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Expression of neural cell adhesion molecule (N-CAM) in perisinusoidal stellate cells of the human liver

Kazuki Nakatani[1], Shuichi Seki[2], Norifumi Kawada[2], Kenzo Kobayashi[2], Kenji Kaneda[1]

[1] Second Department of Anatomy, Osaka City University Medical School, 1–4–54 Asahimachi, Abeno-ku, Osaka, 545 Japan
[2] Third Department of Internal Medicine, Osaka City University Medical School, 1–5–7 Asahimachi, Abeno-ku, Osaka, 545 Japan

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Abstract. Neural cell adhesion molecule (N-CAM) is distributed in most nerve cells and some non-neural tissues. The present immunohistochemical study has revealed, for the first time, the expression of N-CAM in perisinusoidal stellate cells of the human liver. Liver specimens were stained with monoclonal antibody against human Leu19 (N-CAM) by a streptoavidin-biotin-peroxidase-complex method. Light- and electron-microscopic analyses have shown that N-CAM-positive nerve fibers are distributed in the periportal and intermediate zones of the liver lobule. Perisinusoidal stellate cells in these zones are also positive for N-CAM. N-CAM is expressed on the surface of the cell, including cytoplasmic projections. Close contact of N-CAM-positive nerve endings with N-CAM-positive stellate cells has been observed. On the other hand, stellate cells in the centrilobular zone exhibit weak or no reaction for N-CAM. Perivascular smooth muscle cells and fibroblasts in the portal area and myofibroblasts around the central veins are negative for N-CAM. The present results indicate that the perisinusoidal stellate cells in the periportal and intermediate zones of the liver lobule characteristically express N-CAM, unlike other related mesenchymal cells, and suggest that the intralobular heterogeneity of N-CAM expression by stellate cells is related to the different maturational stages of these cells.

Key words: Cell adhesion molecules, neuronal – Stellate cells – Liver – Immunohistochemistry – Human

Introduction

Neural cell adhesion molecule (N-CAM) is a membrane glycoprotein that belongs to the immunoglobulin superfamily. It mediates calcium-independent cell-to-cell adhesion and binds cells together by a homophilic interaction (Rao et al. 1993).

N-CAM was first reported as a cell surface component with an important role in neuronal aggregation in the embryo (Brackenbury et al. 1977). In the adult, it is distributed in most nerve cells and glial cells (Nybroe et al. 1988; Goridis and Brunet 1992).

Non-neural tissues also have N-CAM. For example, skeletal muscle cells express N-CAM, which plays an important role for the proper attachment of axons to skeletal muscle cells during synaptogenesis (Covault and Sanes 1986; Tosney et al. 1986). Once synapses between nerves and muscle cells are established, N-CAM expression of muscle cells disappears except at the site of neuro-muscular junction (Covault and Sanes 1986). N-CAM expression in non-neural tissues is however not always related to innervation. Skeletal muscle cells re-express N-CAM by atrophic changes or regeneration (Miettinen and Cupo 1993). With regard to smooth muscle cells, N-CAM is distributed in gastrointestinal and uterine smooth muscle cells in the adult, whereas it is absent in vascular smooth muscle cells (Miettinen and Cupo 1993). Furthermore, natural killer (NK) cells have a surface marker CD56 (Leu19, NKH1a) that is identical to N-CAM (Lanier et al. 1991). N-CAM expression by Leydig cells has also been reported (Mayerhofer et al. 1992; Davidoff et al. 1993).

In the liver, N-CAM-positive structures thus far known are nerve fibers and NK cells (Scoazec et al. 1993). Nerve fibers are distributed in the portal area and in the liver lobule, making synapses with perivascular smooth muscle cells, stellate cells, and hepatocytes (Bioulac-Sage et al. 1990). They are considered to regulate hepatic metabolism and intrahepatic blood flow. In pathological conditions, proliferating bile ductules are also reported to express N-CAM (Roskams et al. 1990; Volpes et al. 1993).

