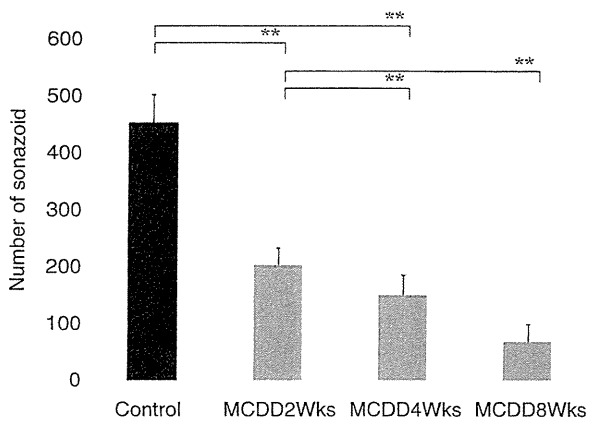

Figure 6 The number of Sonazoid microbubbles phagocytosed by isolated KCs in the control group and the MCDD-2wk, 4wk and 8wk groups were observed by inverted microscopy. After Sonazoid was added, the isolated KCs were cultured before observation. The number of Sonazoid microbubbles phagocytosed by 10 KCs in the control group was 450.5 ± 48.5, whereas 204.1 ± 28.7, 150.9 ± 34.2, and 69.7 ± 29.1 microbubbles were phagocytosed in the MCDD-2wk, 4wk and 8wk groups, respectively (mean ± standard deviation). Significant differences were found between the control group and each week of the MCDD groups, and also between the MCDD-2wk and MCDD-8wk groups and between the MCDD-4wk and MCDD-8wk groups ($P < 0.01$).

microbubbles by phagocytic cells was few after injection. Considering that most of phagocytic cells on sinusoidal wall are KCs, it is reasonable to think contrast agent is phagocytosed by KCs in hepatic sinusoids. Time-course observation also showed the number of phagocytosed microbubbles by phagocytic cells did not increase in the MCDD group (Fig. 4). This finding suggests that the phagocytic capability of KCs may start to decrease during the early stage of NASH, and that could enable the diagnosis of NASH at an early stage of fibrosis. To demonstrate these findings using isolated and cultured KCs, the number of phagocytosed Sonazoid microbubbles decreased in the MCDD rats (Fig. 6). In addition, the number of phagocytosed Sonazoid or latex beads tended to decrease in proportion to the duration of the MCDD administration (Fig. 5). In NASH patients, fibrosis is often detected at a late stage of the disease, because NASH is usually monitored as NAFLD. However, by using Sonazoid CEUS, the diagnosis of NASH could be possible at an early stage, and this represents a groundbreaking development in NASH treatment.

Our study also suggested the clinical usefulness of Sonazoid CEUS in the diagnosis of NASH by demonstrating that: (i) parenchymal enhancement was decreased in the delayed parenchymal phase; and (ii) the phagocytic capacity of Kupffer cells was lowered as the duration of MCDD administration increased. Considering that Sonazoid is specifically phagocytosed by Kupffer cells, the quantification of phagocytic capacity should also be possible.

Some studies have reported the narrowed sinusoids seen in steatosis and steahohepatitis disturb the hepatic microcirculation.[29–31] In particular, the sinusoidal space of a NAFLD animal model was reduced by up to 50 % of the size of healthy control animals.[31] In order to preclude the possibility that the lowered liver parenchymal enhancement was caused by a circulatory disturbance of the contrast agent, latex beads with a diameter of 1 μm, which is smaller than the diameter of Sonazoid (2 μm), were used in the present *in vivo* study, since the width of a normal sinusoid is approximately 5 μm. We performed CEUS with Levovist (4 mL/body) at one minute after Levovist intravenous injection in the early vascular phase to see if decreased parenchymal enhancement was associated with the narrowed sinusoids. Additionally, the parenchymal enhancement of fatty liver patients, NASH patients and healthy volunteers at 1 min after Levovist injection showed a similar intensity in the liver parenchyma in the early vascular phase (Fig. 8). These results demonstrated that the decreased enhancement of liver parenchyma was not due to the narrowed sinusoids or circulatory disturbances.

As shown above, our results suggested decreased Sonazoid-enhanced echogenicity was mainly due to impaired KC phagocytosis, although narrowed sinusoids could be present in MCDD rats due to fatty liver. Sonazoid CEUS could become a useful tool to distinguish NASH patients from many NAFLD patients.

