Short Communication

Immunohistochemical Staining Methods for Connexin32 on Formalin-Fixed Paraffin-Embedded Sections

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Abstract: Connexin32 (Cx32), one of the components of gap junction intercellular communication, is commonly detected by the fluorescent antibody method, although this method has some defects when conducting detailed histopathological investigations. In order to develop a new method to improve the fluorescent antibody method, the optimal immunohistochemical procedure on formalin-fixed paraffin-embedded sections was examined. According to the results, the localization of Cx32 was observed on sections prepared with the Dako catalyzed signal amplification (CSA) method after 0.2% protease digestion for 30 minutes after a 3-day or 6-month fixation. As the CSA method is highly sensitive and based on signal amplification by biotinylated tyramide, the localization of Cx32 was clearly shown on the cell membranes of hepatocytes. In fact, by this CSA method, a decrease in the signals of Cx32 was shown in the liver of rats treated with phenobarbital or in hepatocellular adenoma, which were consistent with previous reports using cryosections. In conclusion, the CSA method is considered an appropriate method for the detection of Cx32 in formalin-fixed paraffin-embedded sections. (J Toxicol Pathol 2006; 19: 151–154)

Key words: connexin32, immunohistochemistry, biotinylated tyramide, catalyzed signal amplification method, gap junction, liver

Gap junctional intercellular communication (GJIC), through which low molecular weight molecules or inorganic ions pass from cell to cell^{1,2}, plays an important role in controlling cell homeostasis^{2–4}. Recently, it has been reported that inhibition of GJIC is deeply involved in carcinogenesis. For example, connexin32 (Cx32) is a major component protein of gap junctions in the liver and Cx32 deficient mice exhibited higher susceptibility to hepatic tumors than normal mice^{5,6}. Furthermore, tumor cells transfected with connexin genes often recovered control of normal cell growth^{7,8}. However, as the biological function of GJIC in pathological processes is not fully understood, more studies are necessary to clarify these functions. Immunohistochemistry (IHC) is an appropriate method to examine the expression of Cx32. Previously, many studies have adopted the fluorescent antibody method for cryosections^{9–12}, but this method has some limitations. For example, it is difficult to detect histological findings using a dark-field microscope, and fluorescence intensity easily fades and cannot be preserved for a long time. Considering

the applicability of this method for routine toxicity studies, these limitations render the fluorescent antibody method inappropriate, since formalin-fixed paraffin-embedded sections are usually made in these studies. On the other hand, the immunoenzymatic method on cryo- or paraffin-sections overcomes these limitations, although special fixation and a precise process to sensitize the signal are needed^{13–16}.

Recently, the catalyzed signal amplification system (CSA, DakoCytomation), or tyramide signal amplification system, has been used to amplify signal detection by IHC¹⁷. Based on the peroxidase catalyzed deposition of a biotinylated tyramide, followed by a secondary reaction with streptavidin peroxidase, even a faint signal can be clearly detected by the CSA method¹⁸.

We investigated two sensitive immunoenzymatic methods, the CSA method and the commonly used labeled streptavidin biotin (LSAB, DakoCytomation) method to establish a stable method to detect Cx32 on formalin-fixed paraffin-embedded sections.

The animals used were male F344/DuCrj rats (Charles River Laboratories Japan Inc.) at 7 weeks of age. A total of 13 rats were individually housed in a taper-type bracket cage in a barrier-sustained room controlled at a temperature of 23 ± 1°C, 55 ± 5% relative humidity, an illumination time of 12 hours/day at an intensity of about 200 lucas, and 10 to 15 ventilation cycles per hour. The animals were fed *ad libitum*

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Fig. 1. Microscopic findings of the signals of Cx32 (arrow) on the cell membranes of hepatocytes in sections after 3 days fixation. Hematoxylin counter staining, $\times 200$. (a) The signals were clearly detected using the CSA method. (b) The signals were not clearly detected using the LSAB method.

Fig. 2. Microscopic findings of the signals of Cx32 in the liver. The CSA method. Hematoxylin counter staining. (a) The signals appeared diffusely in the livers of the control rats, $\times 100$. (b) Decreased signals in the centrilobular zone were detected after PB treatment, $\times 100$. C: central vein of the liver. (c) The signals disappeared in hepatocellular adenoma, $\times 200$. *: hepatocellular adenoma.

with solid feed (Certified Rodent Diet 5002: PMI Nutrition International, Inc.). Five of them were provided with 0.1% PB, which affects the expression of Cx32 in the liver⁹, and 8 of them as control animals were provided with tap water as drinking water for 6 weeks. At the end of the treatment period, all the rats were euthanized by exsanguination under ether anesthesia. The livers of the rats were fixed in phosphate-buffered 10% formalin solution for 3 days (5 control animals and 5 phenobarbital (PB) treated animals) or 6 months (3 control animals), dehydrated, and then embedded in paraffin (melting point 57–60°C). Three-micrometer sections were deparaffinized for use in the subsequent process. In addition, sections of 4 rats' livers, including a hepatocellular adenoma, were made from formalin-fixed (6 months) paraffin-embedded (melting point 57–60°C) blocks which had been made in a previous carcinogenesis study. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Sankyo Co., Ltd.

The procedure for IHC: protease-digestion was performed in 0.2% protease (Sigma-Aldrich Japan) in PBS buffer (0.05 M, pH 7.6) for 30 minutes at room temperature. Anti-rat Cx32 mouse monoclonal antibody (Chemicon International Inc.) at a dilution of 1:1000 was used as the primary antibody. According to manufacturer's instructions, either the LSAB method or the CSA method were utilized. Thereafter, 3,3'-diaminobenzidine tetrahydrochloride was used for visualization.

In the CSA method under this condition, diffuse signals of Cx32 were clearly observed on the cell membranes of the hepatocytes in the sections after 3 days fixation (Fig. 1a), while the signals were obscure using the LSAB method (Fig. 1b). We tried the CSA method without protease digestion or with a microwave process for antigen retrieval, but clear Cx32 signals could not be obtained. In the tissues after 6 months fixation or those from the previous carcinogenicity study, clear signals were still detected by the CSA method in the hepatocytes near the serosa, although the signals of Cx32 tended to decrease around the center of the tissues.

In comparison to non-treated rats (Fig. 2a), a decrease of Cx32 signals was detectable in the centrilobular zone after PB administration (Fig. 2b) and in the hepatocellular adenoma (Fig. 2c) using the CSA method. These changes were consistent with previous reports using cryosections^{9,14,16,19}.

Utilizing the present method on paraffin-embedded sections offers some advantages when compared to the fluorescent antibody method. Previously-made paraffin-blocks can be used, and both the signals and histological structure can be investigated much more clearly using a light microscope than using a dark-field microscope. These advantages would be useful when investigating the relationship between Cx32 expression and pathogenesis or carcinogenesis.

In conclusion, with the CSA method, the signals of Cx32 were easily detectable in formalin-fixed paraffin-embedded sections. We believe that our procedure can

contribute to retrospective studies of Cx32 expression as well as prospective studies.

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