Phagocytosis of Ultrasound Contrast Agents and Diagnostic Low Intensity Insonation Increased the Expression of Heat Shock Protein 70 in Kupffer cells

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Abstract

Purpose
Phagocytizing ultrasound contrast agents by Kupffer cells, which are liver macrophages, enhances clinical diagnostic ability in ultrasound screening for liver tumors. The bioeffects of insonation on the phagocytized ultrasound contrast agent are still not well known. We evaluated the cell stress in this kind of ultrasound examination from diverse aspects.

Methods
We used the ultrasound contrast agent (Sonazoid) in this study. Kupffer cells from 60 rat livers were cultured and assigned randomly to 2 groups. One group was insonated only (US). The other group was insonated after the Kupffer cells phagocytized the Sonazoid microbubbles. Each group was divided into 4 subgroups according to intensity of insonation as follows, mechanical index (MI) 0.2 (n=8), 0.6 (n=8), 1.6 (n=8), and control (n=6). Insonation was applied for 30 seconds by clinical ultrasound diagnostic equipment (Aplio, Toshiba). Microscopic observation was performed before and after insonation to detect morphological changes. The production of heat shock protein 70 (HSP70) and leakage of lactase dehydrogenase (LDH) were determined to evaluate the cell injury.

Results
In both groups, no significant morphologic changes of microbubbles were observed at MI 0.2, while burst and vanishing of the microbubbles were observed at MI 1.6. There were no obviously morphologic changes of Kupffer cells in all groups. In the expression of HSP70, no significant change was observed in the US group, however in the CEUS group, expression significantly increase at MI 0.2 (p<0.05) compared with controls. Intergroup comparisons at the same ultrasound intensity level, the expression of HSP70 in the CEUS group was significantly stronger (p<0.05) than other groups especially at MI 0.2. The leakage of LDH did not significantly differ among any groups.

Conclusion
This study showed that insonation after Kupffer cells phagocytosed the ultrasound contrast agent caused no morphologic change in Kupffer cells. However it is undeniable that cell-stress could be increased.

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Key words: Kupffer cell, HSP, Perflubutane, Ultrasound

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Introduction

Studies have shown that ultrasound (US) contrast agents improve the ability of US examinations to detect hepatocellular carcinoma. It is assumed that the US perflubutane microbubble (Sonazoid™) contrast agent is especially helpful for both liver vascular imaging because of it is activity and also liver parenchyma imaging (Kupffer imaging) by enhancing the contrast of the HCC and metastasis lesions greater degree than other previous ultrasound contrast agents due to the phagocytization perflubutane bubbles in Kupffer cells. However there is concern that the destruction and oscillation of phagocytosis of bubbles during insonation may affect the function and viability of Kupffer cells. A previous paper reported some hepatic cell damage after destruction of microbubbles. In this study, we attempted to observe morphological changes of Kupffer cells and increased HSP 70 expression in vitro after insonation using a clinical ultrasound scanner.

Materials and Methods

Animals

Male Wistar rats aged 18 weeks and weighing 300–400 g were purchased from CLEA Japan Inc. (Tokyo, Japan). All experimental procedures were performed in accordance with experimental protocols approved by the Animal Ethical Committee of Tokyo Medical University.

Kupffer cell isolation

Rat Kupffer cells were isolated. The isolation method was similar to that report in a previous paper described. Anesthesia was performed by intraperitoneal injection of pentobarbital sodium (50 mg/kg, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) in all animals. In all rats after a median line incision the portal vein was exteriorized and catheterized with an indwelling catheter. The vena cavae were also exteriorized and ligated. Rat livers were perfused by indwelling catheters in situ with calcium-free minimum essential medium (Sigma, St Louis, USA), followed by 0.3% pronase (Roche Diagnostics Corp., Indianapolis, IN, USA) and 0.025% type IV collagenase (Sigma) in Dulbecco’s modified Eagle’s medium/F-12 (Sigma). After preparation the livers were carefully removed and minced. The suspension containing Kupffer cells was incubated with 0.035% pronase and 62.5 units/mL DNase (Sigma) in Dulbecco’s modified Eagle’s medium/F-12 in a shaken water bath at 37°C for 20 min. After being centrifuged several times, Kupffer cells were separated and incubated for 48 hours before the experiment.