Recently, much attention has been paid to the contractility of perisinusoidal stellate cells and their regulatory function in hepatic microcirculation. Several hu-

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Correspondence to: K. Nakatani

moral factors such as endothelins have been revealed to induce the contraction of stellate cells (Kawada et al. 1992, 1993). There are however only a few reports concerning neural factors. In order to investigate the interaction between stellate cells and nerve fibers, we have performed immunohistochemical staining of the liver with N-CAM. During the course of this study, we have found, for the first time, that stellate cells also express N-CAM in the human liver.

Materials and methods

Liver specimens

Human liver specimens were obtained from 19 patients (one normal liver, four Gilbert's disease, one mild fatty liver, one recovered chronic hepatitis type C, two hepatocellular carcinoma with mild liver damage, ten with metastatic liver tumors) who underwent surgery, laparoscopy, or percutaneous needle biopsy. Informed consent was provided by the patients and their families before operation. The specimens were taken from the non-tumor portion that looked macroscopically normal. All the materials used here had normal histology in hematoxylin-eosin stained sections.

Immunohistochemistry

Fresh liver specimens were fixed in a periodate-lysine 3% paraformaldehyde (PLP) solution for 6–24 h at 4°C. They were then immersed in 0.1 M phosphate buffer (PB), pH 7.4, containing 8.5%, 15%, and 20% sucrose, successively, each for one day, embedded in OCT compound (Miles Scientific, Ill., USA), and frozen in dry ice and 99.5% ethanol. Frozen sections (5 μm thick) were cut on a cryostat (Bright Instrument Co., Huntingdon, UK) and air-dried immediately. After being washed with 0.01 M phosphate-buffered

