—**Reports on Experiments and Clinical Cases**—

Triple-Staining to Identify Apoptosis of Hepatic Cells *In Situ*

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Abstract

To identify apoptosis of nonparenchymal cells in fibrotic livers, we established a triple staining method which combined immunohistochemistry for cell markers and Masson staining for collagen as well as terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL). Five μm formalin fixed, paraffin-embedded liver sections were prepared for staining. Firstly, TUNEL staining was carried out to detect apoptosis of liver cells. Then, the sections were subjected to immunohistochemistry for a-smooth muscle actin (α-SMA) or KP-1 to identify hepatic stellate cells or Kupffer cells. Finally, Masson staining was performed to show the relationship between apoptosis and collagens. In addition, we optimized different conditions of fixation, digestion and color development which may affect the results. (J Nippon Med Sch 2000; 67: 280—283)

Key words: apoptosis, TUNEL, hepatocyte, hepatic stellate cell, Kupffer cell

Introduction

The importance of cell apoptosis in the physiology and pathophysiology of the liver is increasingly recognized[1,2]. Although apoptosis of cells can be identified *in situ*[3], it is difficult to identify the cell types of apoptotic cells in liver tissue, especially nonparenchymal cells in hepatic sinusoid, such as Kupffer cells and hepatic stellate cells (HSC).

In order to detect the apoptosis of different types of cells in paraffin-embedded sections of liver tissue, we have combined immunohistochemistry and modified Masson staining to show cell identity and fibrogenesis in the liver, as well as terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) staining.

Moreover, different conditions of fixation, digestion and staining procedures were used to validate and optimize this method.

Materials and Methods

(1) *Samples*

Twenty liver biopsy samples were taken from patients with chronic hepatitis B and 2 autopsy samples with chronic alcoholic liver disease.

Six rat liver samples were taken from 4 rats with common bile duct occlusion for 28 days and 2 normal rats.

5 μm thick formalin fixed, paraffin-embedded, liver sections were prepared following routine procedure.

Samples were used after the receiving of informed consent for research.

(2) *Methods*

1) Optimization of conditions of fixation, digestion and staining

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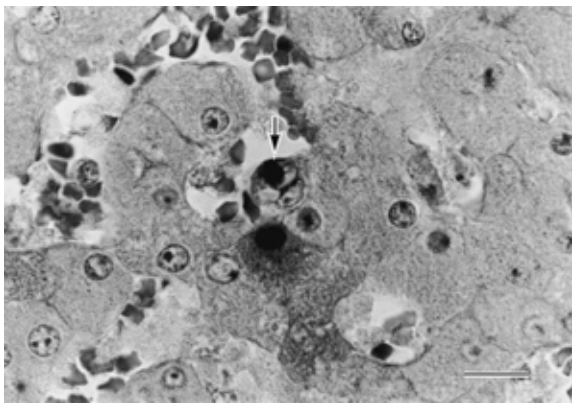

Fig. 1 The apoptotic cells of hepatocytes (arrow) are phagocytized by Kupffer cell in a hepatic sinusoid. TUNEL+Masson ×800

i) Different method of fixation: *In vivo* perfusion fixation was performed after anesthesia of normal rats. The rat chest was opened and 10% buffered formalin was perfused from the rat left ventricle for 30 minutes, Liver tissue was then taken and fixed at room temperature. *In vitro* fixation was performed directly without any *in vivo* perfusion by different fixation solutions: 1% buffered formalin, 10% buffered formalin, formaldehyde, AF fixation solution at room temperature, 10% buffered formalin at 100℃ after heating for 2 minutes by microwave. In addition, a tissue was also embedded without any fixation at room temperature overnight of postmortem autolysis.

ii) Different concentration of proteinase K for digestion: Proteinase K (0 μg/m*l*, 10 μg/m*l*, 20 μg/m*l*, 40 μg/m*l*) were used to digest protein for 30 minutes at 37℃.

iii) Different color development methods: For TUNEL staining, a second antibody was AP-labeled and detected by NBT/BCIP. For immunohistochemistry, a second antibody was POD-labeled and detected by diaminobenzidine (DAB). We also tried the color development the other way around.

