Targeted Ultrasound Imaging of Apoptosis with Annexin A5 Microbubble in Acute Doxorubicin-Induced Cardiotoxicity

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Directed by Professor Namsik Chung

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ABSTRACT

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Recently, apoptosis has been suggested as a common mechanism of myocardial loss in doxorubicin-induced cardiomyopathy. The aim of this study was to assess the feasibility of targeted ultrasound imaging of apoptosis with annexin A5 microbubbles (A5MB) in acute doxorubicin-induced cardiotoxicity. Avidinated and octafluoropropan-filled phospholipid microbubbles were conjugated with biotinylated annexin A5. To confirm the specific binding ability of A5MB, flow cytometry was performed with hydrogen peroxide induced apoptosis of rat aorta smooth muscle cells incubated with fluorescein-5-isothiocyanate (FITC) labeled annexin A5 and A5MB. Adult male rats were injected intraperitoneally with 5 mg/kg doxorubicin weekly for 3 weeks (n=5). Control rats were injected with normal saline (n=2). At 24 h after the final treatment, triggering imaging

was done at a mechanical index of 0.8 using a 14 MHz linear probe 15 min after intravenous bolus injection of A5MB for washout of freely circulating microbubbles. After the contrast echocardiography, the heart was isolated for histological detection of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. In the in vitro tests, fluorescence intensity was low for healthy cells and high for apoptotic cells when incubated with FITC-labeled annexin A5 and A5MB. Rats treated with doxorubicin showed significant contrast opacification of myocardium in contrast echocardiography using A5MB. However, no opacification was observed in control rats. Late apoptosis was confirmed by TUNEL assay in doxorubicin treated rats. In conclusion, acute doxorubicin-induced cardiomyopathy based on early apoptosis can be assessed and imaged with targeted ultrasound imaging using A5MB in rats.

Key words: Doxorubicin; cardiomyopathy; apoptosis; annexin A5; microbubbles; echocardiography

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I. INTRODUCTION

Doxorubicin is a cytotoxic anthracycline that is widely used as a sole chemotherapy or in polychemotherapy regimens for several malignancies. Although the dose-response relationship between anthracycline regimens and remission and event-free survival is well established, cardiotoxicity is the major limiting factor in high-dose schedules.[1] At present, doxorubicin cardiotoxicity is routinely screened noninvasively by measurement of the left ventricular ejection fraction. Despite the fact that ventricular function analysis has continued to prove relevant in defining patient risks, abnormal observations can be made only when cardiac damage already has reached

significant proportions.[2]

Recently, myocardial apoptosis has been suggested as a common mechanism of acute and chronic myocyte loss.[3-5] In the pathophysiology of cardiovascular disease, programmed cell death of cardiomyocytes has been suggested to be an important contributor because apoptotic cardiomyocytes have been identified during hypoxia, ischemia, cardiac overload, acute myocardial infarction, end-stage heart failure in vivo, and anthracycline use.[6, 7] Doxorubicin induces apoptosis in several cell lines and, in a rat model, its kidney, intestine, and cardiomyocytes.[7-9] Therefore, the detection of apoptosis could be an opportunity for the noninvasive exploration of early cardiomyopathy.

The detection methods used in most of the studies evaluating apoptosis of the heart are based on the occurrence of DNA fragmentation, such as the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and DNA laddering. However, because TUNEL and DNA laddering do not detect the early stages of cell death, these techniques are not ideal for the assessment of early apoptosis. In addition, in vivo detection of

cell death is not possible with TUNEL or DNA gel electrophoresis.[10]

One of the earliest events after the triggering of cell death is the externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane of the cell. PS externalization is considered to be in close relation to the activation of key players in the cell death program and can be induced by a variety of cell death triggers. The detection of PS exposure can be easily achieved by the phospholipid binding protein annexin A5. In a number of in vitro and in vivo studies, it have been demonstrated that annexin A5 is a specific marker for the early and late stages of cells undergoing programmed cell death and that the protein is also suitable for the in situ detection of cell death. Therefore, labeled annexin A5 provides a useful tool for in situ detection of cell death in vivo and also, at least potentially, in the clinical setting.[11] Recombinant human annexin A5 has been radiolabeled with ^{99m}Tc, and the feasibility of apoptosis imaging has been demonstrated both in experimental and in clinical cardiovascular disease states. [12-14]