Ultrasound Contrast Agent

Perflubutane (Sonazoid™; Daiichi Sankyo Company Ltd., Tokyo, Japan), consisting of stabilized gas microbubbles 2–3 μm in diameter in aqueous suspension, was used as the ultrasound contrast agent in all experiments.

Microscopic Observation of Ultrasound Contrast Agent Phagocytosis

We evaluate morphological changes in Kupffer cells by microscopy. Kupffer cell phagocytosis of microbubbles was observed under a microscope (IX70, Olympus, Tokyo, Japan) and recorded by a camera (model JU-TU531T, Toshiba Corp., Tokyo, Japan). The small glass plate on which Kupffer cells had been cultured was inverted them placed into another large culture well with 40 ml culture medium. We added 0.5 ml contrast agent at 1/1000 standard concentration under the small glass plates using a 21 G needle. Then the bubbles of perflubutane settled near the Kupffer cells. Six cells were selected at random and recorded by video during the phagocytosis. The 6 cells were photographed before and after insonation of each MI.

Insonation

Insonation was performed with an SSA-770A system (Aplio; Toshiba Medical Systems Co, Ltd, Tokyo, Japan) set on a conventional B mode with a PTV-382M probe, a small convex-shape probe that transmitted and received center frequencies of 3.5 MHz. The insonation conditions were as follows: frame rate 15 frames/sec, duration of insonation 30 sec, single focus, on the bottom of the culture dish containing the Kupffer cells.

Three levels of MI were selected for insonation in the experiments as follows: no insonation, MI 0.2, MI 0.6, and MI 1.6, because MI 0.2 is popular for low MI imaging, MI 1.6 is the maximum intensity of the ultrasound system and MI 0.6 is the midpoint. The MI is useful as an index of acoustic intensity, defined as the maximal acoustic negative pressure on the ultrasound field, divided by the square root of the central transmitted frequency.

Kupffer cells from 60 rat livers were assigned to one of 2 groups. One group was only insonated (US). The other group was insonated after the Kupffer cells phagocytized the ultrasound contrast agent (CEUS). Each group was divided into 4 subgroups according to intensity of insonation as follows MI 0.2 (n=8), 0.6 (n=8), 1.6 (n=8), and control (n=6).

Determination of HSP

The expression of HSP70 was determined to evaluate the cell-stress tolerance. We performed Western blot analysis after insonation as follows: whole cell lysates containing 20 μg of protein boiled in equal volumes of loading buffer protein were separated electrophoretically...
on 4-12% Tris-glycine gradient gels and subsequently transferred to pure nitrocellulose membranes (BOIRAD) which were blocked with 5% nonfat dried milk in TBS for 2 hours. Primary antibody against Rabbit Anti-HSP70 polyclonal antibody (CSA-400 Stressgen Bioreagent, Canada) was applied at 1/1,000 dilutions overnight, followed by washing three times with TBS containing 0.05% Tween 20, secondary antibody IgG: HRP horseradish peroxidase conjugate adsorbed with human IgG (SAB-300, C) was applied at 1/10,000 dilution for 2 hours those blots were washed in TTBS three times and incubated for 10 minutes. Amersham ECL Western blotting detection reagents and analysis system (GE Healthcare, UK) and Versa Doc imaging system model 5000, Nippon Laboratories (BPC-300) bio-rad super signal exposed to photographic film. Anti-β-Actin HSP70 Protein Active HSP70 HeLa Cell Lysate (Stressgen Bioreagent, Canada) was used as an internal control.

**Determination of LDH**

LDH leakage was determined to evaluate cell injury. After insonation quantitative analysis of the concentration of LDH in media was performed by Cytotoxicity Detection Kit PLUS (Roche Diagnostics GmbH, Germany).

**Statistics**

For statistical evaluation, student paired t-test was used to compare the data of HSP and LDH (software: SPSS ver. 16). A p-value less than 0.05 was considered to indicate a statistically significant difference.

**Results**

**Microscopic observation**

The results of microscopic observations were just described because all cases showed same phenomenon.