2) Triple staining of TUNEL, immunohistochemistry (α-SMA or KP-1) and Modified Masson staining

i) Firstly, we carried out TUNEL staining to detect apoptotic cells: The sections were deparaffinized with xylene and subjected to 3% H_2O_2 for 15 minutes to inhibit endogenous peroxidase and then washed in phosphate buffer solution (PBS). TUNEL staining (TUNEL Kit, Boehringer Mannheim) was carried out following the manufacturer's instructions with some minor modifications: Proteinase K 20 μg/m*l* was digested for 30 minutes at 37℃ and then incubated with TdT and dUTP (TUNEL reaction mixture solution) for 60 minutes at 37℃, whereafter, it was incubated with peroxidase-conjugated solution (converter-POD) for 30 minutes at 37℃ with three washes in PBS between each incubation. Finally, freshly prepared diaminobenzidine (DAB) was put onto slides to display the result.

ii) Then, we carried out immunohistochemistry to identify HSCs or Kupffer cells: The sections were washed in PBS and incubated in 10% bovine serum for 10 minutes at room temperature, and immunohistochemistry was began sequentially. Specimens were incubated with either polyclonal anti-α-smooth muscle actin (α-SMA) or KP-1 antibodies (Dako company, both diluted 1: 50 in PBS) for 60 minutes at room temperature and biotin-conjugated second antibody (Ready-to-use) was applied for 15 minutes at room temperature. It was then, incubated with streptavidin-alkaline phosphatase solution for 15 minutes with three washes of PBS between each step. NBT/BCIP was used to display the result.

iii) Finally, we carried out Modified Masson stain[4]

Results

1. Apoptotic cells in the liver were indentifiable by triple staining

Triple staining detected the apoptotic cells in the liver tissue. TUNEL staining helped us to show cell apoptosis, and immunochemistry identified the cell type. Masson staining revealed the structure of hepatic sinusoid and collagens.

On the sections from patients with chronic liver diseases, mild necroinflammation and fibrosis were seen. There were several apoptotic hepatocytes (**Fig 1**). Hepatic stellate cells (HSCs) proliferated and were activated in hepatic sinusoid and the collagens were deposited around them. However, TUNEL positive HSCs (**Fig 2**) were hard to find. Similarly, Kupffer cells proliferated and were activated with enlarged cytoplasms and some of them were enclosed in TUNEL positive bodies (**Fig 3**).

According to common bile duct occlusion small bile

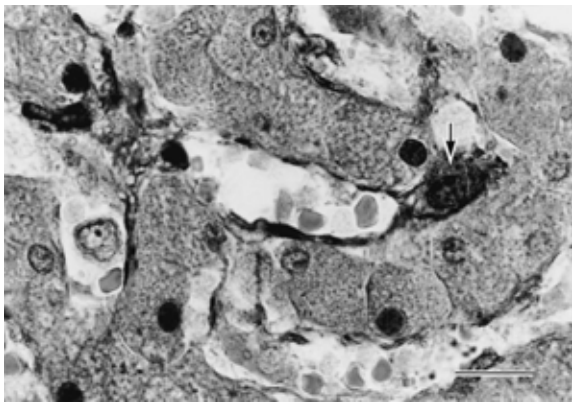

Fig. 2 In hepatic stellate cells, the nuclei are condensed and stained with dark blue (arrow) and the cytoplasm is protuberant through the wall of hepatic sinusoid with fibrosis. TUNEL (NBT/BCIP) + α-SMA (DAB) + Masson stain ×1000

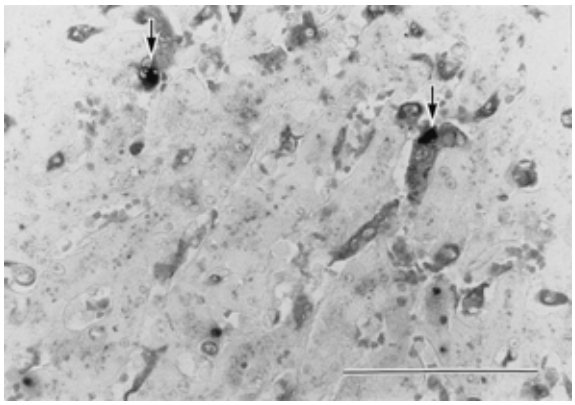

Fig. 3 Proliferated Kupffer cells and enclosed phagocytotic TUNEL positive bodies showing apoptosis (arrow) are seen. TUNEL+KP-1 ×400