Imaging of cellular and molecular events with contrast-enhanced ultrasound has recently been achieved by the use of novel targeted

microbubble contrast agents that are retained within diseased organs.[15] Myocardial contrast echocardiography is an ultrasound imaging technique that uses intravenously injected, gas-filled, acoustically reflective microbubbles that act as red blood cell tracers as they pass unimpeded through the microcirculation.[16] Unlike inert microbubble blood tracers, targeted microbubbles were designed to adhere to specific endothelial surface epitopes to allow ultrasonic detection of these epitopes.[17] As microbubbles are pure intravascular tracers, molecular imaging with contrast-enhanced ultrasound has focused on diseases such as inflammation, thrombus formation, and angiogenesis, which are mediated by pathophysiologic events that occur within the vasculature.[18] The relative advantage of using ultrasound is that it is well balanced in terms of sensitivity and spatial resolution. In comparison to radionuclide imaging, ultrasound is slightly less sensitive, mostly as a result of the influence of background tissue signal, but has superior spatial resolution. Other potential advantages of ultrasound include its low cost, high temporal resolution, and rapid data acquisition.[18]

The aim of this study was to assess the feasibility of targeted ultrasound imaging of apoptosis with microbubbles conjugated with annexin A5 in acute doxorubicin-induced cardiotoxicity model.

II. MATERIALS AND METHODS

1. Annexin A5 Expression in E. coli

A. Materials

The human placental cDNA library was obtained from BD Bioscience (USA). Cloning host cells JM109 were from Promega (USA). The prokaryotic expression vector pET22b(+) and expression host cells, Rosetta (DE3), were obtained from Novagen (USA). Chromatographic resins and Q-Sepharose fast flow were purchased from Amersham Biosciences (USA). Recombinant human annexin A5 was purchased from Hyphen BioMed (France).

B. Cloning of Human Annexin A5

Annexin A5 was cloned by PCR with primers 5-ACATATGGCACAGGTTCTCAGAGGCACT-3 and 5-AAGCTTTTAGTCATCTTCTCCACAGAGCAG-3. The PCR product was subcloned into the prokaryotic expression vector pET22b(+).

C. Expression and Purification

The constructed plasmid was transformed into E.coli Rosetta (DE3) cells. Transformants were grown at 37 ℃ with shaking in LB medium and isopropyl-b-D-thiogalactoside was used as an inducer. The lysed bacterial supernatant was bound onto a Q-Sepharose fast flow column that was previously equilibrated with 50 mM Tris pH 8.0. A step gradient of NaCl was applied to elute the bound proteins.

2. Preparation of Microbubbles Conjugated with Annexin A5

Biotinylated microbubbles were prepared by sonication (35 w, 4 min) of octafluoropropan gas with aqueous dispersion of 5 mg/mL DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine, Avanti Polar Lipids, Inc), 5 mg/mL

PEG-distearate(poly(ethylenglycol) distearate, Sigma chemical Co.), and 2.5 mg/mL DSPE-PEG-biotin (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-Biotinyl-poly (ethyleneglycol) 2000, Avanti Polar Lipids, Inc) in normal saline. After sonication, the microbubbles were placed in a tube and centrifuged for 3min at 2,000 rpm. The bottom saline was drained and 5mL of saline was added to the foam and the centrifugal was washed 2 times.

The prepared microbubbles were combined with NeutrAvidin (PIERCE Biotechnology) for 30 min and washed with saline. Then the microbubbles were combined with biotinylated annexin A5 for 30 min and washed 2 times. FITC- labeled Annexin A5 microbubbles (A5MB) were prepared using FITC-labeled NeutrAvidin (PIERCE Biotechnology).

3. In Vitro Test – Flow Cytometry

Rat aorta smooth muscle cells were cultured over the 70% confluency in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% fetal bovine serum (FBS) and treated with hydrogen peroxide (H_2O_2) (100 μM).

At 3 h after the treatment, the cells were collected and washed with phosphate-buffered saline (PBS). The cells were resuspended in a binding solution of 500$\mu\ell$ (2% BSA, 10 mM HEPES, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$) and blocked for 1 h at room temperature. FITC-labeled Annexin A5 and FITC labeled A5MB were added to cell suspensions. The reaction was incubated in the dark for 20 min at room temperature and the cells were washed 2 times with PBS. Samples were placed on a tube and immediately analyzed on a FACS Calibur (Becton Dickinson) to generate histograms of green fluorescence intensity.

