containing 0.01% hydrogen peroxide in 0.05 M Tris-buffered saline (pH 7.6) as a chromogenic substrate. The sections were then counterstained with hematoxylin and eosin, dehydrated in a graded ethanol series, and mounted in Permount (Fisher Scientific)[23].

For immunofluorescence studies, the sections were incubated with secondary antibodies conjugated with Alexa 488 or Alexa 594 (Molecular Probes). Nuclei were stained with TOTO-3 (Molecular Probes). Slides were observed under a confocal laser-scanning microscope (LSM 510 META; Carl Zeiss). Optical sections were obtained at 1024 × 1024 pixels resolution, and analyzed using LSM software (Carl Zeiss). We substituted nonimmune rabbit serum for primary antibodies as a negative control for each immunostaining experiment. Histological examinations were done at 8 ($n$ = 10), 20 ($n$ = 7), 32 ($n$ = 7), 64 ($n$ = 7) and 90 weeks ($n$ = 12) in wild-type and *Chm1*$^{-/-}$ mice. Quantitative analysis of the stained area was carried out by converting images to monochrome with optimum saturation and counting the black pixels using NIH Image software.

**Animals.** Wild-type ICR mice and Wistar rats were purchased from Japan CLEA. *Apoe*$^{-/-}$ mice, deficient in apolipoprotein E, were used as an atherosclerosis animal model. These mice are also known to show stage-related aortic stenosis[45]. These mice were purchased from the Jackson Laboratory and maintained on a normal diet. The genetic backgrounds of the *Chm1*$^{-/-}$ and age-matched control mice have been described previously[46]. TT2 embryonic stem cell clones[47] harboring the targeted *Chm1* coding region were identified by Southern-blot analysis and were aggregated with CD-1 single eight-cell embryos to generate chimeras as described previously[46]. Chimeras were crossed with C57BL/6 female mice to achieve germline transmission of the targeted allele. Offspring were genotyped either by Southern blotting or by PCR. Heterozygous *Chm1*$^{+/-}$ mice were backcrossed twice to C57/BL6 mice and were maintained. Homozygous *Chm1*$^{-/-}$ strains, kept until 90.2 ± 7.4 weeks old, were used for experimental procedures. Homozygous *Chm1*$^{+/+}$ littermates of the *Chm1*$^{-/-}$ strain were used as controls for experimental procedures to ensure that controls were age-matched and had the same genetic background as the knockout mice. All experimental procedures and protocols were approved by the Animal Care and Use Committee of Keio University, Japan.

**Echocardiography.** Transthoracic echocardiography was carried out with the Sonos 1000 echocardiogram (Hewlett-Packard) equipped with a 10-MHz linear-array transducer. The heart was imaged in two-dimensional and color Doppler mode in the long- and short-axis view at the level of the aortic valve[48]. M-mode scanning along the long axis view was examined, and the thickness of the interventricular septum and left ventricular free wall, left ventricular end-diastolic dimension, left ventricular end-systolic dimension, and ejection fraction were measured. The area trace of the aortic valve was also examined at each time point. An echocardiographic examination was done at 8 ($n$ = 10), 20 ($n$ = 10), 32 ($n$ = 10), 64 ($n$ = 10) and 90 weeks ($n$ = 12) in wild-type and *Chm1*$^{-/-}$ mice.

**Isolation of adult rat aortic VICs.** The hearts were dissected from anesthetized 5-week-old Wistar rats. The leaflet of the aortic valves was rapidly removed, chopped under a stereomicroscope, and used for the explant culture as previously described[5]. Pieces that measured 5 × 5 mm were cut from the tissue, placed in 12-well collagen coated dishes (Iwaki), and grown in M199 medium (Sigma-Aldrich) containing 10% fetal bovine serum. Conditioned medium was obtained from confluent VICs 3 d after changing the medium and used in further analyses.

**Human samples.** Samples comprising 24 aortic and 11 mitral valves (from 35 men; mean age, 59.9 ± 17.1 y) were collected from patients undergoing valve replacement due to valvular stenosis or regurgitation. Samples were fixed immediately after removal in formaldehyde and then embedded in paraffin. For controls, six micro- and macroscopically normal, noncalcified, smooth and pliable aortic and mitral valves were collected from three autopsied patients (mean age, 53.2 ± 19.2 years). The use of autopsied and surgical specimens of human tissue was approved by the institutional review board of Keio University.

**Statistical analysis.** Values are presented as the mean ± SEM. Statistical significance was evaluated using the unpaired student's $t$-test for comparisons between two mean values. Multiple comparisons between more than three groups were carried out using an ANOVA test. A value of $P < 0.05$ was considered significant.

**Other methods.** Other methods are described in the **Supplementary Methods** online.

**GenBank accession numbers.** Mouse *Chm1*, NM_010701; rat *Chm1*, NM_030854.

*Note: Supplementary information is available on the Nature Medicine website.*

ACKNOWLEDGMENTS

We thank Y. Nishizaki and S. Kondo for technical support. This study was supported in part by research grants from the Ministry of Education, Science and Culture, Japan, and by the Program for Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation.

AUTHOR CONTRIBUTIONS

This study was designed by K.F.; experiments were performed by M.Y., S.Y., K.M., K.K. and N.K.; the *Chm1* knockout mouse and antibody to Chm-I were made by C.S. and Y.H.; surgical specimens were collected by M.M., H.S. and R.Y.; the MMP experiment was performed by T.S. and Y.O.; specimens from *Apoe* knockout mice were distributed by M.S.; S.O. contributed to the writing of the paper.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/naturemedicine/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Hammon, J.W., Jr., O'Sullivan, M.J., Oury, J. & Fosburg, R.G. Allograft cardiac valves. A view through the scanning electron microscope. *J. Thorac. Cardiovasc. Surg.* **68**, 352–360 (1974).
2. Soini, Y., Salo, T. & Satta, J. Angiogenesis is involved in the pathogenesis of nonrheumatic aortic valve stenosis. *Hum. Pathol.* **34**, 756–763 (2003).
3. Yamauchi, R. *et al.* Upregulation of SR-PSOX/CXCL16 and recruitment of CD8+ T cells in cardiac valves during inflammatory valvular heart disease. *Arterioscler. Thromb. Vasc. Biol.* **24**, 282–287 (2004).
4. Filip, D.A., Radu, A. & Simionescu, M. Interstitial cells of the heart valves possess characteristics similar to smooth muscle cells. *Circ. Res.* **59**, 310–320 (1986).
5. Lester, W., Rosenthal, A., Granton, B. & Gotlieb, A.I. Porcine mitral valve interstitial cells in culture. *Lab. Invest.* **59**, 710–719 (1988).
6. Gotlieb, A.I., Rosenthal, A. & Kazemian, P. Fibroblast growth factor 2 regulation of mitral valve interstitial cell repair *in vitro*. *J. Thorac. Cardiovasc. Surg.* **124**, 591–597 (2002).
7. Akiyama, H. *et al.* Essential role of Sox9 in the pathway that controls formation of cardiac valves and septa. *Proc. Natl. Acad. Sci. USA* **101**, 6502–6507 (2004).
8. Ranger, A.M. *et al.* The transcription factor NF-ATc is essential for cardiac valve formation. *Nature* **392**, 186–190 (1998).
9. Rajamannan, N.M. *et al.* Human aortic valve calcification is associated with an osteoblast phenotype. *Circulation* **107**, 2181–2184 (2003).
10. Chan-Thomas, P.S., Thompson, R.P., Robert, B., Yacoub, M.H. & Barton, P.J. Expression of homeobox genes Msx-1 (Hox-7) and Msx-2 (Hox-8) during cardiac development in the chick. *Dev. Dyn.* **197**, 203–216 (1993).
11. Sugi, Y., Yamamura, H., Okagawa, H. & Markwald, R.R. Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice. *Dev. Biol.* **269**, 505–518 (2004).
12. Camenisch, T.D. *et al.* Temporal and distinct TGFbeta ligand requirements during mouse and avian endocardial cushion morphogenesis. *Dev. Biol.* **248**, 170–181 (2002).
13. Ikeda, T. *et al.* The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. *Arthritis Rheum.* **50**, 3561–3573 (2004).
14. Hiraki, Y. [Molecular cloning of a novel cartilage-specific functional matrix, chondromodulin-I, and its role in endochondral bone formation]. *Seikagaku* **63**, 1449–1454 (1991).
15. Funaki, H. *et al.* Expression and localization of angiogenic inhibitory factor, chondromodulin-I, in adult rat eye. *Invest. Ophthalmol. Vis. Sci.* **42**, 1193–1200 (2001).
16. Azizan, A., Holaday, N. & Neame, P.J. Post-translational processing of bovine chondromodulin-I. *J. Biol. Chem.* **276**, 23632–23638 (2001).
17. Hiraki, Y. *et al.* Identification of chondromodulin I as a novel endothelial cell growth inhibitor. Purification and its localization in the avascular zone of epiphyseal cartilage. *J. Biol. Chem.* **272**, 32419–32426 (1997).
18. Inoue, H., Kondo, J., Koike, T., Shukunami, C. & Hiraki, Y. Identification of an autocrine chondrocyte colony-stimulating factor: chondromodulin-I stimulates the

