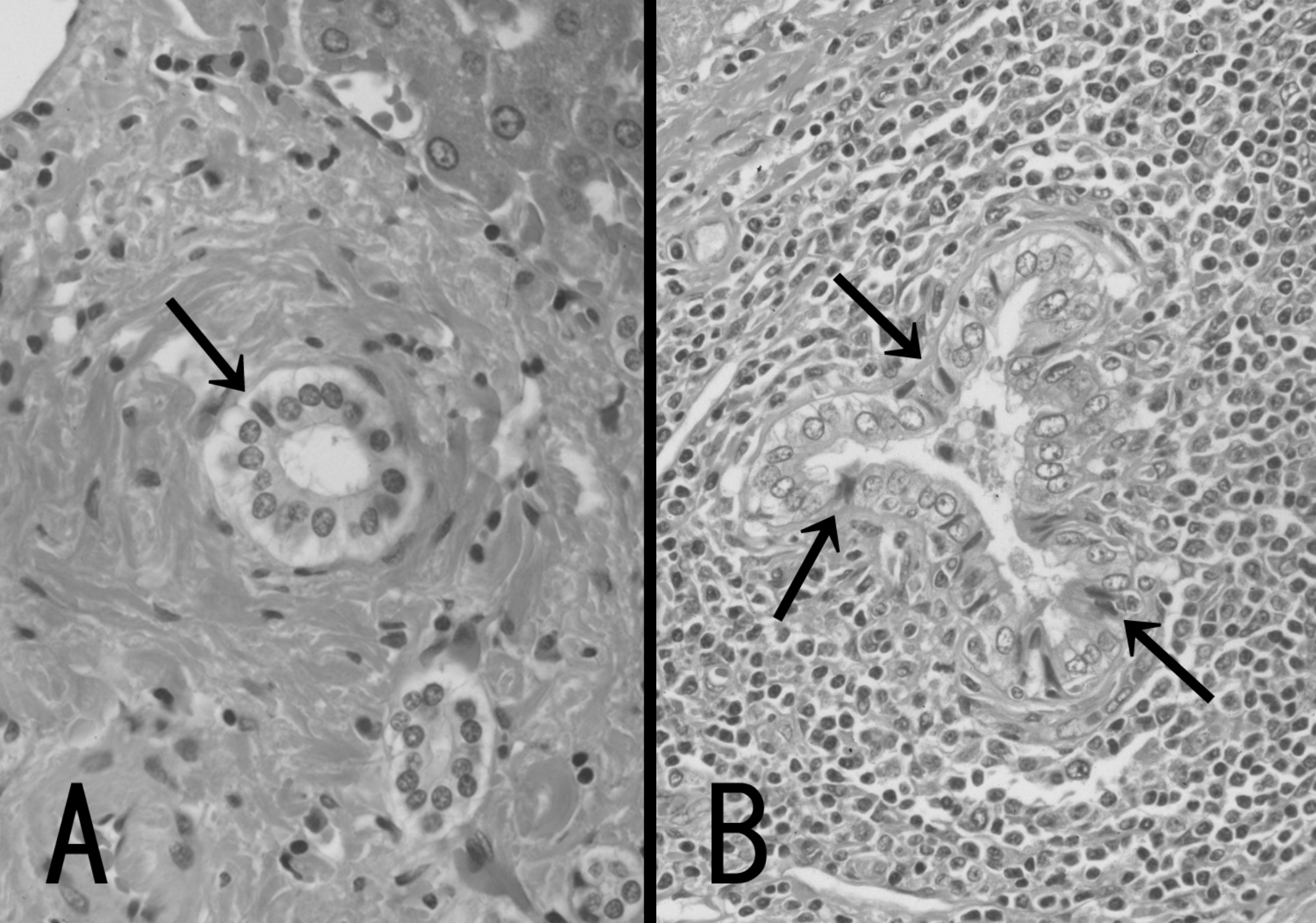


Fig 5 Primary biliary cirrhosis. (A) Injured bile ducts show definite membranous staining for Fas. (B) Injured bile ducts (arrows) are completely