4. Doxorubicin Cardiotoxicity Model

Adult male Sprague-Dawley rats weighing 250±10 g were purchased from Harlan and maintained under standard conditions at the animal care facility. The rats had free access to standard rodent chow and water. Doxorubicin (Dong-A Pharm., Seoul, Korea) 5mg/kg was injected intraperitoneally every week for 3 weeks, resulting in a total cumulative dose of 15mg/kg per animal (n=5). Control rats were injected with the same volume of

physiological saline instead of doxorubicin (n=2). At 24 h after the final treatment, myocardial contrast echocardiography was performed. Immediately after the echocardiography, the rats were killed and the entire heart was excised for histological detection of apoptosis.

5. Contrast Echocardiography Using Microbubbles

Rats were sedated with zoletil (50 mg/kg) and xylazine (5 mg/kg) given intraperitoneally. Once sedated, a catheter was placed in a femoral vein for microbubble administration. Imaging was performed at 14 MHz with a linear-array transducer interfaced with an ultrasound system (Vivid 7, GE Vingmed Ultrasound, Horten, Norway). Images were acquired in a parasternal short axis view with the transducer fixed in position with a free-standing clamp. Before microbubble injection, baseline images were acquired. Gain settings, depth, and focus were initially optimized and maintained throughout the experiment. Ultrasound transmission was then suspended, and 4 x 10^6 A5MB were injected as an intravenous bolus. Imaging was not resumed until 15 min after the injection for retention of

microbubbles in apoptotic tissue and clearance of freely circulating microbubbles from the blood pool. Intermittent ECG-triggering imaging (mechanical index of 0.8) was then initiated at a pulse interval of 1 cardiac cycle for several frames. Images were recorded digitally and analyzed offline.

6. Histological Examination

The TUNEL Assay was performed according to the instructions of the manufacturer (Chemicon, Temecula, CA, USA). In brief, the excised heart tissues were fixed in 3.7% buffered formaldehyde and embedded in paraffin. Tissue sections 5 μm thick were deparaffinized, rehydrated, and rinsed with PBS. A positive control sample was prepared from normal heart tissue by treating with DNase I (10 U/mL, 10 min at room temperature). The sections were pretreated with 3.0% H_2O_2, subjected to the reaction with TdT enzyme for 37 °C for 1 h and incubated in digoxigenin-conjugated nucleotide substrate at 37 ℃ for 30 min. Nuclei exhibiting DNA fragmentation were performed by 3,3-diamino benzidine for 5 min. The nuclei of apoptotic cardiomyocytes was stained dark brown. Lastly, the sections were

counterstained with methyl green and then coverslipped. The sections were observed by light microscopy.

III. RESULTS

1. Expression and Purification of Human Annexin A5

The expression of annexin A5 was confirmed by SDS-PAGE. As shown in Figure 1, annexin A5 with 36 kDa molecular weight was highly expressed by the induction of IPTG.

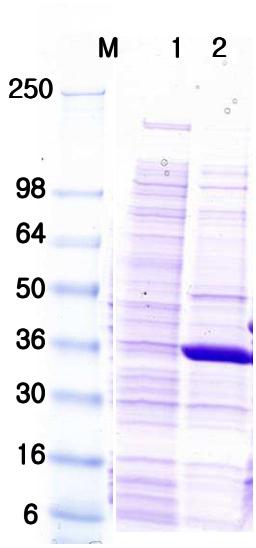

Fig 1. Expression of recombinant human annexin A5

Lane M, molecular weight makers; lane 1, total bacterial protein before IPTG induction; lane 2, bacterial lysate after IPTG induction

Ion-exchange chromatography with Q-Sepharose fast flow was performed for purification of recombinant human annexin A5. The recombinant protein was eluted at about 150 mM NaCl (Fig 2). The final yield of recombinant human annexin A5 was approximately 40-50 mg/L of culture. The purified

annexin A5 was confirmed by Western blot analysis with a polyclonal antibody against human annexin A5 (Fig 3).