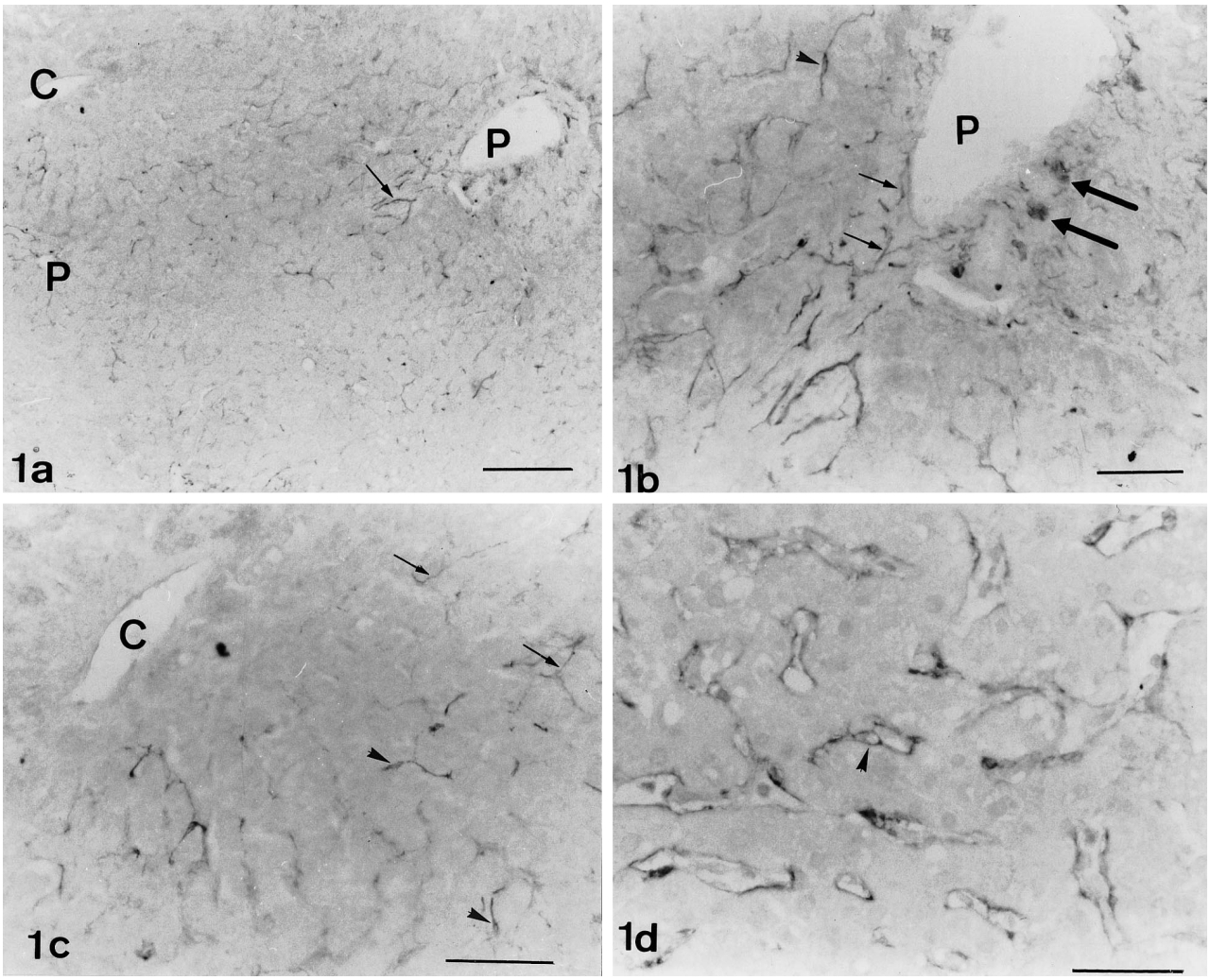


Fig. 1a–d. Light microscopy of N-CAM expression in the liver. **a** N-CAM-positive structures (*arrow*) are seen in the portal area and in the periportal and intermediate zones of the liver lobule, but are rare in the centrilobular zone. ×25. *Bar*: 0.5 mm. **b** In the portal area, thick nerve bundles (*large arrows*) are seen near the portal branches. Thin fibers (*small arrows*) are detected both around the vascular wall and within the connective tissue. Nerve fibers enter the liver lobule and run along the sinusoidal wall, often making branches. Spindle-shaped sinusoidal cells (*arrowhead*) with an obvious nucleus are also positive for N-CAM. ×60. *Bar*: 0.2 mm. **c** In the intermediate zone of the liver lobule, N-CAM-positive sinusoidal cells (*arrowheads*) and nerve fibers (*arrows*) are detected; they are absent in the centrilobular zone. ×75. *Bar*: 0.2 mm. **d** Higher magnification of N-CAM-positive sinusoidal cells (*arrowhead*) that extend several projections along the sinusoidal wall. ×150. *Bar*: 0.1 mm. *C* Central veins; *P* interlobular portal branches

saline (PBS), pH 7.4, three times for 10 min at 4°C, and being treated with 10% normal rabbit serum for 20 min at room temperature, they were incubated with 0.2% mouse monoclonal antibody against human Leu19 (Becton-Dickinson, Calif., USA) overnight at 4°C. This antibody, produced by My31 hybridoma cell lines, recognizes Leu19 antigen on the surface of human NK cells (Lanier et al. 1986). As negative controls, all specimens were incubated with 1% normal mouse serum instead of anti-Leu19 overnight at 4°C. They were rinsed with PBS twice for 5 min at 4°C, and endogenous peroxidase was then blocked by incubating the sections in methanol containing 0.03% hydrogen peroxide for 20 min at room temperature. For the following reactions, we used a Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan). After rinsing with PBS, five times for 5 min at 4°C, sections were treated with biotinylated rabbit polyclonal antibody against mouse immunoglobulins for 40 min at room temperature. They were rinsed with PBS, three times for 5 min at 4°C, and incubated with peroxidase-labeled streptoavidin for 20 min at room temperature. After being rinsed with PBS, three times for 5 min at 4°C, they were treated with 0.25 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB) in the presence of 0.003% hydrogen peroxide in 0.05 M TRIS-buffered saline, pH 7.4, for 3–5 min. They were then counterstained for nuclei with 10% methyl green.

