**NOTE** *Laboratory Animal Science*

# Localization of connexin 32 in spontaneous liver lesions of mice

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(Received 26 August 2014/Accepted 28 July 2015/Published online in J-STAGE 11 August 2015)

ABSTRACT. We examined the localization of connexin 32 (Cx32), a component of gap junctions, in 24-month-old male B6C3F1 mice with spontaneously occurring hepatocellular altered foci or tumors. Immunohistochemically, Cx32-staining intensity in cell-to-cell membranes of altered hepatocytes was decreased in eosinophilic foci and increased in basophilic foci as compared to those in intact hepatocytes. These alterations were enhanced in adenomas and carcinomas with both eosinophilic and basophilic cytoplasm. In cell membranes facing on the sinusoidal portions, the intensities increased in all lesions. Image analyses confirmed that the spot areas of Cx32 were decreased in eosinophilic foci, but increased in basophilic foci, adenomas and carcinomas. These results demonstrate that Cx32 shows different expression in different types of hepatic lesions.

KEY WORDS: connexin, immunohistochemistry, liver lesion, mouse

doi: 10.1292/jvms.14-0437; *J. Vet. Med. Sci.* 78(1): 113–116, 2016

A gap junction on the plasma membrane in liver cells is a channel connecting adjacent cells and is comprised of connexons which are hexamers of connexins (Cxs) [15, 18]. The Cxs are known to play a crucial role in cell-to-cell communications (gap junctional intercellular communication: GJIC) by transportation of small molecules including inorganic ions and low-molecular-weight metabolites of less than 1–2 kDa [6, 11, 13]. In addition, GJIC is recognized to control cell growth, differentiation and tumor formation. The transfusion of activated oncogenes into cells inhibits GJIC, whereas overexpression of Cxs or incubation of cells with GJIC stimulating compounds inhibits tumorigenicity of certain tumor-derived cell lines [7, 16]. Among the Cxs, Cx32 contributes to tissue homeostasis, and suppression of tumor promotion and progression in the liver [2, 3, 8]. Immunohistochemically, Cx32 was found to be down-regulated, inactivated or incorrectly localized in hepatic tumors of rodents. To the best of our knowledge, however, few reports are available on Cx32 in hepatic lesions, such as eosinophilic or basophilic foci, adenoma or carcinoma. In the current study, we examined the location pattern of Cx32 in altered and neoplastic lesions in the liver of 24-month-old male B6C3F1 mice. We selected this strain, because they are reported to develop several types of hepatocellular foci or neoplasms with aging at a high frequency [12, 14].

Four-week-old male B6C3F1 mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). Animals were individually housed until 24 months of age in suspended stainless wire-mesh cages in a barrier-sustained room controlled at a temperature of 23 ± 2°C, relative humidity of 55 ± 10%, illumination time of 13 hr/day at an intensity of about 200 luces and ventilation at 10–15 cycles/hr. Basal diet (NMF: Oriental Yeast Co., Ltd., Tokyo, Japan) and fresh tap water were given *ad libitum*. At termination, all mice were euthanized by exsanguination under ether anesthesia. All experimental procedures were performed in accordance with the Guidelines for Animal Experimentation issued by the Japanese Association for Laboratory Animal Science [5]. The experimental protocol was approved by the Animal Experimental Committee of Daiichi Sankyo Co., Ltd. (Tokyo, Japan).

For histopathology, the livers were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 3 $\mu$m in thickness, stained with hematoxylin-eosin (H&E) and examined with a light microscope. The histological evaluation was performed by a pathologist to identify specific structures, namely, altered hepatocellular foci (clear cell, eosinophilic and basophilic foci) or neoplasms (hepatocellular adenoma and carcinoma). In addition, hepatocellular adenomas and carcinomas were subclassified as those with eosinophilic cytoplasm (large cytoplasm and eosinophilic staining) or basophilic cytoplasm (small cytoplasm and basophilic staining).

For Cx32 immunohistochemistry, 3 $\mu$m sections were prepared from paraffin blocks and stained by the catalyzed signal amplification method according to a previous report [4]. Rabbit anti-rat Cx32 antibody (Zymed Laboratories Inc., South San Francisco, CA, U.S.A., 1:2,000 dilution) was used as the primary antibody, and biotin-labeled goat anti-rabbit immunoglobulin (Dako Cytomation, Kyoto, Japan, 1:400 dilution) was utilized as the linking antibody. The immunostaining was conducted by an automated machine (Ventana XT, Roche Diagnostics, Tokyo, Japan) to standard-