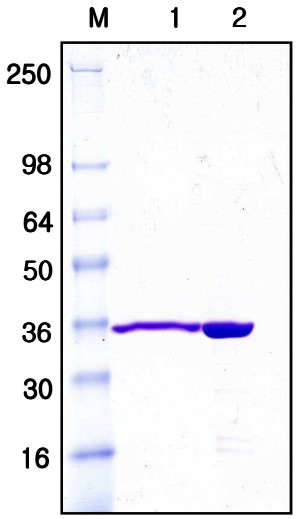

Fig 2. SDS-PAGE analysis of purified recombinant human annexin A5

Lane M, molecular weight marker; lane 1, commercial recombinant human annexin A5; lane 2, purified recombinant human annexin A5

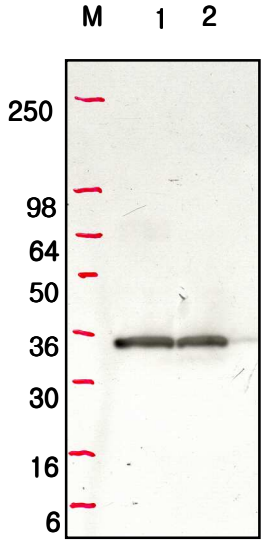

Fig 3. Western blotting analysis of purified recombinant human annexin A5

Lane M, molecular weight marker; lane 1, commercial recombinant human annexin A5; lane 2, purified recombinant human annexin A5

2. In Vitro Test – Flow Cytometry

The histograms of fluorescence intensity obtained by flow cytometry represent specific binding of FITC labeled annexin-5-microbubbles to apoptotic SMC (Fig 4). Compared to healthy cells, apoptotic cells incubated with FITC-labeled A5MB were characterized by a higher percentage of

fluorescence staining positive (3.7% versus 91.4%). Fluorescence intensity was low for healthy cells and increased when apoptotic cells were incubated with FITC-labeled Annexin A5 and FITC-labeled A5MB. These results indicate that the A5MB bind specifically to apoptotic cells.

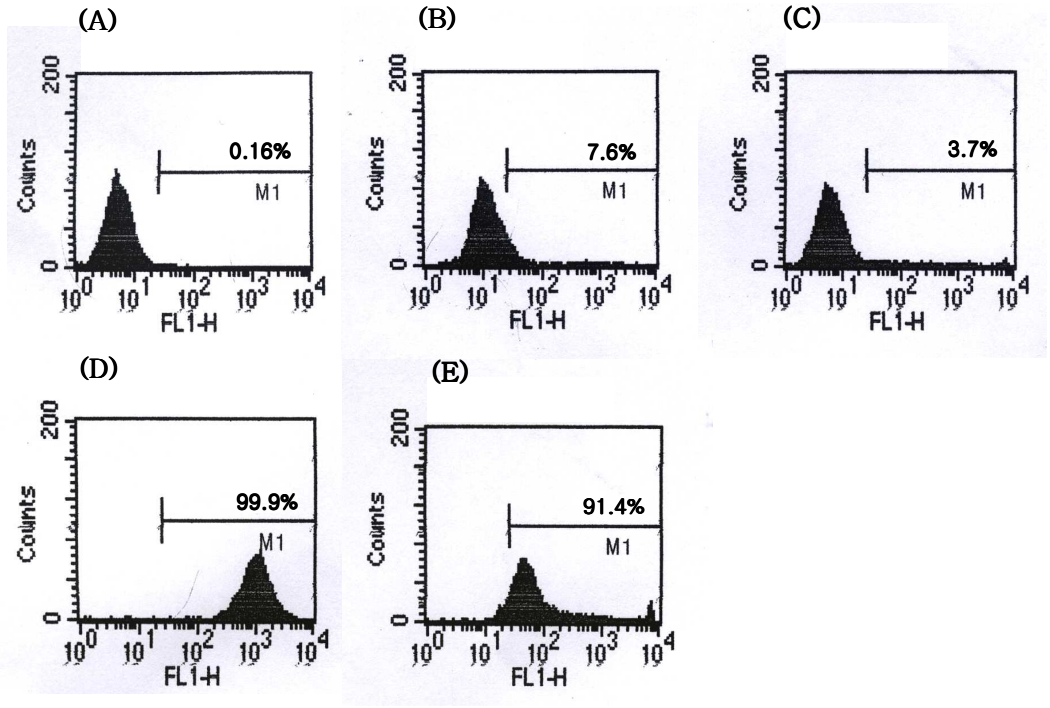

Fig 4. Specific binding of FITC-labeled Annexin A5 to apoptosis cells

(A) Healthy cells

(B) Healthy cells incubated with FITC-labeled Annexin A5
(C) Healthy cells incubated with FITC-labeled annexin A5 conjugated microbubbles
(D) Apoptotic cells incubated with FITC-labeled Annexin A5
(E) Apoptotic cells incubated with FITC-labeled annexin A5 conjugated microbubbles