For electron microscopy, liver specimens fixed with the PLP solution were cut into 50-μm-thick sections by a Microslicer (Dosaka, Kyoto, Japan). Sections were stained for N-CAM in a similar way to that described above, although blocking of endogenous peroxidase reaction was not perfomed. After colorization with DAB and hydrogen peroxide, sections were postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in polybed (Polyscience, Pa., USA). Ultrathin sections were stained with saturated lead citrate and observed under a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Results

Light microscopy

N-CAM-positive structures were found both in the portal area and inside the liver lobule (Fig. 1a). In the portal area, nerve fibers that run along interlobular portal veins and hepatic arteries were stained with N-CAM. Thick nerve bundles were located at the perivascular regions, whereas thin fibers were distributed throughout the connective tissue of the portal area (Fig. 1b). In the liver lobule, N-CAM-positive nerve fibers entered the liver lobule from the portal area and ran along the sinusoidal wall, often branching during their course. They did not however reach the central zone of the liver lobule.

We further observed N-CAM-positive cells, each with an elliptic nucleus, lying along the sinusoid. They extended well-developed cytoplasmic processes along the sinusoidal wall (Fig. 1b–d) and contained several small vacuoles in the cytoplasm. These morphological features were consistent with those reported for perisinusoidal stellate cells (Wake 1980). However, it was often difficult clearly to differentiate the processes of stellate cells from nerve fibers by light microscopy. Both N-CAM-positive stellate cells and N-CAM-positive nerve fibers were frequently seen in the periportal and intermediate zones of the liver lobule, but were rare in the centrilobular zone (Fig. 1a–c). No N-CAM-positive structures were present in the connective tissue around the central vein (Fig. 1c). Some lymphocytes in the portal area and in the sinusoids were stained with N-CAM.

All liver specimens from the 19 patients used in this study showed similar results, although the staining intensity of the stellate cells varied among the specimens. There were no detectable immunoreactive structures in the negative control specimens, which were treated with normal mouse serum instead of primary antibody (data not shown).

EIectron microscopy

Immuno-electron microscopic analyses have revealed that N-CAM-positive sinusoidal cells exist outside the sinusoid and contain many lipid droplets (Fig. 2a, b). Thus, they have been identified as perisinusoidal stellate cells. Immunoprecipitates are seen all along the cell surface, including the cytoplasmic projections that extend between sinusoidal endothelial cells and hepatocytes (Fig. 2a, c). Tiny projections are however difficult to differentiate from nerve fibers unless characteristic features of stellate cells, such as lipid droplets and dilated rough endoplasmic reticulum, or those of nerve fibers, such as neurofilaments, are present in the section. Kupffer cells, endothelial cells, and hepatocytes are negative for N-CAM (Fig. 2a–c), whereas large granular lymphocytes are positive (data not shown).

We have observed that N-CAM-positive nerve endings make close contact with N-CAM-positive stellate cells at the side facing the hepatocytes (Fig. 2b). This N-CAM expression is detected not only at the neuro-stellate cell junctions, but also at the other surface of the stellate cells. There are several small vesicles in the nerve endings but obvious synaptic structures have not been identified because of the immunoprecipitates. N-CAM-positive nerve endings are also closely apposed to the N-CAM-negative hepatocytes (Fig. 3). Reaction products exist on the contact surface of the hepatocytes with nerve endings, although it is difficult to determine whether these products are the result of the true reaction on the hepatocytes or merely diffusion from apposed nerve cells.

Intralobular heterogeneity of N-CAM expression by the stellate cells, as observed under a light microscope, could be confirmed ultrastructurally. We prepared thin sections from the centrilobular zone, which included the central vein, and observed weak or almost no N-CAM expression in the stellate cells in this zone (Fig. 4). On the other hand, stellate cells obtained from the periportal and intermediate zones were mostly positive (Fig. 2a–c). Myofibroblasts around the central vein were negative for N-CAM (Fig. 5).

In the portal area, several N-CAM-positive axons were embraced by N-CAM-negative Schwann cells (data not shown). Perivascular smooth muscle cells of the portal branches (Fig. 6) and fibroblasts in the portal area were negative for N-CAM. The results obtained in this study are summarized in Fig. 7.