© 2006 Nature Publishing Group http://www.nature.com/naturemedicine

colony formation of growth plate chondrocytes in agarose culture. *Biochem. Biophys. Res. Commun.* **241**, 395–400 (1997).
19. Shukunami, C. & Hiraki, Y. Expression of cartilage-specific functional matrix chondromodulin-I mRNA in rabbit growth plate chondrocytes and its responsiveness to growth stimuli *in vitro*. *Biochem. Biophys. Res. Commun.* **249**, 885–890 (1998).
20. Hiraki, Y., Kono, T., Sato, M., Shukunami, C. & Kondo, J. Inhibition of DNA synthesis and tube morphogenesis of cultured vascular endothelial cells by chondromodulin-I. *FEBS Lett.* **415**, 321–324 (1997).
21. Zacks, S. *et al.* Characterization of Cobblestone mitral valve interstitial cells. *Arch. Pathol. Lab. Med.* **115**, 774–779 (1991).
22. Oshima, Y. *et al.* Expression and localization of tenomodulin, a transmembrane type chondromodulin-I-related angiogenesis inhibitor, in mouse eyes. *Invest. Ophthalmol. Vis. Sci.* **44**, 1814–1823 (2003).
23. Hiraki, Y. *et al.* Molecular cloning of human chondromodulin-I, a cartilage-derived growth modulating factor, and its expression in Chinese hamster ovary cells. *Eur. J. Biochem.* **260**, 869–878 (1999).
24. Shukunami, C., Iyama, K., Inoue, H. & Hiraki, Y. Spatiotemporal pattern of the mouse chondromodulin-I gene expression and its regulatory role in vascular invasion into cartilage during endochondral bone formation. *Int. J. Dev. Biol.* **43**, 39–49 (1999).
25. Shukunami, C., Yamamoto, S., Tanabe, T. & Hiraki, Y. Generation of multiple transcripts from the chicken chondromodulin-I gene and their expression during embryonic development. *FEBS Lett.* **456**, 165–170 (1999).
26. Dietz, U.H., Ziegelmeier, G., Bittner, K., Bruckner, P. & Balling, R. Spatio-temporal distribution of chondromodulin-I mRNA in the chicken embryo: expression during cartilage development and formation of the heart and eye. *Dev. Dyn.* **216**, 233–243 (1999).
27. Sachdev, S.W. *et al.* Sequence analysis of zebrafish chondromodulin-1 and expression profile in the notochord and chondrogenic regions during cartilage morphogenesis. *Mech. Dev.* **105**, 157–162 (2001).
28. Chalajour, F. *et al.* Angiogenic activation of valvular endothelial cells in aortic valve stenosis. *Exp. Cell Res.* **298**, 455–464 (2004).
29. Takeda, S., Bonnamy, J.P., Owen, M.J., Ducy, P. & Karsenty, G. Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes Dev.* **15**, 467–481 (2001).
30. Dawson, D.W. *et al.* Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science* **285**, 245–248 (1999).
31. Shworak, N.W. Angiogenic modulators in valve development and disease: does valvular disease recapitulate developmental signaling pathways? *Curr. Opin. Cardiol.* **19**, 140–146 (2004).
32. Mohler, E.R., III. *et al.* Bone formation and inflammation in cardiac valves. *Circulation* **103**, 1522–1528 (2001).
33. Mazzone, A. *et al.* Neoangiogenesis, T-lymphocyte infiltration, and heat shock protein-60 are biological hallmarks of an immunomediated inflammatory process in end-stage calcified aortic valve stenosis. *J. Am. Coll. Cardiol.* **43**, 1670–1676 (2004).
34. O'Brien, K.D., McDonald, T.O., Chait, A., Allen, M.D. & Alpers, C.E. Neovascular expression of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 in human atherosclerosis and their relation to intimal leukocyte content. *Circulation* **93**, 672–682 (1996).
35. Moulton, K.S. *et al.* Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. *Proc. Natl. Acad. Sci. USA* **100**, 4736–4741 (2003).
36. Otto, C.M., Kuusisto, J., Reichenbach, D.D., Gown, A.M. & O'Brien, K.D. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. *Circulation* **90**, 844–853 (1994).
37. O'Brien, K.D. *et al.* Osteopontin is expressed in human aortic valvular lesions. *Circulation* **92**, 2163–2168 (1995).
38. Agmon, Y. *et al.* Aortic valve sclerosis and aortic atherosclerosis: different manifestations of the same disease? Insights from a population-based study. *J. Am. Coll. Cardiol.* **38**, 827–834 (2001).
39. O'Brien, K.D. *et al.* Association of angiotensin-converting enzyme with low-density lipoprotein in aortic valvular lesions and in human plasma. *Circulation* **106**, 2224–2230 (2002).
40. Pohle, K. *et al.* Association of cardiovascular risk factors to aortic valve calcification as quantified by electron beam computed tomography. *Mayo Clin. Proc.* **79**, 1242–1246 (2004).
41. Rabkin, E. *et al.* Activated interstitial myofibroblasts express catabolic enzymes and mediate matrix remodeling in myxomatous heart valves. *Circulation* **104**, 2525–2532 (2001).
42. Fondard, O. *et al.* Extracellular matrix remodelling in human aortic valve disease: the role of matrix metalloproteinases and their tissue inhibitors. *Eur. Heart J.* **26**, 1333–1341 (2005).
43. Enomoto, H. *et al.* Vascular endothelial growth factor isoforms and their receptors are expressed in human osteoarthritic cartilage. *Am. J. Pathol.* **162**, 171–181 (2003).
44. Fujimoto, N. & Iwata, K. Use of EIA to measure MMPs and TIMPs. *Methods Mol. Biol.* **151**, 347–358 (2001).
45. Tanaka, K. *et al.* Age-associated aortic stenosis in apolipoprotein E-deficient mice. *J. Am. Coll. Cardiol.* **46**, 134–141 (2005).
46. Nakamichi, Y. *et al.* Chondromodulin I is a bone remodeling factor. *Mol. Cell. Biol.* **23**, 636–644 (2003).
47. Kyuwa, S., Xiao, Y., Toyoda, Y. & Sato, E. Characterization of embryonic stem-like cell lines derived from embryoid bodies. *Exp. Anim.* **46**, 11–16 (1997).
48. Litwin, S.E., Katz, S.E., Morgan, J.P. & Douglas, P.S. Serial echocardiographic assessment of left ventricular geometry and function after large myocardial infarction in the rat. *Circulation* **89**, 345–354 (1994).