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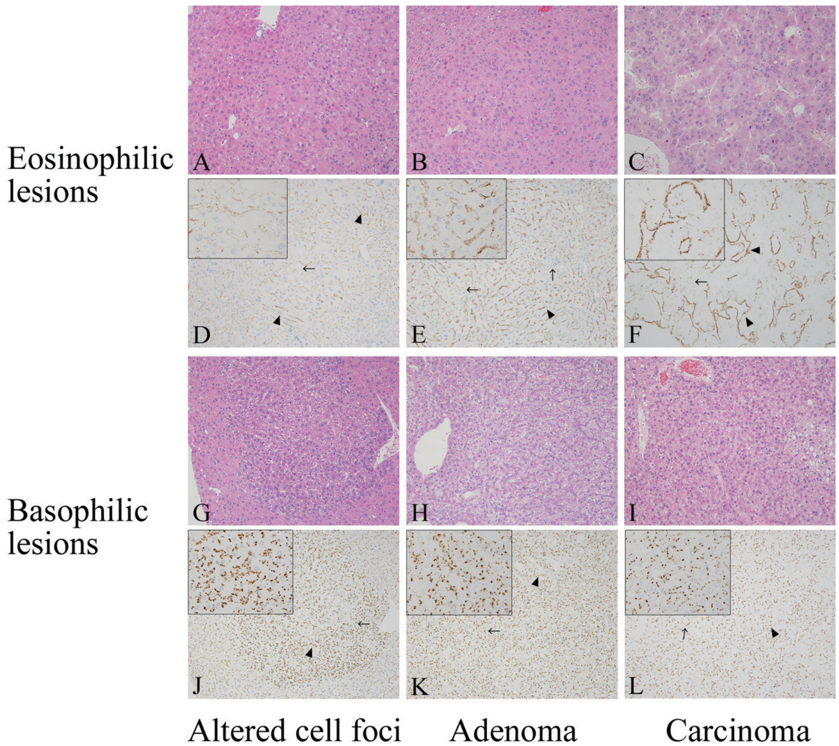


Fig. 1. Morphological and Cx32-immunohistochemical appearance in eosinophilic (A and D) and basophilic (G and J) foci of altered cells, eosinophilic (B and E) and basophilic (H and K) cytoplasm of adenomas and eosinophilic (C and F) and basophilic (I and L) cytoplasm of hepatic carcinomas in 24-month-old male B6C3F1 mice. Immunohistochemically, reduced Cx32 positive spots at the cell-to-cell membrane of the hepatocytes (arrows) and strong positive staining in the cell membrane facing the sinusoidal space (arrow heads) were noted in eosinophilic lesions. Increased number and size of Cx32 positive spots were also observed in the cell membrane of basophilic lesions (arrows, cell-to-cell membrane; arrowheads, sinusoidal space). A, B, C, G, H and I show H&E stain; D, E, F, J, K and L show Cx32 stain. Original magnification: 120 ×.

ize the staining conditions. The scoring of immunostaining intensity was as follows: –, negative; +, weak; 2+, moderate; 3+, severe; and 4+, very severe. Then, Cx32 positive stains were measured with an image analyzer (IPAP-WIN, Sumika Technoservice Corporation, Osaka, Japan). The areas of positive spots ($\mu m^2$) and the number of nuclei of hepatocytes were measured in non-lesions (intact area of the same sections) or lesion area, and the total spot area per hepatocyte ($\mu m^2$/cell) was also calculated.

Quantitative data are expressed as the group mean and standard deviation (SD), and were statistically analyzed between the intact and lesion areas by Student's *t*-test. A *P* value of less than 5% was considered statistically significant.

Representative morphological lesions, such as eosinophilic and basophilic foci, and hepatocellular adenomas and carcinomas, are shown in the figure (Fig. 1A, 1B, 1C, 1G, 1H and 1I). Immunohistochemically, the Cx32-staining intensity in cell-to-cell membranes of altered hepatocytes decreased in clear cell foci and eosinophilic foci, and increased in basophilic foci, compared to that in intact cells (Fig. 1D and 1J). In the cell membranes facing the sinusoidal space, increases in intensity were observed histopathologically in both eosinophilic and basophilic foci with increased number and size of positive spots. These Cx32-staining intensities were enhanced in hepatic adenomas and carcinomas (Fig. 1E, 1F, 1K, 1L and Table 1). The staining pattern of Cx32 was different between the eosinophilic type (eosinophilic foci, adenoma and carcinoma with eosinophilic cytoplasm) and basophilic type (basophilic foci, adenoma and carcinoma with basophilic cytoplasm), implying that tumors with eosinophilic and basophilic cytoplasm developed from eosinophilic and basophilic foci, respectively. According to previous reports [9, 10], Cx32 expression was observed in hepatocellular carcinoma induced by diethylnitrosamine (sinusoidal localization) or carbontetrachloride (large spot), although they did not examine the relationships between the type of Cx32 expression and morphological change in the hepatocytes. In intact hepatocytes, Cx32 expression was found in the cell membrane, suggesting that it played an important role in GJIC [17]. In this study, decrease in the Cx32-staining intensity in cell-to-cell membranes or increased size of positive spots suggesting aggregation of Cx32 was observed. Therefore, it was considered that normal GJIC function may not be maintained in hepatocyte lesions.