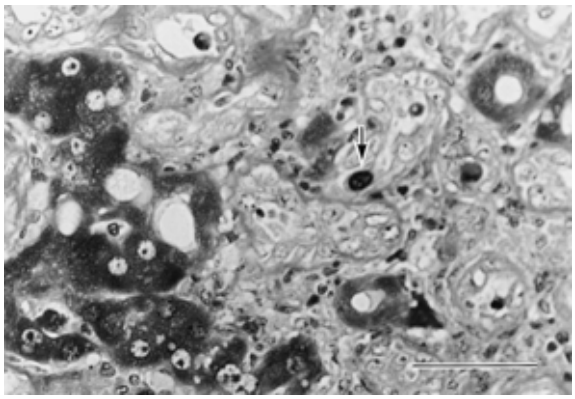

Fig. 4 The apoptosis of epithelial cells (arrow) is seen in the proliferated small bile ducts. TUNEL+Masson ×400

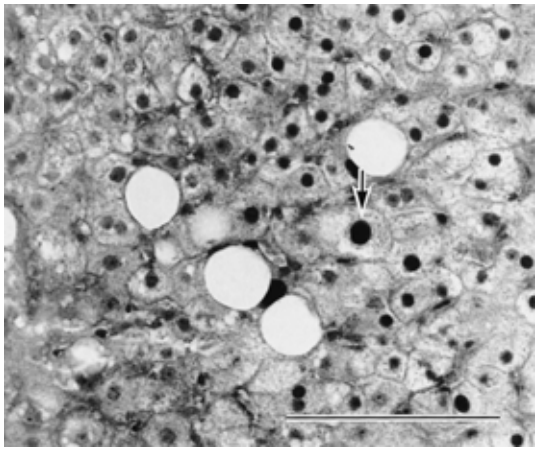

Fig. 5 Liver biopsy is fixed by low concentrated fixation and many false positive nuclei (arrow) are seen. TUNEL+Masson ×100
Abbreviations: α-SMA: α-smooth muscle actin; DAB: diaminobenzidine; TUNEL: terminal deoxynucleotidyl transferase UTP nick end labeling

ducts were proliferated and their epithelial cells were cubec shaped with big nuclei. An TUNEL positive cells were noted in 1 to 3 epithelial cells and their normal constructions disappeared with cytoplasmic change. Moreover, some apoptotic bodies could be seen to secrete into the lumen of small bile ducts (**Fig 4**).

2. Effects of different conditions on fixation, digestion and staining

i) 10% buffered formalin perfusion or fixing method were best to show apoptosis of liver cells: The sections, fixed by 10% buffered formalin perfusion and fixation, were best to identify the apoptotic cells in the liver, and nonspecific staining was not seen. In 1% buffered formalin and formaldehyde fixed sections, although the background was clear at the edge of the liver tissue, many false positive nuclei were found in clusters in the center. The cells of microwave and AF solution fixed sections were stained both in the nuclei and cytoplasm. Similarly, the cells of the autolytic tissue were all stained with damaged cell structure. These positive cells were different from the apoptotic cells in the morphologic changes. It should be mentioned that these were thought to be false positive cells (**Fig 5**).

ii) No different results were seen in different methods of digestion: TUNEL positive cells were clearly

seen with different concentrations of proteinase K, even in the sections without any digestion.

iii) Different cell types were be detected clearly by DAB: DAB and NBT/BCIP were both good to show a TUNEL positive nucleus, and the nuclei were brown red with DAB and dark blue with NBT/BCIP on a cut edge. However, NBT/BCIP was weak to show α-SMA and KP-1 by immunohistochemistry on the cloud edge. As a result, we chose NBT/BCIP to identify an apoptotic cellular nucleus and DAB to show the different cell type in hepatic sinusoid by immunohistochemistry.

Discussion

Although nonparenchymal cells represent up to one third of all hepatic cells, their specific roles of apoptosis in various liver diseases are important[5,6]. It has been realized that activated HSCs are a major cellular source of liver fibrosis. From this respect, the established triple staining method, which can clearly identify fibrosis and apoptosis of different cell types on one section of liver tissue *in situ*, might help us advance our understanding of various pathologic changes in liver diseases, such as chronic hepatitis and fibrosis.

It has been reported that necrotis and autolystic cells also show positive reactions in nuclei by TUNEL staining because of their broken DNA strands[7,8]. In order to get clear and reliable histological results, we should optimize the conditions to prevent false positive results. Our studies indicate that good results depend on appropriate fixation and pretreatment[9-11]. In addition, the morphologic features of apoptotic cells such as chromatin condensation, blebbing and fragmentation and loss of contact with their neighboring cells, may help to confirm the phenomena of apoptosis and distinguish them from necrotic cells.

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