© 2006 Nature Publishing Group http://www.nature.com/naturemedicine

特　集
心筋症
―新しく見直された
病態・治療

心筋症の発症機序と病態

# たこつぼ型心筋症の成因と診断

順天堂大学医学部附属順天堂医院循環器内科　山田　京志
*Yamada, Atsushi*
同　　助教授　河合　祥雄
*Kawai, Sachio*

**Takotsubo (Ampulla) cardiomyopathy**

*KEY WORDS*
たこつぼ心筋症／ST上昇／左室収縮異常

## はじめに

たこつぼ心筋症（たこつぼ心筋障害）とは，1990年に広島市民病院の佐藤らによって名付けられた病態で，「急性心筋梗塞に類似した胸痛と心電図所見を有し，左室心尖部を中心とした無収縮・つぼ型拡張を示すが，急性期の冠状動脈造影で有意な狭窄はなく，心電図や壁運動異常が短期間で正常化する病態を指すもの」とされている[1]。近年，冠状動脈造影および左室造影検査の普及および検査施行年齢の高齢化に伴い多くの本例が見い出され，稀な疾患ではないことが判明している。

また，これまで統一の診断基準は存在せず，個々の施設での症例報告が主であったが，近年わが国だけでなく，欧米においても多数報告例がみられるようになっている[2-6]。2004年，特発性心筋症調査研究班「たこつぼ心筋症（たこつぼ心筋障害）調査研究グループ」により診断の手引き[7]（表）が発表され，病態生理の究明，治療方法・予後の解明などへの利用が期待されている。

## 疫　学

これまで本疾患の統一された診断基準は存在しなかったため，正確な頻度は不明であるが，2000年わが国における学会での約250症例の報告をまとめた河合らの報告[8]によると，急性心筋梗塞の約1～2％に認められ，性比は1：

- 精動ストレスなどの臨床背景を有し，急性心筋梗塞に類似した臨床経過をとることが多い。
- 中年から高齢女性に好発する。

表　たこつぼ心筋障害(たこつぼ心筋症)診断の手引き

Ⅰ．定　義

たこつぼ心筋障害(たこつぼ心筋症)：takotsubo (ampulla) cardiomyopathy とは，急性発症の原因不明の左室心尖部バルーン状拡張(無収縮)を呈する症例を指す。本症では左心室はあたかも「たこつぼ」様の形態をとる。心尖部の無収縮は，数週～1ヶ月以内に，大部分の症例において，ほぼ正常化する。
心室収縮異常は主に左心室に生じるが，右心室にも認められる例がある。心室流出路機能性狭窄(圧格差，血流速度亢進，心雑音)も観察される。

(注)他の原因，たとえば脳血管障害患者が，本疾患と同様の心室収縮異常を呈する場合には「脳血管障害に合併したたこつぼ心筋障害」として，特発性と区別して扱う。

Ⅱ．除外項目

たこつぼ心筋障害(たこつぼ心筋症)の診断にあたっては，以下の病変，疾患による異常を除外しなければならない。

a．冠状動脈の器質的有意狭窄または攣縮。特に左心室心尖部を含めて広範に還流する左前下降枝病変による急性心筋梗塞(冠状動脈造影は，急性期の造影が望ましいが，慢性期に行い有意狭窄病変がないか，心室収縮異常形態に関する病変がないことを確認することが必要である)
b．脳血管障害
c．褐色細胞腫
d．ウイルス性もしくは特発性心筋炎

(注)冠状動脈病変の除外には冠状動脈造影が必須である。脳血管疾患，褐色細胞腫などでたこつぼ様の心筋障害を合併することがある。

Ⅲ．診断の参考事項

1．症　状：急性冠症候群に類似の胸痛，呼吸困難，症状なく発症することがある。
2．契　機：精神的ストレス，身体的侵襲，明らかな契機なしに発症することもある。
3．年齢・性差：高齢者ことに女性に多い傾向が知られる。
4．心室形態：心室造影または心エコー図における心尖部バルーン状拡張とその速やかな改善。
5．心電図：発症直後はST上昇がみられることがある。その後，典型例では広範な誘導でT波が陰転化し，次第に陰性部分が深くなりQT延長を伴う。この変化は徐々に回復するが，陰性T波は数ヵ月続くことがある。急性期に異常Q波やQRS電位差の変化を認めることもある。
6．検査項目：典型例においては，心筋逸脱酵素値上昇は中程度以下に留まる。
7．心臓核医学検査：心臓核医学検査において，異常を認める例がある。
8．予　後：大部分が速やかに快復するが，肺水腫や他の後遺症を呈する例，死亡例がある。

7と女性に多く，発症年齢は男性65.9±9.1歳，女性68.6±12.2歳であり，女性で3年年長であるとしている。また，2004年2月土橋らの報告[3]では，急性冠症候群150～200例に1例(0.5～0.75%)で地域差はなく，性比は12：76で女性に多い。好発年齢は67±13歳で，特に女性では高齢発症であるとされている。さらに，発症契機および関連する事象として，①劇的で著しい感情変化が予想される精神的背景(20%)，②何らかの苦痛を伴うであろう非心臓

■ 冠状動脈多枝攣縮，カテコラミン心筋症，冠状動脈微小循環障害，心筋炎などの成因が考えられている。

■ 多くは胸痛で発症するが，胸部不快感など軽微なものから呼吸困難，ショック状態など重症例も存在する。

手術・処置ないし前処置(12%)，③疾患の増悪(13%)などが報告されている。一方，④明確な誘因が明らかでないものも少なからず報告(26%)されている。

## 成　因

佐藤ら[1]は，本疾患を「急性心筋梗塞に類似した胸痛と心電図変化を有しながら，急性期の冠状動脈造影で有意な狭窄はなく，左室の壁運動異常が1つの冠動脈の支配領域を超えて心尖部を中心とした広範囲に及び，左室造影において「たこつぼ型」を呈し，かつその壁運動異常は1－2週間でほぼ正常化する」とし，その成因を多枝攣縮による気絶心筋(stunned myocardium)に求めているが，その後，同様の症例が多数報告されるに伴い，いくつかの成因が考えられている。

### 1．冠状動脈多枝攣縮

一過性の心筋虚血により，心筋壊死は生じずに収縮力低下を後遺する状態を気絶心筋というが，左前下降枝を含む多枝の冠状動脈が同時に攣縮が起これば，心尖部を中心とした広範囲で壁運動異常を生じうる。佐藤ら[1]はたこつぼ型心筋障害の急性期に冠状動脈攣縮誘発試験を行い，多くの症例で多枝攣縮が証明されたと報告し，①欧米に比べわが国での報告が多いこと，②情動ストレスとの関連が多いことから，本疾患の成因を冠状動脈多枝攣縮に求めている。

安部らは急性期に造影検査を施行した9例中7例にアセチルコリン負荷誘発試験を施行し，2例で陰性，4例でびまん性冠状動脈攣縮，1例に右冠状動脈局所的攣縮を認めたが，5例の冠状動脈攣縮例において，症状や心電図変化を欠いたと報告[2]している。現在，たこつぼ型心筋障害慢性期の攣縮誘発率は10～30%である。

河合らは本例における心筋生検所見において，発症早期に細胞浸潤，次いで心筋脱落，巣状の線維症の変化がみられたことより，定義上，組織所見を欠く気絶心筋ではないと主張[3]している。

### 2．カテコラミン心筋症

内因性カテコラミン増加により心筋障害が生じるが，精神的あるいは身体的ストレスが誘因であることが多いことから，本例を成因とする報告もある。しかしながら，心臓神経の分布は心基部に多く，①左室収縮異常の説明不可能であること，②褐色細胞腫での合併例が少ないこと，③急性期のカテコラミン値も正常であることが多いなど，さらなる補助仮説を要する。

### 3．冠状動脈微小循環障害

本例において，①急性期の冠状動脈造影で冠血流速度が低下する例がみられること，②Flow wire studyで冠血流予備能が低下する例が報告[4]されていること，③心筋コントラストエコーで可逆性の陰影欠損をみる例がある[5]ことから，筋層内の循環障害による気絶心筋であるとする説も存在する。しかし，臨床的に細動脈レベルの攣縮とそれに基づく気絶心筋は仮説の域を出ず，実証されていない。

### 4．心筋炎

急性心筋炎の中には，たこつぼ型心筋障害に類似した壁運動障害を呈するものも報告されているが，先行感染やウイルス抗体価の上昇を欠き，病理組織学的にも心筋炎像を認めるものも少ない。

