Localization of connexin 32 in spontaneous liver lesions of mice

Isao ICHARASHI1)*, Toshihiko MAKINO1), Kiyonori KAI1), Munehiro TERANISHI1), Wataru TAKASAKI1), Hiroshi SATOH2) and Kazuhisa FURUHAMA2,3)

1) Medicinal Safety Research Laboratories, Daiichi Sankyo Co., Ltd., Kitakasai, Edogawa, Tokyo 134–8630, Japan
2) Cooperative Department of Veterinary Medicine, Iwate University, Morioka, Iwate 020–8550, Japan
3) United Graduate School of Veterinary Science, Gifu University, Gifu 501–1193, Japan

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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 or basophilic foci, adenoma and carcinoma. These results demonstrate that Cx32 shows different expression in different types of hepatic lesions.

KEY WORDS: connexin, immunohistochemistry, liver lesion, mouse

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 transfection 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 spontaneously occurring hepatocellular altered foci or tumors. 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 luxes 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 µm 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 µm 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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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 (µm²) 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 (µm²/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, 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 ×.

![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 ×.](image-url)

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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 may partially due to large variations among the lesions. In tumor lesions, increases in spot areas were observed in adenoma and carcinoma with basophilic cytoplasm. As for the difference in spot areas between eosinophilic foci and tumors with basophilic 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.

REFERENCES

1. Bannasch, P. 1986. Preneoplastic lesions as end points in carcinogenicity testing. I. Hepatic preneoplasia. Carcinogenesis 7: 689–695. [Medline] [CrossRef]

2. Hokaiwado, N., Asamoto, M., Futakuchi, M., Ogawa, K., Takahashi, S. and Shirai, T. 2007. Both early and late stages of hepatocarcinogenesis are enhanced in Cx32 dominant negative mutant transgenic rats with disrupted gap junctional intercellular communication. J. Membr. Biol. 218: 101–106. [Medline] [CrossRef]

3. Igarashi, I., Makino, T., Suzuki, Y., Kai, K., Teranishi, M., Takasaki, W. and Furuhama, K. 2013. Background lesions during a 24-month observation period in connexin 32-deficient mice. J. Vet. Med. Sci. 75: 207–210. [Medline] [CrossRef]
4. Igarashi, I., Shirai, M., Suzuki, Y., Atsumi, F., Sehata, S., Maejima, T., Manabe, S. and Teranishi, M. 2006. Immunohistochemical staining methods for connexin32 on formalin-fixed paraffin-embedded sections. J. Toxicol. Pathol. 19: 151–154. [CrossRef]
5. Japanese Association for Laboratory Animal Science 1987. Guidelines for animal experimentation. Exp. Anim. 3: 285–288.
6. Kumar, N. M. and Gilula, N. B. 1996. The gap junction communication channel. Cell 84: 381–388. [Medline] [CrossRef]
7. Mesnil, M. 2002. Connexins and cancer. Biol. Cell 94: 493–500. [Medline] [CrossRef]
8. Mesnil, M., Crespin, S., Avanzo, J. L. and Zaidan-Dagli, M. L. 2005. Defective gap junctional intercellular communication in the carcinogenic process. Biochim. Biophys. Acta 1719: 125–145. [Medline] [CrossRef]
9. Nakata, Y., Iwai, M., Kimura, S. and Shimazu, T. 1996. Prolonged decrease in hepatic connexin32 in chronic liver injury induced by carbon tetrachloride in rats. J. Hepatol. 25: 529–537. [Medline] [CrossRef]
10. Neveu, M. J., Hully, J. R., Babcock, K. L., Hertzberg, E. L., Nicholson, B. J., Paul, D. L. and Pitot, H. C. 1994. Multiple mechanisms are responsible for altered expression of gap junction genes during oncogenesis in rat liver. J. Cell Sci. 107: 83–95. [Medline]
11. Nielsen, M. S., Axelsen, N. L., Sorgen, P. L., Verma, V., Delmar, M. and Holstein-Rathlou, N. H. 2012. Gap Junctions. Compr. Physiol. 2: 1981–2035. [Medline]
12. Reynolds, S. H., Stowers, S. J., Maronpot, R. R., Anderson, M. W. and Aaronson, S. A. 1986. Detection and identification of activated oncogenes in spontaneously occurring benign and malignant hepatocellular tumors of the B6C3F1 mouse. Proc. Natl. Acad. Sci. U.S.A. 83: 33–37. [Medline] [CrossRef]
13. Sáez, J. C., Connor, J. A., Spray, D. C. and Bennett, M. V. 1989. Hepatocyte gap junctions are permeable to the second messenger, inositol 1, 4, 5-trisphosphate, and to calcium ions. Proc. Natl. Acad. Sci. U.S.A. 86: 2708–2712. [Medline] [CrossRef]
14. Takahashi, M., Dinse, G. E., Foley, J. F., Hardisty, J. F. and Maronpot, R. R. 2002. Comparative prevalence, multiplicity, and progression of spontaneous and vinyl carbamate-induced liver lesions in five strains of male mice. Toxicol. Pathol. 30: 599–605. [Medline] [CrossRef]
15. Trosko, J. E. and Ruch, R. J. 1998. Cell-cell communication in carcinogenesis. Front. Biosci. 3: 208–236. [Medline]
16. Trosko, J. E. and Ruch, R. J. 2002. Gap junctions as targets for cancer chemoprevention and chemotherapy. Curr. Drug Targets 3: 465–482. [Medline] [CrossRef]
17. Vinken, M., Vanhaecke, T., Papeleu, P., Snykers, S., Henkens, T. and Rogiers, V. 2006. Connexins and their channels in cell growth and cell death. Cell. Signal. 18: 592–600. [Medline] [CrossRef]
18. Yamasaki, H. 1996. Role of disrupted gap junctional intercellular communication in detection and characterization of carcinogens. Mutat. Res. 365: 91–105. [Medline] [CrossRef]