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**Table 1** Grouping Phagocytosis of ultrasound contrast

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Control</th>
<th>MI 0.2</th>
<th>MI 0.6</th>
<th>MI 1.6</th>
<th>Control</th>
<th>MI 0.2</th>
<th>MI 0.6</th>
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<tr>
<td>Sonazoid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>US intensity</td>
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<td>0.6</td>
<td>1.6</td>
<td></td>
<td>0.2</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Animal²</td>
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<td>8</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>8</td>
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* : MI (mechanical index)

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**Fig. 1** Representative microscopic images under microscopy with 400-fold magnification. A) Before injecting microbubble solution, B) 30 seconds after, C) 15 minutes after, and D) 30 minutes after the injection. It appeared that all Kupffer cells completed phagocytization within 30 minutes.
Fig. 2  Representative microscopic images under microscopy with 400-fold magnification. Before A), and after B) phagocytizing the perflubutane bubbles. The primary cultured Kupffer cells 24 hours after phagocytizing the perflubutane bubbles returned their shape as did the bubbles, under phase-contrast microscopy.

Fig. 3  Representative microscopic images under microscopy with 400-fold magnification. A) The primary cultured Kupffer cells 24 hours after phagocytizing the perflubutane bubbles before insonation. B) After insonation at MI 0.2. C) After insonation at MI 0.6, the shrunk free bubbles were desfroget and disappeared. D) After insonation at MI 1.6, most of the bubbles were not detectable.

The adhesion of the ultrasound contrast agent microbubbles to the Kupffer cells was completed within 30 seconds after filling with perflubutane. All cells phagocytized one to eight microbubbles within forty minutes. In the fastest case about 10 minutes after the adhesion, the microbubbles moved to the area adjacent to the nucleus of the Kupffer cells. All phagocytized microbubbles retained their shape. All Kupffer cells phagocytizing the microbubbles also maintained their morphology.

Even 24 hours after phagocytization the primary cultured Kupffer cells and the microbubbles retain their morphological appearance.

After insonation at MI 0.2 no change was detected. However, after insonation at MI 0.6, the free perflubutane bubbles in the culture solution began to show oscillation and some of them shrank. After insonation at MI 1.6, most of the free and phagocytized bubbles were fragmented or vanished and the leaked gas from the destroyed agents' shell agglutinated. On the
other hand, Kupffer cells were morphologically stable in all groups.

The analysis of the expression of HSP70

The β-Actin lines show the loading control. Analysis showed no significant change in the lines of β-Actin. No significant change in the expression of HSP70 was observed within the US only group, however in the CEUS only groups, the expression significantly increased at MI 0.2 (p<0.05) compared with control. Intergroup comparison at the same ultrasound intensity level the expression of the group of CEUS was significantly higher (p<0.05) especially at MI 0.2.

The concentration of LDH

No significant increase in LDH concentration was detected in any group or subgroup.

Discussion

We set out to determine whether phagocytosis of ultrasound contrast agents and diagnostic low intensity insonation affects cell function.

The Kupffer cell is a macrophage in the liver. They naturally phagocytize the microbubbles of perflubutane and the phagocytized bubbles even react under insonation. Hepatocellular carcinoma (HCC) lesions are not enhanced by the ultrasound contrast agent in delayed phase images on clinical ultrasound contrast enhanced examination. The lesions have few Kupffer cells3−5. Therefore lesions of HCC should appear as hypoechoic areas compared to bright noncancerous lesion. This effect remarkably improves screening for detection of HCC6 lesions.

However a previous report suggested might that it should make adverse effects on liver cells2. In our study, to conform as much as possible to the situation of the insonated Kupffer cells during ordinary clinical ultrasound examinations, we employed the same, clinically used ultrasound contrast agent and diagnostic ultrasound equipment and probe as used in the clinical studies. However microenvironments surrounding Kupffer cells like serum, oxygen saturation, and other kinds of liver cells naturally differed from the in vivo situation, because they were isolated. It is undeniable that the differences could affect ultrasound attenuation and cell damage. However the experimental condition was able to evaluate responses from only Kupffer cells and to achieve a homogeneous insonation dose distribution.

The microscopic observation showed the stability of the perflubutane microbubble and the low interaction between the microbubbles and Kupffer cells. The phagocytizing Kupffer cells showed no morphological change for at least 24 hours. This result showed that
phagocytizing perflubutane would cause no acute toxicity for Kupffer cells. Moreover the cells showed no morphological change after various levels of insonation, even at the bubble destruction insonation level (MI 1.6).