3. Targeted Ultrasound Imaging

Figure 5 demonstrated the results of contrast echocardiography using A5MB. In rats treated with doxorubicin, the 1st frame showed significant contrast opacification of myocardium – the signal coming from the adhered microbubbles (Fig 5A). These retained microbubbles were destroyed during subsequent high mechanical index imaging. Therefore, no opacification was observed in the 10th frame (Fig 5B). In control rats, unbound microbubbles were dissipated during the waiting period and no contrast enhancement was observed in the 1st frame (Fig 5C) as well as the 10th frame (Fig 5D).

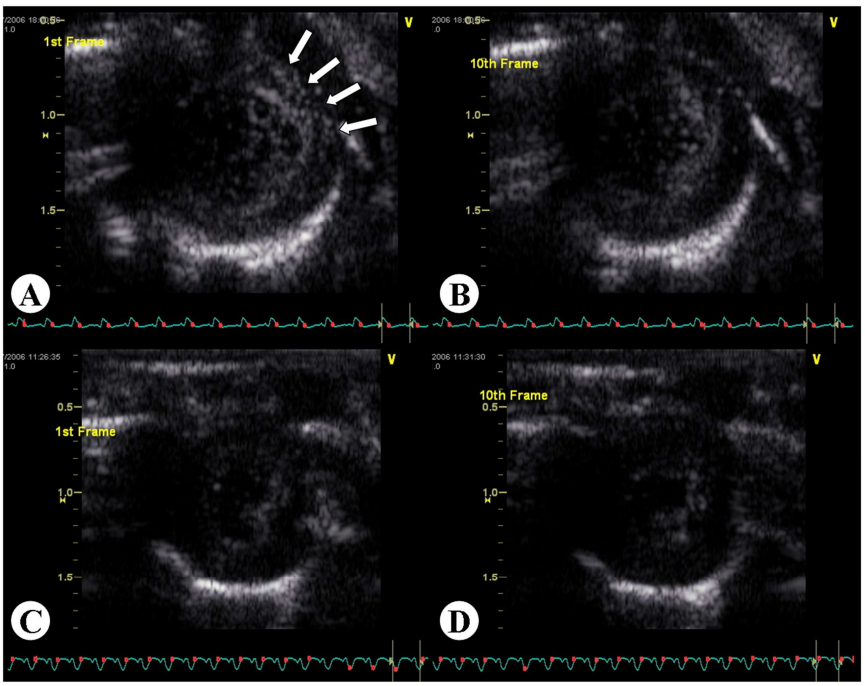

Fig 5. Targeted ultrasound imaging with microbubbles conjugated with annexin A5 in doxorubicin-treated rat (A,B) and control rat (C,D)

(A) Significant contrast opacification of myocardium (arrows) was observed in the 1st frame.

(B) Initial contrast enhancement was dissipated during subsequent high mechanical index imaging in the 10th frame.

(C) No contrast opacification was observed in the myocardium in the 1st frame.

(D) No contrast opacification was observed in the myocardium in the 10th frame.

4. TUNEL Assay

The result of the TUNEL assay revealed increased apoptosis frequencies for both the cardiomyocytes and endothelial cells of the doxorubicin-treated hearts compared to the control hearts (Fig 6). The ratio of the TUNEL-positive cardiomyocytes and endothelial cells were significantly higher in the doxorubicin-treated group than in the control group indicating that doxorubicin induced apoptosis of cardiomyocyte and endothelial cells (Fig 7).