Table 1. Distribution of Cx32 positive spots of hepatic lesions in male B6C3F1 mice

| Pathological findings | | n | Cell-to-cell membrane | | | | | Sinusoidal space[b)] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | – | + | 2+ | 3+ | 4+ | – | + | 2+ | 3+ | 4+ |
| Intact areas | | 44 | | | 44[a)] | | | | 44 | | | |
| Altered cell | Clear cell foci | 4 | | 4 | | | | | 4 | | | |
| | Eosinophilic foci | 4 | | 4 | | | | | | 4 | | |
| | Basophilic foci | 4 | | | | 4 | | | | 4 | | |
| Adenoma | Eosinophilic cytoplasm | 8 | 3 | 5 | | | | | | | 8 | |
| | Basophilic cytoplasm | 8 | | | | 8 | | | | | 8 | |
| Carcinoma | Eosinophilic cytoplasm | 8 | 8 | | | | | | | | | 8 |
| | Basophilic cytoplasm | 8 | | | | 8 | | | | | 8 | |

a) Number of animals showing the identical staining intensity. b) Cell membrane facing on the sinusoidal portions. Blank indicates zero. Staining intensity: –, negative; +, weak; 2+, moderate; 3+, severe; and 4+, very severe.

Table 2. Image analysis for Cx32 stainings in hepatic lesions in male B6C3F1 mice

| Pathological findings | | n | Total spot area/hepatocyte ($\mu m^2$/cell) | |
|---|---|---|---|---|
| | | | Intact area | Lesion area |
| Altered cell | Clear cell foci | 4 | 25.2±7.8 | 29.7±7.1 |
| | Eosinophilic foci | 4 | 14.8±2.7 | 7.4±5.7* |
| | Basophilic foci | 4 | 22.2±8.3 | 34.0±11.0* |
| Adenoma | Eosinophilic cytoplasm | 8 | 20.1±8.4 | 31.6±16.2* |
| | Basophilic cytoplasm | 8 | 23.3±9.3 | 42.1±21.2* |
| Carcinoma | Eosinophilic cytoplasm | 8 | 21.2±9.7 | 70.9±90.6* |
| | Basophilic cytoplasm | 8 | 17.8±6.9 | 39.9±23.2* |

Significant difference from non-tumor areas. *$P<0.05$ by the Student's *t*-test. Each value represents the mean ± SD of the respective groups.

By image analyses (Table 2), no changes in spot areas with Cx32 stains were noted in clear cell foci, compared to those in the intact area. Meanwhile, significant decreases in spot area in eosinophilic foci and increases in spot area in basophilic foci were seen. The discrepancy between the immunohistochemical findings and image analyses in clear cell foci may be partially due to large variations among the lesions. In tumor lesions, increases in spot areas were observed in adenoma and carcinoma with eosinophilic cytoplasm. Likewise, increases in the spot areas were noted in adenoma and carcinoma with basophilic cytoplasm. As for the difference in spot areas between eosinophilic foci and tumors with eosinophilic cytoplasm, the possibility is raised that the imaging analysis may have low sensitivity in the fine structure because imaging analysis does not differentiate the Cx32 localization between sinusoidal area and cell-to-cell membrane. It is considered that decreased spot areas observed in the eosinophilic foci mainly reflect decreased expression of Cx32 in the cell-to-cell membranes, and increased spot areas observed in adenoma or carcinoma reflect increased expression in the sinusoidal spaces. The reasons for the difference in Cx32 localization between the eosinophilic and basophilic lesions remain unknown. Generally, eosinophilic cytoplasm contains lots of peroxisomes or smooth endoplasmic reticulum, and basophilic cytoplasm includes mainly rough endoplasmic reticulum [1]. Taken together with our results, proliferation of subcellular organelles may relate to the deviations from the normal condition of the cell membrane construction of Cx32 and the difference in Cx32 localization pattern. Cx32 localization patterns observed in the eosinophilic and basophilic lesions did not differ as the lesions progressed, and therefore, these patterns were considered not to suggest progression of the lesions. These expression patterns may reflect the morphological characteristics of hepatocytes.

In conclusion, these results demonstrate that Cx32 shows different expression in different types of hepatic lesions.

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