その他に，心臓交感神経過剰反応(障害と亢進)，心臓心筋炎に起因する冠攣縮に心筋脂肪酸代謝障害が加わる可能性などが想定されている。

## 症　状

「急性心筋梗塞を疑わせる胸痛」が一般的とされるが，胸部不快感，圧迫感，背部痛など軽微な症状であることも多く，疾患の増悪などに際しては呼吸困難，血圧低下，心電図異常として発症，発見されることがある。また，ショック状態，肺水腫で発症する重症例も存在することが知られており，原因不明の突然死に含まれる外傷性ストレスと関連したものがこの病態であっ

- 心電図ではST上昇，Q波出現を示し，陰性T波に移行する。
- 左心室心尖部を中心とする収縮低下と心基部過収縮を呈する。
- 冠状動脈造影では有意狭窄を認めない。

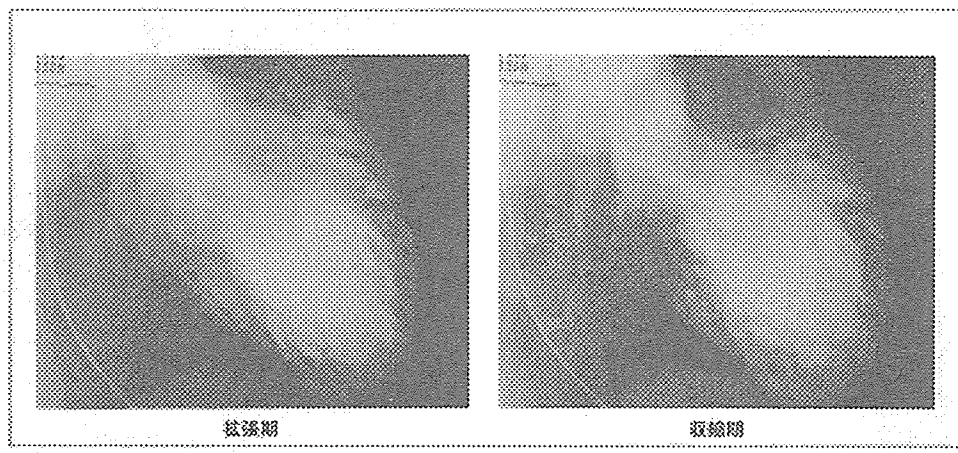


**図1 左心室造影像**
急性期の左心室造影検査において，AHA分類#2～#4の広範囲に無収縮，心基部(#1, #5)に過収縮を認める。
AHA：American Heart Association

たとも考えられる。

## 検査所見

### 1．心電図

本疾患では，発症早期に急性心筋梗塞と類似したST上昇とQ波の出現を示すことが知られている。大多数で広範な前壁中隔梗塞を示す誘導部位でのST上昇がみられているが，発症翌日や2～3日後から比較的長期にわたって左右対称性の陰性T波を示すことが知られ，広範・巨大陰性T波のみを認める例も報告されている。本症の経時的変化ではST上昇の後陰性T波に移行する。

土橋らの報告[2]でも，その90％がST上昇を，27％が異常Q波を認めている。ST上昇に関してみると，広範な胸部誘導においてST上昇を認める例がほとんどであった。また少数ではあったが，下壁誘導でのみST上昇を示す例や，V1，V2ではST上昇を認めない例などが報告されている。

### 2．左心室造影(図1)/心臓超音波検査

左心室心尖部を中心とする収縮低下と心基部の過収縮により，収縮期左室造影においてたこ漁に用いられる"たこつぼ"のような形態を示すため，「たこつぼ」の名称が使われている。これまでわが国では「たこつぼ型心筋症」，「たこつぼ心筋」，「たこつぼ心筋障害」，「たこつぼ心機能障害」など多様な名称が用いられていたが，特発性心筋症調査研究班「たこつぼ心筋症(たこつぼ心筋障害)調査研究グループ」の調査では，和文名称として「たこつぼ心筋症」より「たこつぼ心筋障害」が適切とされた。しかし，欧米人に対しては「たこつぼ」では想像困難と思われたため，ampulla cardiomyopathyが提唱されている[3]。

こうした壁運動異常は必ずしも緊急左心室造影でなく，心臓超音波検査においても確認することができる。

### 3．冠状動脈造影検査

本疾患は，急性心筋梗塞に類似した症状と心電図所見をもって発症するため，緊急心臓カテーテル検査が施行されることが多く，その結果，冠状動脈は正常あるいは有意狭窄を認めないことが特徴である。しかし，多数の症例報告がなされるにつれ，冠状動脈攣縮

■ 心筋障害が個々の心筋細胞を標的とし，病変は単一心筋細胞の障害およびその集合体から成る。
■ 病理組織学的に気絶心筋や心筋炎とは異なる。

やslow flowの存在が示されてきている。

土手ら[30]は，急性期にエルゴノビンやアセチルコリンを用いた誘発試験で，多枝攣縮の存在を証明している。また，Kurisuら[30]は本例において，3枝とも冠状動脈血流速度が低下［TIMI (thrombolysis in myocardial infarction) frame count増加］していたと報告している。

### 4．核医学検査

Tl (thallium) 心筋シンチグラムでは急性期に壁運動の低下している部分で集積欠損がみられ，慢性期に正常化する。BMIPP (β-methyl-p-iodophenyl pentadecanoic acid) 心筋シンチグラムでは，Tl心筋シンチグラムより広範な集積低下を示す例が多い。MIBG (meta-iodobenzyl guanidine) 心筋シンチグラムでは壁運動低下部位での集積低下がみられる例と，収縮の低下していない心基部を含めて心臓全体の取り込みが消失している例がある。いずれも経過とともに改善し，慢性期には正常化するのが一般的である。

### 5．心室内圧較差

心臓超音波検査におけるドップラー計測や心室造影検査時に心室内圧較差の存在する例も報告され，壁運動改善とともに心室内圧較差も減少することが確認されている。

## 病理所見

現在，本疾患の成因として前述のごとく，冠状動脈多枝攣縮，冠状動脈微小循環障害，カテコラミン心筋症，心筋炎の関与など多数挙げられているが，河合らは病理組織学的所見についてまとめ，さらにその成因について考察を加えている[29]。本症の心筋病変の特徴は，「心筋障害が個々の心筋細胞を標的として生じており，病変は単一心筋細胞の障害およびその集合体より成ることである」（図2）とし，心筋内微小循環攣縮による気絶心筋や，通常の心筋炎とは異なると報告している。さらに，視床下部電気刺激ネコ急死モデルにみられる心筋病変に類似するとし，本症での原因不明突然死の外傷ストレスとの関連を示唆した。

## まとめ

本症の存在は，注意深い臨床医により古くから気付かれており[30-38]，近年でも巨大陰性T波を呈する疾患の中で論じられていたが，1990年広島市民病院の佐藤らによる（たこ）つぼ型とのnamingにより一般臨床医の注目を集めた。現在，逆たこつぼ現象も知られており，心室収縮異常部位に関してもまとまりがなく，報告者によりその内容はさまざまである。2005年までに特発性心筋症調査研究班「たこつぼ心筋症（たこつぼ心筋障害）調査研究グループ」により診断の手引きが発表される

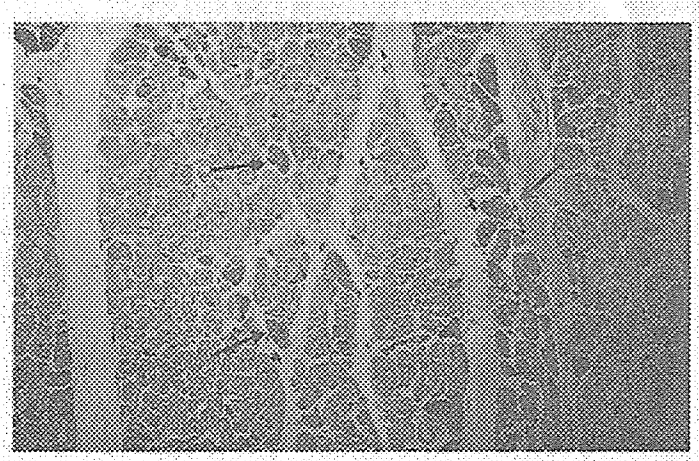

**図2　心筋生検組織像**
正常心筋は桃色に分別され，障害された心筋細胞のみ染まる。個々の心筋細胞障害像を認める（→）。
luxol fast blue染色，200倍。

- 心電図ではST上昇，Q波出現を示し，陰性T波に移行する。
- 左心室心尖部を中心とする収縮低下と心基部過収縮を呈する。
- 冠状動脈造影では有意狭窄を認めない。