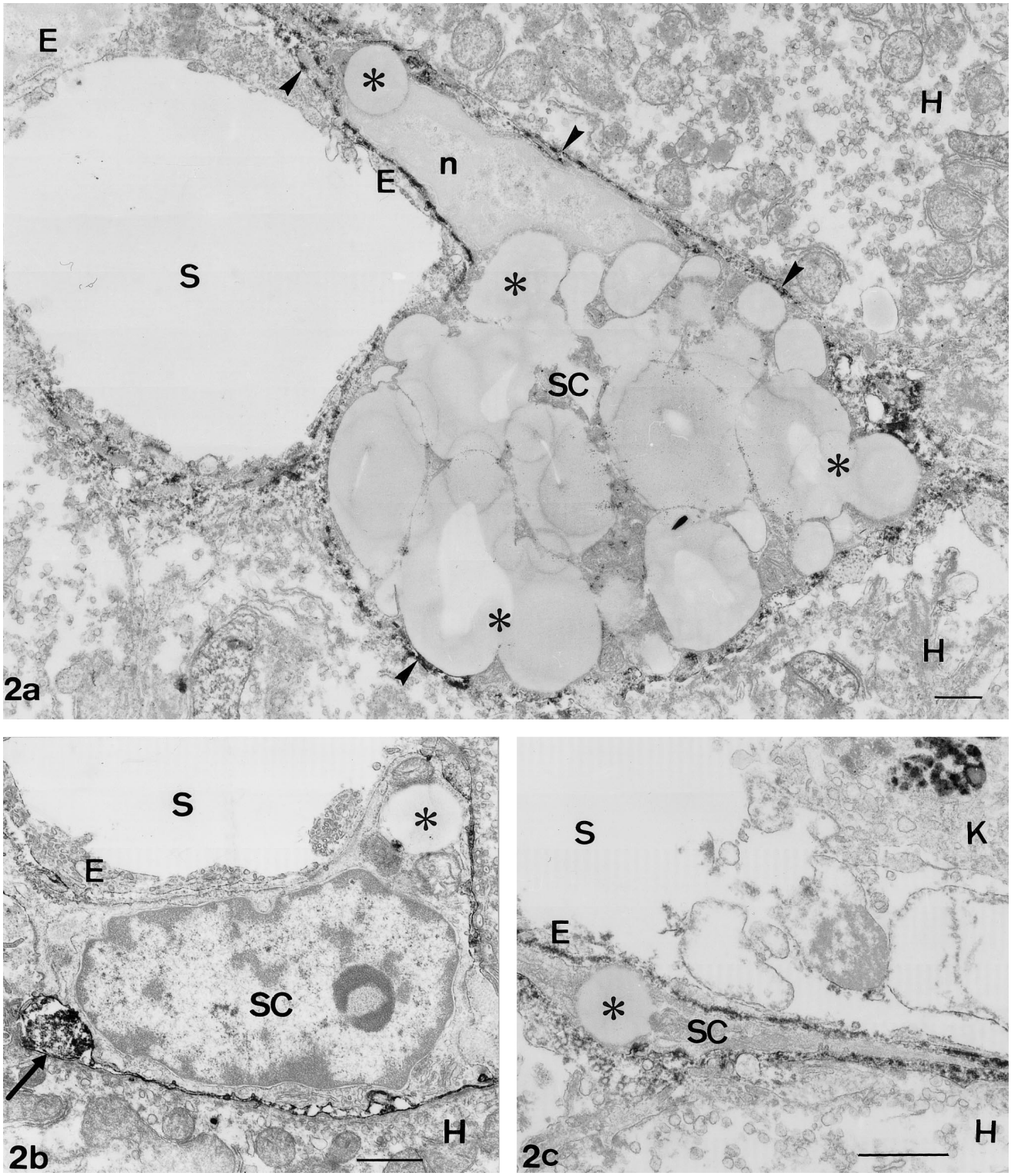
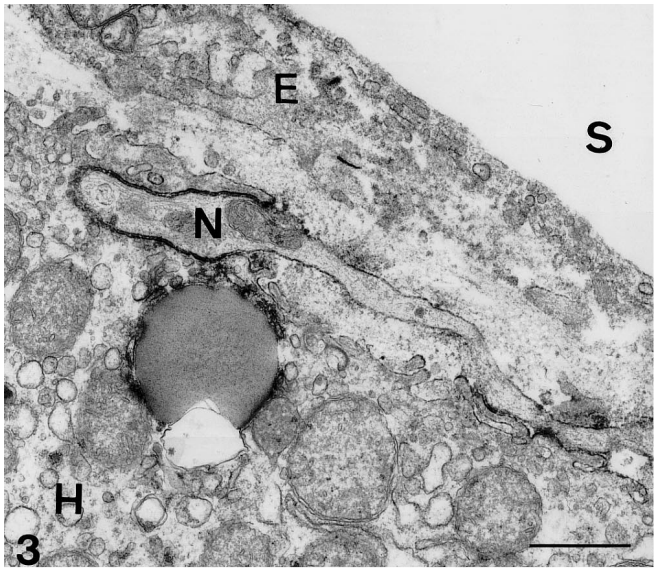