HSP 70 accomplishes a key role to maintain the physiological function of the cell after many kinds of stress. We therefore decided to use HSP 70 as the marker of cell stress in this study, because so far no common marker for the bioeffects for insonation with microbubble ultrasound contrast agents has been established.

Interestingly, in the group of insonation with phagocytizing perflubutane, the expression of HSP 70 declined as the insonation intensity increase. On the other hand the insonation-only group showed no significant change. A paper by Nollen and Morimoto stated that in non-stress conditions HSP would bind with heat shock factor and be inactivated, and that immediately after stress, HSP would be released and activated. It was assumed that US alone causes no stress for cells. If it did cause cell damage, the HSP expression would increase at higher insonation levels.

However it is a fact that low intensity insonation with phagocytizing perflubutane enhances some bioeffects. More intriguingly, low ultrasound intensity was more stressful for microbubble-phagocytized cells than high ultrasound intensity.

In next step we would like to make sure the interesting phenomenon. If just enhances the expression of HSP and causes no cell damage, preconditioning of insonation after phagocytizing the microbubbles of ultrasound contrast agent could reduce the adverse effects of liver ischemia-reperfusion injury resulting from partial hepatectomy. In short, it is possible that a hepatic induced HSP level could induce higher hepatic cell-tolerance.

LDH is known as a stable enzyme contained in the cytoplasm of all cells and released promptly when the cell membrane is damaged. This is the reason that LDH was selected as a marker of cell injury in this study. However no case showed elevation of LDH level. Even insonation with phagocytizing perflubutane seems to cause no irreparable harm which is compatible with the results of previous studies. However another report showed the microbubble burst of another ultrasound contrast agent at the surface of cell membrane under insonation causing perforations penetrating holes in just microseconds. In our study most bubbles were inside the cell. This difference should decrease deleterious the harmful effects.

**Conclusion**

This study showed insonation after the Kupffer cells phagocytosed the ultrasound contrast agent caused no morphologic change of Kupffer cell. However there is an undeniable possibility that cell-stress could be increased.

**References**


Kupffer 細胞による超音波造影剤 perfluorobutane の取り込みと
超音波照射による HSP70 の発現誘導作用について

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目的：びまん性肝疾患、肝膵病性変病に対する画像診断に、造影超音波検査は検出感度が高い低侵襲性検査方法である。SONAZOID 造影超音波では、Kupffer 細胞内に取り込まれた造影剤が超音波照射を受けて、反射、共振、崩壊することで画像が得られる。これらの造影剤の変化による刺激が Kupffer 細胞内の HSP70 発現にどのような影響を及ぼすのかについて検討した。方法：雄性 Wistar ラット 18 週 60 匹を超音波法と造影超音波 2 群に分け、さらに各群は音圧により、コントロール (n=6)、MI 0.2 (n=8)、MI 0.6 (n=8)、MI 1.6 (n=8) の 4 グループ (計 8 グループ) に分けして実験を行なった。肝組織より分離し、48 時間培養後の Kupffer 細胞の培養液中で、造影剤溶液を加えて気泡を処理し、顕微鏡で観察した。Kupffer 細胞がマイクロバブルを食事したこと確認した後、Toshiba Apio XY 造影モードで、30 秒間照射し、細胞を 24 時間培養後、細胞を剝がして Western blot analysis HSP の誘導を検討した。結果：いずれの条件においても HSP の誘導が確認された。超音波のみ照射群では音圧の増加に連れて増加し MI 1.6 においてピークとなった。MI 1.6 においてコントロールと比べて有意な HSP の産生亢進を認めた。perfluorobutane＋超音波群において、MI 0.2 においてコントロールと比べて HSP の産生は著しく有意に亢進したが、音圧の増加に連れて低下し MI 1.6 では有意差がみられなかった。ピークは MI 0.2 の時点であった (p<0.05)。

Kupffer 細胞による超音波造影剤 perfluorobutane の取り込みと超音波照射による HSP70 の発現誘導作用について

（キーワード） Kupffer 細胞、HSP、perfluorobutane、超音波