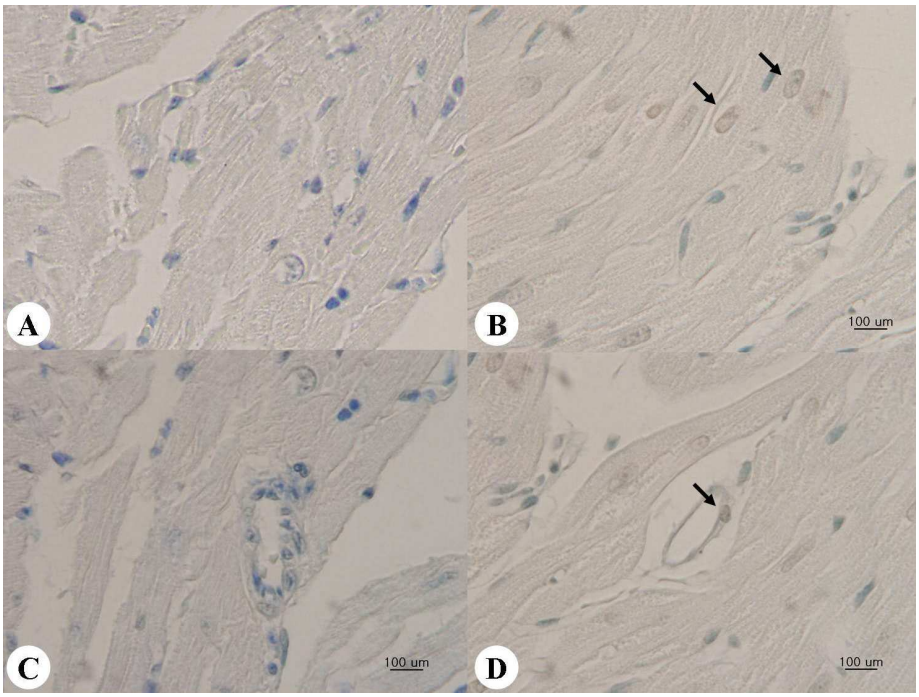

Fig 6. In situ labeling of DNA fragmentation in rat myocardium from TUNEL assay

Fragmented DNA was labeled in brown, and all nuclei were counter-stained with methyl-green. Myocardial sections from rats in the control group reveal no evidence of DNA fragmentation in cardiomyocytes (A) or endothelial cells (C). A heterogenous pattern is revealed for the isolated apoptotic nuclei in the myocardium of doxorubicin-treated rats, as indicated by arrows, for both the cardiomyocytes (B) and endothelial cells (D).

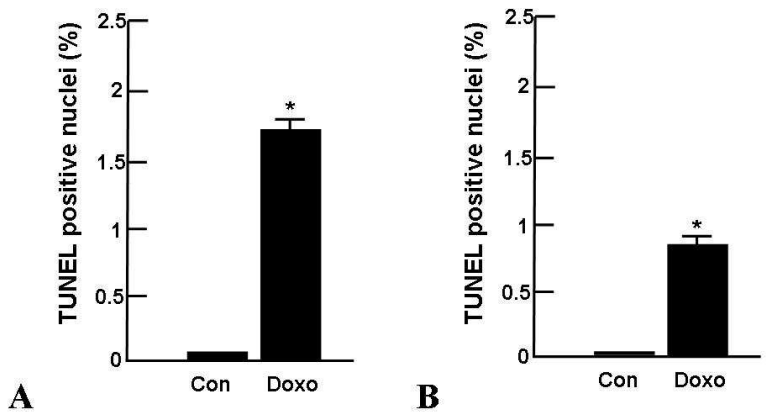

Fig 7. The quantitative results of positive TUNEL-staining cardiomyocytes and endothelial cells

The ratio of the TUNEL-positive cardiomyocytes (A) and endothelial cells (B) were significantly higher in the doxorubicin-treated group (Doxo) than in the control group (Con). (*$p<0.01$ vs. control)

IV. DISCUSSION

The main finding of this study is that targeted ultrasound imaging using A5MB is capable of identifying apoptosis in acute doxorubicin induced cardiomyopathy. This study is the first description of an ultrasound approach

for detecting apoptosis using a targeted contrast agent and has major implications for the noninvasive detection of early apoptosis in doxorubicin induced cardiomyopathy.

Molecular imaging is a rapidly evolving discipline with the goal of developing tools to display and quantify molecular and cellular targets in vivo.[19] Although techniques for molecular imaging have been developed for essentially every form of medical imaging, there are significant differences that influence the choice of imaging method in both research and clinical settings. For imaging of apoptosis, recombinant human annexin A5 has been radiolabeled with ^{99m}Tc, and the feasibility of imaging apoptosis has been demonstrated both in experimental and clinical cardiovascular disease states.[12-14] Bennink et al. recently showed that acute doxorubicin induced cardiomyopathy based on early apoptosis can be imaged with ^{99m}Tc-annexin A5 scintigraphy in murine model.[20]