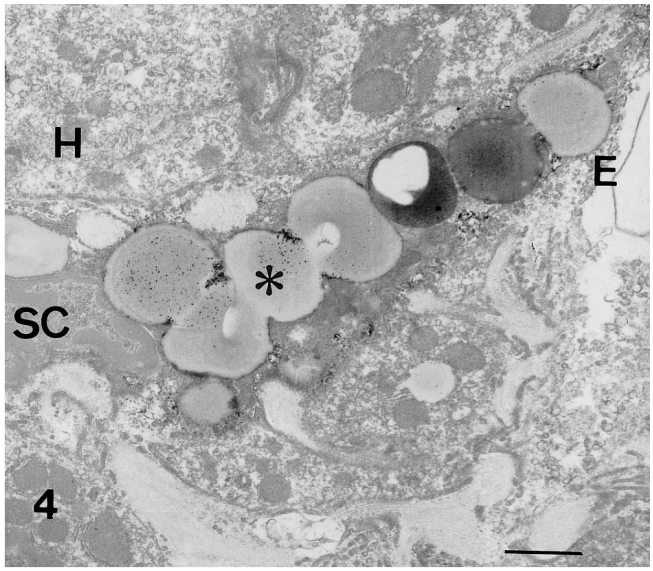
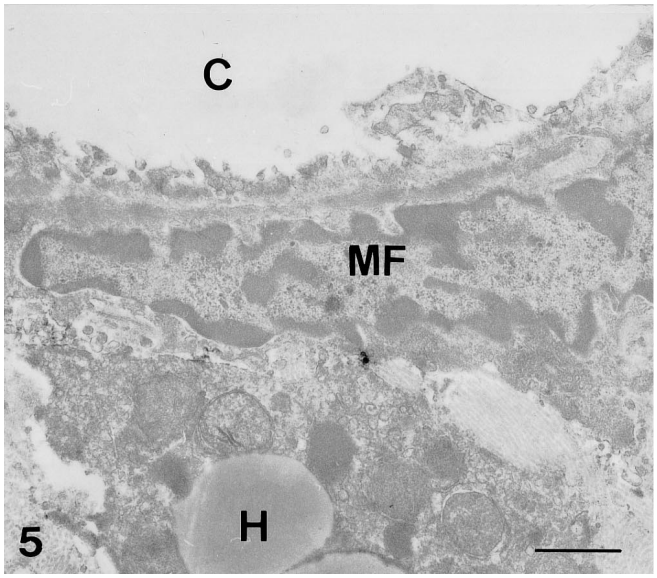