negative for Bcl-2, though infiltrating mononuclear cells are positive. (Immunohistochemical staining for Fas (A) and Bcl-2 (B) counterstained with hematoxylin. Original magnification, x200)

Gra




A
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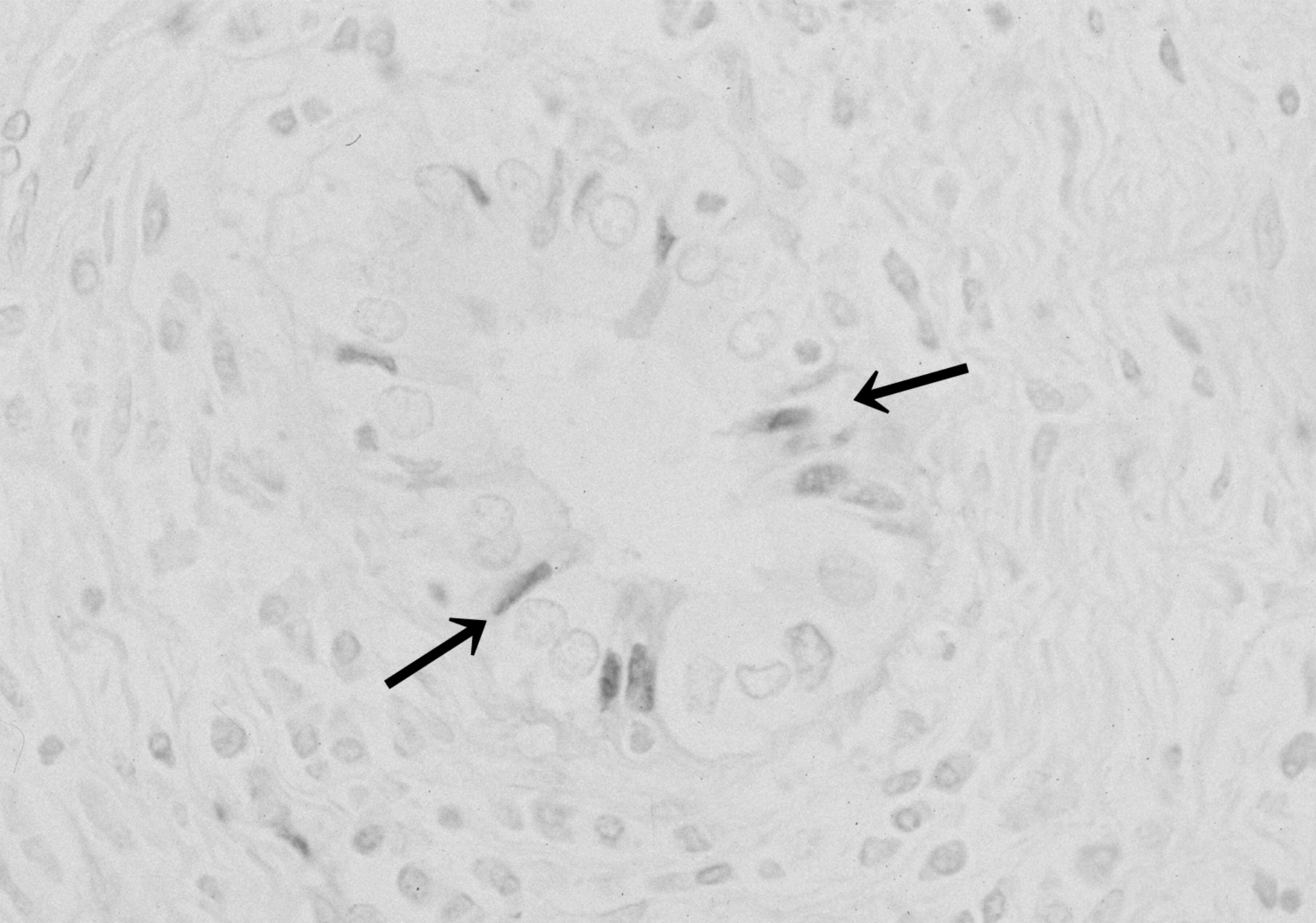

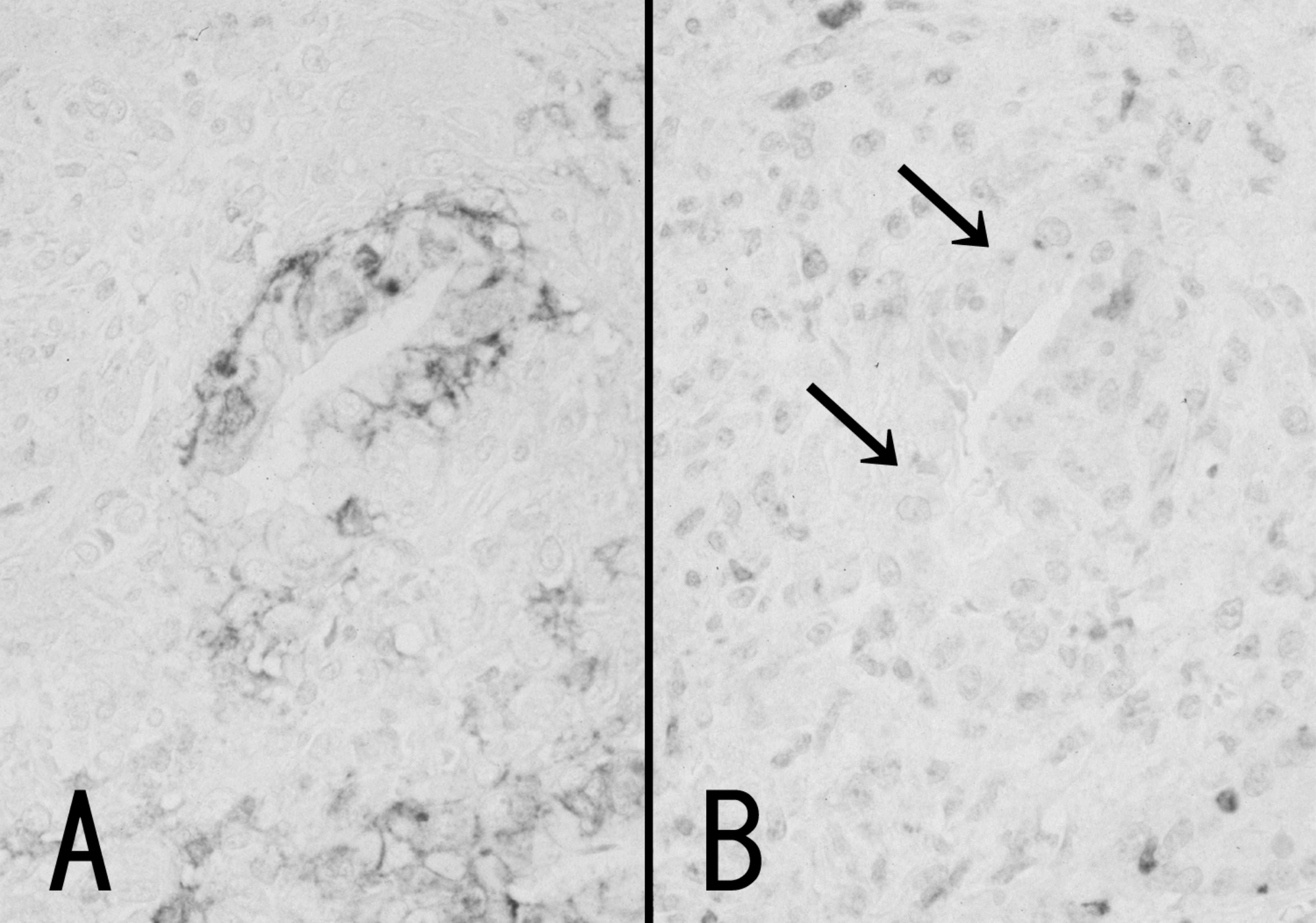
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