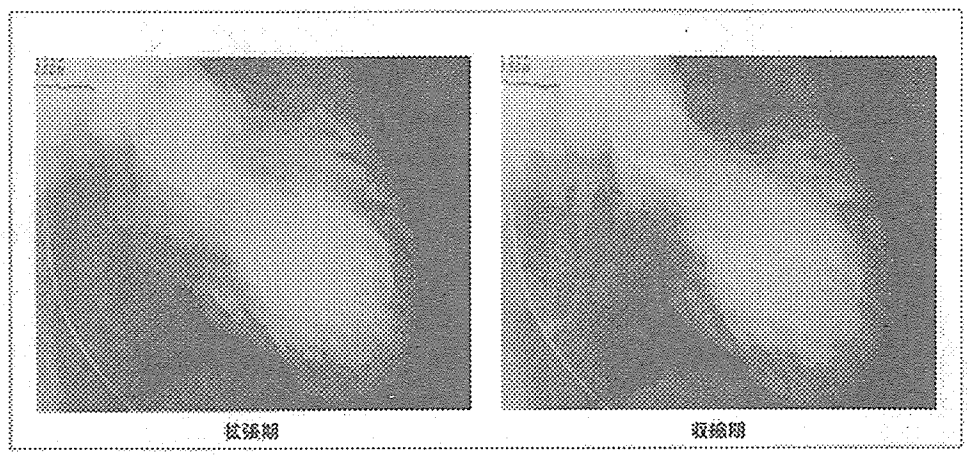


**図1 左心室造影像**
急性期の左心室造影検査において，AHA分類#2～#4の広範囲に無収縮，心基部(#1, #5)に過収縮を認める。
AHA；American Heart Association

たとも考えられる。

## 検査所見

### 1．心電図

本疾患では，発症早期に急性心筋梗塞と類似したST上昇とQ波の出現を示すことが知られている。大多数で広範な前壁中隔梗塞を示す誘導部位でのST上昇がみられているが，発症翌日や2～3日後から比較的長期にわたって左右対称性の陰性T波を示すことが知られ，広範・巨大陰性T波のみを認める例も報告されている。本症の経時的変化ではST上昇の後陰性T波に移行する。

土橋らの報告[2]でも，その90％がST上昇を，27％が異常Q波を認めている。ST上昇に関してみると，広範な胸部誘導においてST上昇を認める例がほとんどであった。また少数ではあったが，下壁誘導でのみST上昇を示す例や，V1，V2ではST上昇を認めない例などが報告されている。

### 2．左心室造影(図1)/心臓超音波検査

左心室心尖部を中心とする収縮低下と心基部の過収縮により，収縮期左室造影においてたこ壺に用いられる"たこつぼ"のような形態を示すため，「たこつぼ」の名称が使われている。これまでわが国では「たこつぼ型心筋症」，「たこつぼ心筋」，「たこつぼ心筋障害」，「たこつぼ心機能障害」など多様な名称が用いられていたが，特発性心筋症調査研究班「たこつぼ心筋症(たこつぼ心筋障害)調査研究グループ」の調査では，和文名称として「たこつぼ心筋症」より「たこつぼ心筋障害」が適切とされた。しかし，欧米人に対しては「たこつぼ」では想像困難と思われたため，ampulla cardiomyopathyが提唱されている[3]。

こうした壁運動異常は必ずしも緊急左心室造影でなく，心臓超音波検査においても確認することができる。

### 3．冠状動脈造影検査

本疾患は，急性心筋梗塞に類似した症状と心電図所見をもって発症するため，緊急心臓カテーテル検査が施行されることが多く，その結果，冠状動脈は正常あるいは有意狭窄を認めないことが特徴である。しかし，多数の症例報告がなされるにつれ，冠状動脈攣縮

■ 診断の手引き作成により，今後統一された疾患概念のもと調査が進むことが望まれる。

予定であり，今後統一された疾患概念のもと，調査研究が進むことが望まれている。

●文 献


1) 佐藤 光，立石博信，内田俊明，他：多枝spasmにより特異な左室造影「ク ボ型」を示したstunned myocardium．児玉和久，土屋一夫，堀 正二編．臨床からみた心筋細胞障害―虚血から心不全まで．東京，春学評論社，1990
2) Seth PS, Aurigemma GP, Krasnow JM, et al : A syndrome of transient left ventricular apical wall motion abnormality in absence of coronary disease : a perspective from the United States. Cardiology 100 : 61-66, 2003
3) Desmet WJ, Adriaenssens BF, Dens JA : Apical ballooning of the left ventricle : first series in white patients. Heart 89 : 1027-1031, 2003
4) Turnage WS, Zeller FA, Divertie GD : Dynamic left ventricular outflow tract obstruction. Diagnosis by transesophageal echocardiography in a critically ill patient. Chest 104 : 1629-1631, 1993
5) Villareal RP, Achari A, Wilansky S, et al : Anteroapical stunning and left ventricular outflow tract obstruction. Mayo Clin Proc 76 : 79-83, 2001
6) Landesman KA, Sadaniantz A : Left ventricular outflow tract obstruction after myocardial infarction due to a hyperdynamic basal septum. Echocardiography 18 : 291-294, 2001
7) 特発性心筋症調査研究班「たこつぼ心筋症(たこつぼ心筋障害)調査研究グループ」(厚生労働省特定疾患特発性心筋症調査研究班)．
8) 河合祥雄：たこつぼ心筋症，またはたこつぼ(Ampulla or Amphora)心筋症―本邦学会報告例の検討―．呼吸と循環 46：1237-1248，2000
9) Tsuchihashi K, Ueshima K, Uchida T, et al : Transient left ventricular apical ballooning without coronary artery stenosis : a novel heart syndrome mimicking acute myocardial infarction. Angina Pectoris-Myocardial Infarction Investigations in Japan. J Am Coll Cardiol 38 : 11-18, 2001
10) 安部美輝，近藤真言，松岡良太，他：たこつぼ型心筋障害の核医学的・冠血流的・組織学的評価．心臓 34：210-216，2002
11) Yanagi S, Nagae K, Yoshida K, et al : Evaluation of coronary flow reserve using Doppler guide wire in patients with ampulla cardiomyopathy : three case reports. J Cardiol 39 : 305-312, 2002
12) 宇野感成，中村文隆，阿古浮哉，他：急性期に心筋コントラストエコーを施行したたこつぼ心筋障害の一例．第14回超音波血流研究会抄録集．演題9．田辺製薬株式会社東京事業所，東京，2001
13) 土手慶五，佐藤 光，立石博信，他：経動脈的PTCA後急性冠閉塞により広範な心筋stunningを呈した5例．J Cardiol 21：203-214，1991
14) Kurisu S, Inoue I, Kawagoe T, et al : Myocardial perfusion and fatty acid metabolism in patients with Tako-Tsubo-like left ventricular dysfunction. J Am Coll Cardiol 41 : 743-748, 2003
15) 河合祥雄，山田享志，鈴木宏昌：たこつぼ心筋障害の病理．Heart View 18：159-166，2004
16) Kuramoto K, Matsushita S, Murakami M : Acute reversible myocardial infarction after blood transfusion in the aged. Jpn Heart J 18 : 191-201, 1977
17) 佐々貴己，岸井美之，曽根孝仁，他：術後症例にみられる一過性心筋梗塞様心電図の臨床的意義．心臓 15：669-678，1983
18) 時岡正明，三浦意人，正岡圭子，他：手術後に認められた一過性心筋梗塞様心電図と断層心エコー図上の広範なアシナジー．J Cardiography 15：639-653，1983