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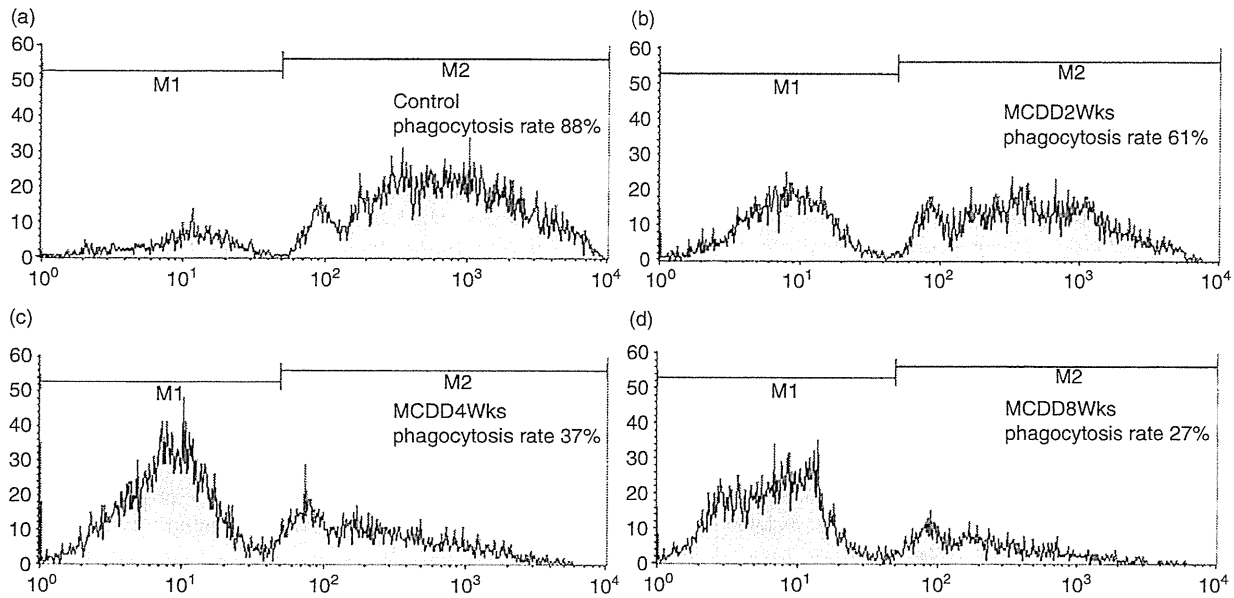

Figure 7 Flow cytometric analysis of isolated and cultured KCs after being treated with fluorescent latex beads. The vertical axis is the KC count and the horizontal axis is the fluorescent intensity. M1 is the number of KCs which did not phagocytose any beads, and M2 is the number of KCs which phagocytosed beads. The phagocytosis rate was calculated by M2/M1 + M2 (the total number of KCs). The phagocytosis rate in the control group was 88% and many latex beads were ingested, whereas the rate was 61 % in the MCDD-2wk (B), 37% in the MCDD-4wk (C) and 27% in the MCDD-8wk (D) groups, where the phagocytic capacity was decreased in proportion to the duration of MCDD administration.

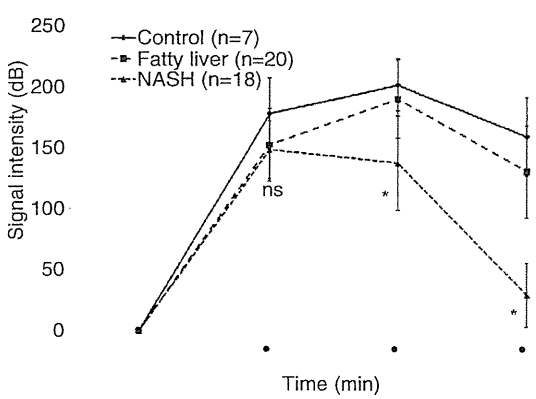

Figure 8 Parenchymal signal intensity in the early vascular phase and the delayed parenchymal phase of Levovist CEUS was evaluated in seven controls (healthy volunteers), 20 fatty liver patients and 18 NASH patients. At 1 min after the Levovist injection, the signal intensity was 178.1 ± 29.3 in the controls, 152.4 ± 30.0 in the fatty liver patients and 148.5 ± 23.6 in the NASH patients (mean ± standard deviation) and no significant differences were observed. However, at 5 and 20 min after injection, there was a significant decrease in the signal intensity in the NASH group.

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