Fig. 2a–c. Immuno-electron microscopy of stellate cells for N-CAM. **a** The stellate cell (*SC*) in the space of Disse contains many lipid droplets (*asterisks*) and exhibits positive N-CAM expression. Immunoprecipitates (*arrowheads*) are seen on the plasma membrane of the stellate cell. Endothelial cells (*E*) are negative for N-CAM. ×8000. *Bar*: 1 μm. **b** An N-CAM-positive stellate cell (*SC*) with a lipid droplet (*asterisk*) makes intimate contact with an N-CAM-positive nerve ending (*arrow*) that contains several small vesicles in the cytoplasm. ×11 000. *Bar*: 1 μm. **c** A cytoplasmic projection of a stellate cell (*SC*) contains a lipid droplet (*asterisk*) and shows a positive reaction for N-CAM. A Kupffer cell (*K*) and endothelial cells (*E*) are negative. ×15 000. *Bar*: 1 μm. *H* Hepatocyte; *S* sinusoid; *n* nucleus







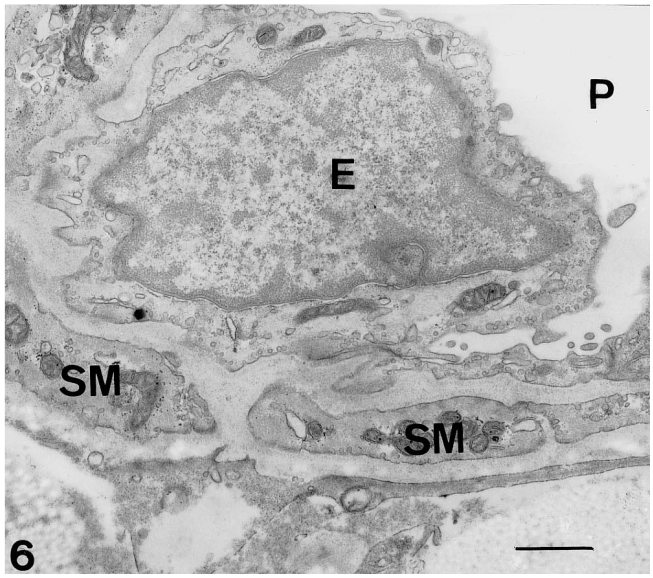


Fig. 3. An N-CAM-positive nerve fiber (*N*) runs in the space of Disse and makes contact with an N-CAM-negative hepatocyte (*H*). *E* Endothelial cell; *S* sinusoid. ×13 000. *Bar*: 1 μm

Fig. 4. An N-CAM-negative stellate cell (*SC*) in the centrilobular zone. *E* Endothelial cell; *H* Hepatocyte; *asterisk* lipid droplets. ×10 000. *Bar*: 1 μm

Fig. 5. A myofibroblast (*MF*) around the central vein (*C*) exhibits no reaction for N-CAM. *H* Hepatocyte. ×11 000. *Bar*: 1 μm

Fig. 6. Perivascular smooth muscle cells (*SM*) of an interlobular portal branch (*P*) are negative for N-CAM. *E* Endothelial cell. ×10 000. *Bar*: 1 μm

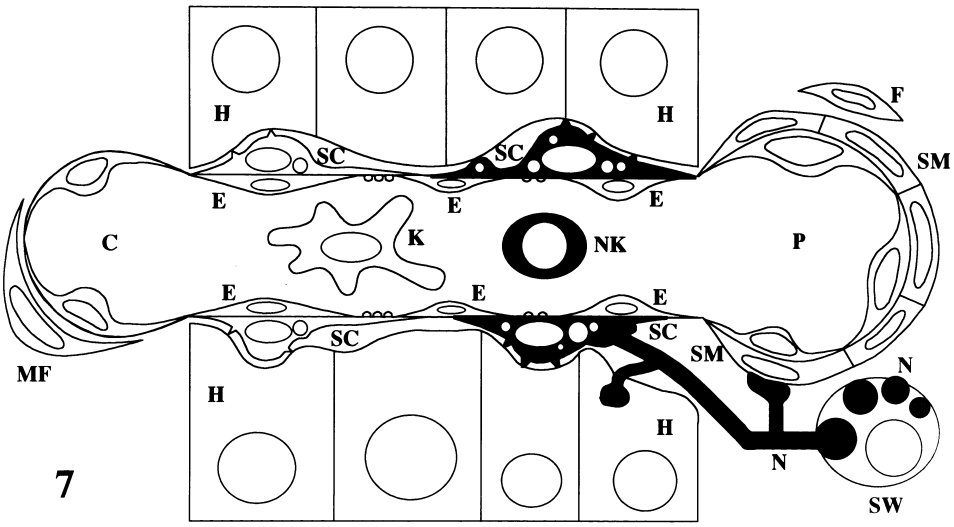


Fig. 7. A schema of N-CAM expression in the normal human liver. Nerve fibers (*N*), stellate cells (*SC*) in the periportal and intermediate zones of the liver lobule, and NK cells (*NK*) are positive for N-CAM (*shaded*), whereas stellate cells (*SC*) in the centrilobular zone, hepatocytes (*H*), Kupffer cells (*K*), endothelial cells (*E*), vascular smooth muscle cells (*SM*) of the interlobular portal veins (*P*), fibroblasts (*F*) in the portal area, myofibroblasts (*MF*) around the central vein (*C*), and Schwann cells (*SW*) are negative