Circulation
JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®
Learn and Live℠

# Mineralocorticoid Receptor Antagonism Ameliorates Left Ventricular Diastolic Dysfunction and Myocardial Fibrosis in Mildly Symptomatic Patients With Idiopathic Dilated Cardiomyopathy: A Pilot Study

Hideo Izawa, Toyoaki Murohara, Kohzo Nagata, Satoshi Isobe, Hiroyuki Asano, Tetsuya Amano, Sahoko Ichihara, Tomoko Kato, Satoru Ohshima, Yosuke Murase, Shigeo Iino, Koji Obata, Akiko Noda, Kenji Okumura and Mitsuhiro Yokota

*Circulation* 2005;112;2940-2945
DOI: 10.1161/CIRCULATIONAHA.105.571653
Circulation is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
Copyright © 2005 American Heart Association. All rights reserved. Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/cgi/content/full/112/19/2940

Subscriptions: Information about subscribing to Circulation is online at http://circ.ahajournals.org/subsriptions/

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, 351 West Camden Street, Baltimore, MD 21202-2436. Phone 410-5280-4050. Fax: 410-528-8550. Email: journalpermissions@lww.com

Reprints: Information about reprints can be found online at http://www.lww.com/static/html/reprints.html

Downloaded from circ.ahajournals.org at Nagoya Univ Medi on January 28, 2007

# Mineralocorticoid Receptor Antagonism Ameliorates Left Ventricular Diastolic Dysfunction and Myocardial Fibrosis in Mildly Symptomatic Patients With Idiopathic Dilated Cardiomyopathy

## A Pilot Study

Hideo Izawa, MD, PhD; Toyoaki Murohara, MD, PhD; Kohzo Nagata, MD, PhD; Satoshi Isobe, MD, PhD; Hiroyuki Asano, MD; Tetsuya Amano, MD, PhD; Sahoko Ichihara, MD, PhD; Tomoko Kato, MD, PhD; Satoru Ohshima, MD; Yosuke Murase, MD; Shigeo Iino, MD, PhD; Koji Obata, PhD; Akiko Noda, PhD; Kenji Okumura, MD, PhD; Mitsuhiro Yokota, MD, PhD

*Background*—Mineralocorticoid receptor antagonism reduces mortality associated with heart failure by mechanisms that remain unclear. The effects of the mineralocorticoid receptor antagonist spironolactone on left ventricular (LV) function and chamber stiffness associated with myocardial fibrosis were investigated in mildly symptomatic patients with idiopathic dilated cardiomyopathy (DCM).

*Methods and Results*—Twenty-five DCM patients with a New York Heart Association functional class of I or II were examined before and after treatment with spironolactone for 12 months. LV pressures and volumes were measured simultaneously, and LV endomyocardial biopsy specimens were obtained. Serum concentrations of the carboxyl-terminal propeptide (PIP) and carboxyl-terminal telopeptide (CITP) of collagen type I were measured. The patients were divided into 2 groups on the basis of the serum PIP/CITP ratio (≤35, group A, n=12; >35, group B, n=13), an index of myocardial collagen accumulation. LV diastolic chamber stiffness, the collagen volume fraction, and abundance of collagen type I and III mRNAs in biopsy tissue were greater and the LV early diastolic strain rate (tissue Doppler echocardiography) was smaller in group B than in group A at baseline. These differences and the difference in PIP/CITP were greatly reduced after treatment of patients in group B with spironolactone, with treatment having no effect on these parameters in group A. The collagen volume fraction was significantly correlated with PIP/CITP, LV early diastolic strain rate, and LV diastolic chamber stiffness for all patients before and after treatment with spironolactone.

*Conclusions*—Spironolactone ameliorated LV diastolic dysfunction and reduced chamber stiffness in association with regression of myocardial fibrosis in mildly symptomatic patients with DCM. These effects appeared limited, however, to patients with increased myocardial collagen accumulation. (*Circulation.* **2005;112:2940-2945.**)

**Key Words:** biopsy ■ cardiomyopathy ■ collagen ■ drugs ■ heart failure

Dilated cardiomyopathy (DCM) is characterized by progressive left ventricular (LV) dilation and greatly impaired LV systolic function, eventually culminating in end-stage congestive heart failure and death.[1,2] Despite recent improvements in medical management, the overall prognosis of individuals with DCM is still poor. The Randomized Aldactone Evaluation Study (RALES) demonstrated a 30% reduction in the mortality of patients with a New York Heart Association (NYHA) functional class of III or IV after treatment with the mineralocorticoid receptor antagonist spironolactone in addition to standard therapy for congestive heart failure.[3] More recently, treatment with another mineralocorticoid receptor antagonist, eplerenone, was found to reduce cardiovascular mortality by 17% in patients with post–acute myocardial infarction.[4] However, the possible therapeutic efficacy of mineralocorticoid receptor antagonists in patients with mildly symptomatic heart failure such as those with an NYHA functional class of I or II has not been previously examined.

The mechanisms of the beneficial effect of mineralocorticoid receptor antagonism in patients with heart failure also remain to be fully elucidated. Given that aldosterone pro-

Received September 27, 2004; de novo received June 27, 2005; revision received August 9, 2005; accepted August 22, 2005.

From the Department of Cardiology, Nagoya University Graduate School of Medicine, Nagoya (H.I., T.M., S. Isobe, H.A., T.K., S.O., Y.M., S. Iino, K. Okumura); Nagoya University School of Health Sciences, Nagoya (K.N., A.N.); Department of Cardiovascular Genome Science, Nagoya University School of Medicine, Nagoya (K. Obata, M.Y.); and Department of Human Functional Genomics, Life Science Research Center, Mie University, Tsu (S. Ichihara), Japan.

Correspondence to Hideo Izawa, MD, PhD, Department of Cardiology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466–8550, Japan. E-mail izawa@med.nagoya-u.ac.jp

© 2005 American Heart Association, Inc.

*Circulation* **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.105.571653**

motes myocardial fibrosis in animals and humans,[5–9] one of the underlying mechanisms of the therapeutic effect of mineralocorticoid receptor antagonism in chronic heart failure is thought to be prevention of this action of aldosterone.[10] Although mineralocorticoid receptor antagonism was found to induce a marked reduction in the serum concentrations of markers of myocardial fibrosis in patients with chronic heart failure, direct clinical evidence of regression of myocardial fibrosis induced by such treatment has been lacking.[11,12] Regression of myocardial fibrosis in response to treatment with a mineralocorticoid receptor antagonist would be expected to result in amelioration of LV diastolic dysfunction, which has been commonly detected in patients with DCM by Doppler echocardiography, especially tissue Doppler imaging.[13,14]

We have now investigated whether treatment with the mineralocorticoid receptor antagonist spironolactone induces regression of myocardial fibrosis in DCM patients with an NYHA functional class of I or II by 3 independent methods: histomorphometric assessment of myocardial collagen content (collagen volume fraction [CVF]), examination of the expression of collagen types I and III, and biochemical measurement of myocardial collagen accumulation. We also determined whether regression of myocardial fibrosis is associated with improvement in LV diastolic function.

## Methods

### Patients

The study protocol was approved by the Ethics Review Board of the Nagoya University School of Medicine, and written informed consent was obtained from each patient before entry into the study. The study population comprised 25 ambulatory patients with idiopathic DCM (19 men, 6 women) and a mean age of 49 years (range, 27 to 65 years). The patients included 17 individuals with an NYHA functional class of I and 8 with class II. All patients had previously been admitted to hospital as a result of congestive heart failure, but they had been in a stable condition for a mean of 10±4 months (range, 6 to 21 months) before enrollment in the study. DCM was defined by an LV ejection fraction of <45% (as determined by contrast ventriculography) in the absence of coronary artery stenosis >50% (as determined by coronary angiography), valvular heart disease, arterial hypertension, or cardiac muscle disease secondary to any known systemic condition.[15] Patients with atrial fibrillation were excluded from the study to facilitate the evaluation of diastolic function. Individuals with conditions associated with changes in the serum concentrations of markers of myocardial fibrosis (connective tissue disorders, pulmonary fibrosis, liver cirrhosis, osteoporosis, renal insufficiency, malignancy at any site) also were excluded. All study patients had been treated with both ACE inhibitors and loop diuretics for >3 months at the baseline condition. No patients had received β-blockers or angiotensin II receptor blockers.