In this study, we used microbubbles conjugated with annexin A5 for the targeted ultrasound imaging of apoptosis. In comparison to radionuclide imaging, the high temporal resolution, availability, rapid execution of

imaging protocols, and relatively low cost are features of targeted molecular imaging with ultrasound that make this technique attractive.[15] As microbubbles are pure intravascular tracers, molecular imaging with contrast-enhanced ultrasound has focused on diseases such as inflammation, thrombus formation, and angiogenesis, which are mediated by pathophysiologic events that occur within the vasculature.[18] In doxorubicin induced cardiotoxicity, both cardiomyocyte and endothelial cell death can occur via apoptosis.[21] Therefore, in this study, retained microbubble to produce contrast enhancement was thought to be adhered to the apoptotic endothelium, not to the apoptotic cardiomyocyte. However, we could not entirely exclude the possibility of direct attachment of A5MB to apoptotic cardiomyocyte by extravasation through the disrupted microvessel. In this study, the direct visualization of A5MB binding to apoptotic cell could not be performed in in vitro tests nor in in vivo experiments.

The results of this study indicate that site-targeted ultrasound has potential for non-invasive investigation of early apoptosis in doxorubicin induced cardiomyopathy. However, a destructive imaging protocol that includes a 15-

minute delay for wash-out may be difficult to apply clinically in the future, because precisely locating the heart before the first destructive pulse may be challenging. In addition, contrast opacification was assessed by visual estimation rather than by a quantitative method in this study, and a newer quantification method may improve the reproducibility and the practicability of the targeted ultrasound imaging.

V. CONCLUSION

Acute doxorubicin-induced cardiomyopathy based on early apoptosis can be assessed and imaged with targeted ultrasound imaging using microbubbles conjugated with annexin A5 in rats. This investigation model may lead to interesting applications in the in vivo evaluation of cardiomyocyte apoptosis in cardiomyopathy.

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2002 Dec;34:1595-607.

ABSTRACT (IN KOREAN)

독소루비신 유발 급성 심장독성에서 Annexin A5 결합 미세기포와 심근조영 심초음파를 이용한 세포고사의 평가

<지도교수 정남식>

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민 필 기

최근 들어 독소루비신 유발 심근병증에서 심근손상의 공통 기전으로서 세포고사에 관한 연구가 활발히 이루어지고 있는데, 본 연구에서는 독소루비신에 의한 급성 심근독성에서 annexin A5 결합 미세기포를 이용한 표적 심근조영 심초음파를 이용하여 세포고사를 평가해 보고자 하였다. 인지질막을 갖는 옥타플루오로프로판 미세기포를 annexin A5와 결합시킨 뒤 flow cytometry를 이용하여 이렇게 생성된 표적 미세기포가 세포고사를 유발시킨 백서 대동맥 평활근 세포와 결합하는지 확인하였다. 독소루비신 유발 급성 심근독성 모델은 백서에 일주일에 한 번씩 독소루비신을 5mg/kg 복강 내 주사하였으며 3주간 처치하였으며, 대조군 백서에는 생리 식염수를 같은 방법으로 주사하였다. 마지막 처치 24시간 후에

심근조영 심초음파를 실시하였다. 14 MHz probe를 이용하여 annexin 결합 미세기포를 정맥 내 주사하고 15분간 기다린 후 high MI 영상을 얻었다. 초음파 검사가 끝나면 백서를 안락사하고 심장을 적출하여 세포고사를 확인하기 위한 TUNEL assay를 시행하였다. Flow cytometry 검사에서 정상 심근 세포에서 형광 강도는 낮았으나 세포고사를 유발시킨 평활근 세포에서는 annexin A5와 annexin A5 결합 미세기포를 이용하였을 때 두 군에서 모두 형광 강도가 증가함을 확인하였다. 심근조영 심초음파 검사에서는 대조군에서는 조영 증강이 관찰되지 않았으나 세포고사가 유발시킨 군에서는 심근의 조영 증강이 관찰되었다. 실험군에서 세포고사는 TUNEL assay로 확인되었다. 결론적으로, 백서를 이용한 독소루비신에 의한 급성 심근 독성 모델에서 annexin 결합 미세기포를 이용한 표적 심근조영 심초음파 검사로 세포 고사를 평가할 수 있었다.

핵심 되는 말: Doxorubicin; cardiomyopathy; apoptosis; annexin A5; microbubbles; echocardiography