Discussion

Previous immunohistological studies using human liver specimens have shown that N-CAM is positive in nerve fibers, NK cells, and proliferating bile ductules (Roskams et al. 1990; Scoazec et al. 1993; Volpes et al. 1993). The present study has, for the first time, demonstrated positive N-CAM expression in perisinusoidal stellate cells of the normal human liver.

The antibody used in this study was produced by the My31 hybridoma cell lines. Lanier et al. (1986) have previously demonstrated that the mouse monoclonal antibody against human Leu19, obtained from the same cell line, recognizes Leu19 antigen on the surface of human NK cells. Moreover, we have not detected immunoreactive structures in any of the specimens treated with normal mouse serum instead of primary antibody, as the negative control. Therefore, we consider that the antibody used by us recognizes the N-CAM antigen, which is identical to the Leu19 antigen.

Perisinusoidal stellate cells are considered to be liver-specific pericytes (Pinzani et al. 1992). They encircle endothelial lining with dendritic processes (Wake and Sato 1993) and show contraction in vitro in response to vasoactive agents such as endothelin-1 (Kawada et al. 1992, 1993). These observations indicate that these cells belong to the same cell lineage as vascular smooth muscle cells. Stellate cells are also related to myofibroblasts and fibroblasts. When activated under certain pathological conditions, they are transformed into myofibroblasts that lack vitamin A lipid droplets and that synthesize a large amount of collagen fibers (Blomhoff and Wake 1991).

The present study has demonstrated that perivascular smooth muscle cells of interlobular portal branches, myofibroblasts around the central vein, and fibroblasts in the portal area exhibit no staining for N-CAM. Miettinen and Cupo (1993) have also reported that N-CAM is generally negative in perivascular smooth muscle cells. The evidence that these three mesenchymal cell species, which are related to stellate cells, are all negative for N-CAM indicates that the expression of N-CAM in hepatic stellate cells is a specific feature.

The reason that only stellate cells express N-CAM and that other related cells such as perivascular smooth muscle cells and myofibroblasts do not express N-CAM is not known, but may be related to their unique anatomical location. Stellate cells exist in the space of Disse, which contains only a little connective tissue and which allows direct contact of stellate cells with hepatocytes. Suzuki formerly termed them “interstitial cells”, which means that they are interposed between nerve fibers and hepatocytes (for a review, see Wake 1980). Wake (1995) has revealed, by the Golgi method and electron microscopy, that stellate cells extend thorn-like projections that adhere to the hepatocytes with a membrane specialization.

The functional significance of N-CAM expression in stellate cells is not known. It is reported that N-CAM plays an important role in the attachment of axons to muscle cells (Covault and Sanes 1986; Tosney et al. 1986). In the present study, N-CAM is expressed not only at the neuro-stellate cell junction, but also at the other surface of the stellate cell. We therefore consider that the expression of N-CAM in stellate cells may have another function than the attachment to neurone structures. N-CAM is also distributed in some non-neural tissues, such as NK cells (Lanier et al. 1991) and Leydig cells (Mayerhofer et al. 1992; Davidoff et al. 1993).

We have further observed an intralobular heterogeneity of N-CAM expression in stellate cells. N-CAM is not or only weakly expressed in the stellate cells located in the centrilobular zone, whereas it is intense in periportal and intermediate zones. A probable explanation for this heterogeneity is the different maturational stages of stellate cells between the central zone and the periportal and intermediate zones of the liver lobule. Functional and morphological heterogeneity of stellate cells in the liver lobule has been reported previously (Wake and Sato 1993); stellate cells in the centrilobular zone have a lower vitamin A content and more slender dendritic projections than those in the periportal zone. The functional significance of N-CAM expression by stellate cells and its relationship to the differentiation or maturation of stellate cells are to be further elucidated.

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