### Study Protocol

Physical examination, laboratory measurements, echocardiography, and LV catheterization, including determination of LV pressure with a micromanometer-tipped catheter and LV endomyocardial biopsy, were performed at baseline for all patients. The patients were then treated with spironolactone (25 mg/d) in addition to their existing drug regimen for 12 months, after which they were subjected to the same procedures as performed at baseline.

### Laboratory Measurements

Peripheral venous blood samples were collected from patients after they had been in the supine position for 30 minutes. Serum was prepared and stored at −80°C until analysis. The serum concentrations of the carboxyl-terminal propeptide of procollagen type I (PIP), the carboxyl-terminal telopeptide of collagen type I (CITP), and the amino-terminal propeptide of procollagen type III (PIIINP) were measured with radioimmunoassay kits as markers of collagen type I synthesis, collagen type I degradation, and collagen type III synthesis, respectively. The lower detection limits of the assays were 1.2 μg/mL for PIP, 0.5 μg/mL for CITP, and 0.2 U/mL for PIIINP. The PIP/CITP ratio, an index of coupling between the synthesis and degradation of collagen type I, also was calculated as a serum marker of myocardial collagen accumulation.[16] The serum concentration of brain natriuretic peptide (BNP) also was determined with a radioimmunoassay kit.

### Echocardiography

We performed M-mode, 2D, pulsed Doppler and tissue color Doppler echocardiography with a phased-array electronic ultrasound system (Vivid Seven, GE VingMed Ultrasound). The peak flow velocities at the mitral level during rapid filling (E) and during atrial contraction (A), as well as the E/A ratio, were calculated from the pulsed Doppler echocardiographic data. For tissue color Doppler imaging, scanning was performed longitudinally from the apex to acquire the 4-chamber view. The LV myocardium was divided into 8 segments (anterior base, anterior apex, inferior base, inferior apex, septal base, septal apex, lateral base, lateral apex), and longitudinal strain and strain rate were estimated for each segment by measuring the spatial velocity gradient over a computation area of 8 to 10 $mm^2$.[17] The LV early diastolic strain rate, a relatively load-independent estimate of LV diastolic function,[18] was then calculated from the average strain and strain rate. All echocardiographic evaluations were performed by an operator blinded to the results of other examinations.

### Catheterization

An externally balanced and calibrated 6F pigtail angiographic micromanometer-tipped catheter was advanced into the left ventricle for measurement of LV pressure. We evaluated the maximum first derivative of LV pressure (LV $dP/dt_{max}$) as an index of contractility and the pressure half-time ($T_{1/2}$) as an index of relaxation, as previously described.[19] Left ventriculography was performed immediately after measurement of LV pressure, and LV volume was calculated by the area-length method.

### Quantitative Morphometry

LV endomyocardial biopsy specimens were obtained with a 6F bioptome. The tissue was fixed immediately in 10% buffered formalin and embedded in paraffin. Three or 4 specimens were analyzed for each patient. Tissue sections were stained with the collagen-specific dye picrosirius red. Using an automated image analysis system (Win ROOF 5.0, Mitani), we calculated the CVF as the sum of all connective tissue areas divided by the sum of all connective tissue and muscle areas in all fields analyzed for each section.[20] These histological evaluations were performed without knowledge of which patient provided the tissue sections.

### Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis

Total RNA was isolated from 1 to 2.5 mg frozen LV biopsy specimens and subjected to quantitative reverse transcription and polymerase chain reaction (PCR) analysis as previously described.[21] The amounts of the mRNAs for collagen types I and III were thus determined with a fluorogenic 5′-nuclease PCR assay and an ABI PRISM 7700 sequence detector (Perkin-Elmer). All PCR assays were performed in triplicate. TaqMan control reagents (Perkin-Elmer) were used to detect human GAPDH mRNA as an internal standard.

### Statistical Analysis

Data are presented as mean±SD. The baseline characteristics of 2 groups of patients were compared by Student's *t* test for unpaired

**TABLE 1. Clinical Characteristics of Study Subjects and Comparison of Hemodynamic Parameters Between Baseline and After Treatment With Spironolactone**

| | Group A | | Group B | |
|---|---|---|---|---|
| | Baseline | After Treatment | Baseline | After Treatment |
| Gender, M/F | 9/3 | | 10/3 | |
| Age, y | 47±11 | | 50±9 | |
| NYHA functional class, I/II | 9/3 | 11/1 | 8/5 | 11/2 |
| Heart rate, bpm | 72±7 | 71±7 | 73±9 | 72±10 |
| SBP, mm Hg | 120±16 | 120±10 | 117±13 | 116±14 |
| DBP, mm Hg | 78±6 | 76±6 | 77±6 | 76±6 |
| Wall thickness, mm | 7±2 | 7±2 | 7±1 | 7±2 |
| LV end-diastolic diameter, mm | 62±9 | 56±5* | 63±10 | 60±7*† |
| LVEF, % | 33±5 | 34±7 | 33±8 | 36±8 |
| LVEDV, mL | 184±29 | 168±12* | 195±20 | 180±17*† |
| LVEDP, mm Hg | 19±3 | 17±2* | 22±2† | 19±2*† |
| LVEDP/LVEDV, mm Hg/mL | 0.102±0.010 | 0.100±0.008 | 0.116±0.010† | 0.107±0.010*† |
| LV $dP/dt_{max}$, mm Hg/s | 1140±347 | 1272±377* | 1031±243 | 1108±238* |
| $T_{1/2}$, ms | 42±6 | 39±4* | 46±6† | 41±6* |
| E/A | 0.83±0.16 | 0.89±0.15 | 0.78±0.14 | 0.81±0.08 |
| LV early diastolic strain rate, 1/s | 0.78±0.16 | 0.80±0.22 | 0.51±0.13† | 0.66±0.10*† |

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; and LV EF, LV ejection fraction. Data are mean±SD.

*$P<0.05$ vs corresponding baseline value; †$P<0.05$ vs corresponding value for group A.

data or the $\chi^2$ test for categorical data. The effects of spironolactone treatment for 12 months were evaluated with Student's $t$ test for paired data. The relation between continuous variables was assessed by linear regression analysis. A value of $P<0.05$ was considered statistically significant.

## Results

No adverse effects or complications occurred during treatment of the study patients with spironolactone, and all subjects completed the study protocol. We divided the patients into 2 groups on the basis of the biochemical index of coupling between the synthesis and degradation of collagen type I at baseline: 12 patients with a serum PIP/CITP ratio of ≤35 (group A) and 13 patients with a corresponding value of >35 (group B). A PIP/CITP ratio of 35 corresponded to the median value of this parameter and thus was used as the cutoff to separate the 2 groups.

### Clinical and Hemodynamic Characteristics Before and After Treatment

There were no significant differences in age, gender, or NYHA functional class between groups A and B, and heart rate, LV ejection fraction, LV $dP/dt_{max}$, and the E/A ratio at baseline did not differ significantly between the 2 groups (Table 1). In contrast, the ratio of LV end-diastolic pressure to LV end-diastolic volume (LVEDP/LVEDV), an indicator of LV diastolic chamber stiffness, was significantly increased at baseline in group B compared with group A. The $T_{1/2}$ at baseline was significantly greater and LV early diastolic strain rate was significantly smaller in group B than in group

**TABLE 2. Comparison of Biochemical Parameters Between Before and After Treatment With Spironolactone**

| | Group A | | Group B | |
|---|---|---|---|---|
| | Baseline | After Treatment | Baseline | After Treatment |
| PIP, μg/mL | 68.3±10.0 | 64.6±10.6 | 85.9±6.6† | 75.0±4.6*† |
| CITP, μg/mL | 3.35±1.04 | 3.52±0.84 | 2.09±0.35† | 2.75±0.59*† |
| PIP/CITP | 21.9±6.6 | 19.1±4.5 | 42.0±6.8† | 28.3±5.6*† |
| PIIINP, U/mL | 0.45±0.14 | 0.41±0.08 | 0.59±0.20† | 0.44±0.18* |
| Aldosterone, pg/mL | 186±37 | 222±151 | 161±35 | 189±41* |
| Norepinephrine, pg/mL | 294±120 | 271±109 | 340±108 | 282±95 |
| BNP, pg/mL | 92±46 | 77±42* | 133±44† | 88±37* |

Data are mean±SD.

*$P<0.05$ vs corresponding baseline value; †$P<0.05$ vs corresponding value for group A.

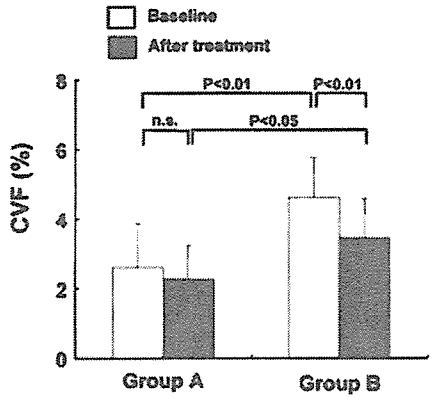


**Figure 1.** CVF values at baseline and after treatment with spironolactone for patients in groups A and B. Data are mean±SD.

A. After treatment with spironolactone, LV $dP/dt_{max}$ was significantly increased although LVEDV was significantly decreased in both groups. $T_{1/2}$ was also significantly decreased in both groups after treatment. LVEDP/LVEDV was reduced and LV early diastolic strain rate was increased in group B after treatment with spironolactone, whereas no significant changes in these variables were apparent in group A.

## Biochemical and Histological Parameters Before and After Treatment

The serum concentrations of PIP (a marker of collagen type I synthesis), PIIINP (a marker of collagen type III synthesis), and BNP were significantly higher and that of CITP (a marker of collagen type I degradation) was significantly lower at baseline in group B than in group A (Table 2). Marked perivascular and interstitial fibrosis, as revealed by staining with the collagen-specific dye picrosirius red, was detected in the endomyocardial biopsy specimens obtained at baseline from patients in group B but not in those from patients of group A (data not shown). The CVF at baseline was also significantly greater in group B than in group A (Figure 1). The extent of perivascular and interstitial fibrosis and the CVF in patients of group B were reduced after treatment with spironolactone, and these effects were accompanied by a significant reduction in the PIP/CITP ratio and the serum

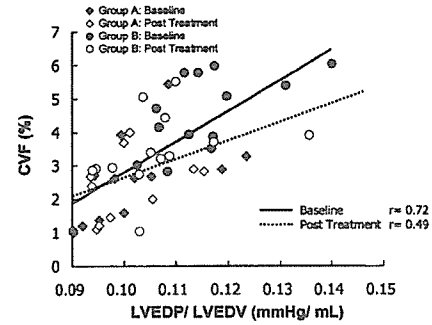


**Figure 5.** Correlation between CVF and LV diastolic chamber stiffness at baseline and after treatment with spironolactone for all patients.

levels of PIP, PIIINP, and BNP, as well as by a significant increase in that of CITP.

## Expression of Collagen Types I and III Before and After Treatment

The amounts of collagen type I and III mRNAs in endomyocardial tissue at baseline were significantly higher in group B than in group A (Figure 2). Those in group B, but not those in group A, were significantly reduced after treatment with spironolactone, consistent with the biochemical and histological findings.

## Relations Between CVF and Myocardial Collagen Accumulation, LV Diastolic Function, and LV Diastolic Chamber Stiffness

The CVF was significantly correlated with the serum PIP/CITP ratio (Figure 3), LV early diastolic strain rate (Figure 4), and LVEDP/LVEDV (Figure 5) values obtained before and after treatment with spironolactone in all patients.

# Discussion

We have evaluated the effects of treatment with the mineralocorticoid receptor antagonist spironolactone on LV diastolic function and myocardial collagen content in mildly symptomatic patients with DCM (NYHA functional class I or II). The main findings of our study include the following: (1) The PIP/CITP ratio in serum, a marker of myocardial colla-

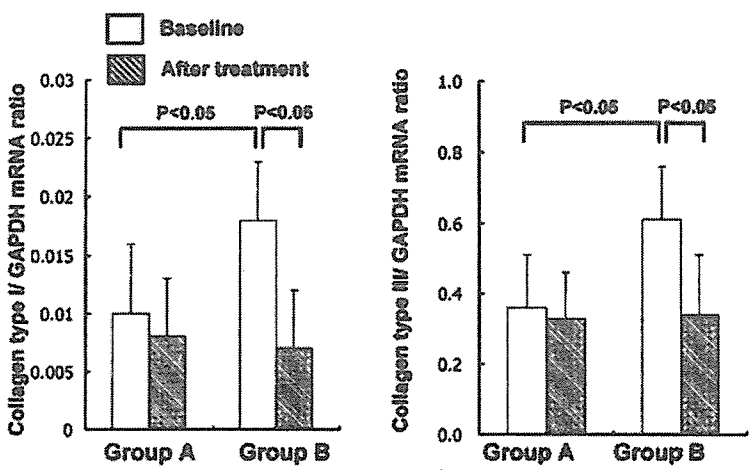


**Figure 2.** Reverse-transcription PCR analysis of the abundance of collagen type I (left) and type III (right) mRNAs in endomyocardial biopsy specimens obtained from patients in groups A and B at baseline and after treatment with spironolactone. Data are normalized by the amount of GAPDH mRNA and are mean±SD.

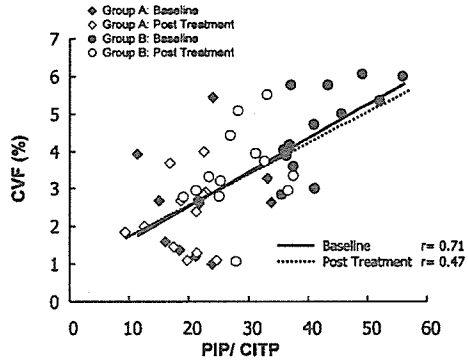


**Figure 3.** Correlation between CVF and PIP/CITP values at baseline and after treatment with spironolactone for all patients.

gen accumulation, was significantly correlated with myocardial collagen content as assessed by histomorphometric analysis; (2) LV diastolic function was inversely related to myocardial collagen content; (3) spironolactone induced both a marked reduction in the extent of myocardial fibrosis and a significant improvement in LV diastolic function, and these effects were accompanied by a reduction in the PIP/CITP ratio; (4) the effects of spironolactone were limited to patients with an increased PIP/CITP ratio; and (5) spironolactone induced a significant improvement in LV systolic function (as evaluated by LV $dP/dt_{max}$) and in LV relaxation (as evaluated by $T_{1/2}$) in all patients.

### Serum Levels of Procollagen Peptide Fragments Are Markers of Collagen Turnover

The PIP fragment is released during the extracellular processing of procollagen type I before the formation of collagen fibers and serves as a marker of fibrogenesis.[22] CITP is a pyridinoline–cross-linked telopeptide produced as a result of the hydrolysis of collagen type I fibrils by matrix metalloproteinase-1 and is a marker of collagen type I degradation.[23] The PIP/CITP ratio, an index of coupling between the synthesis and degradation of collagen type I, was found to be higher in hypertensive patients with increased collagen accumulation in myocardial tissue than in those with normal collagen accumulation.[20] The PIP/CITP ratio thus serves as a serum marker of myocardial collagen accumulation. Indeed, both the CVF and the abundance of collagen type I and III mRNAs in the LV myocardium were higher in the patients with an increased PIP/CITP ratio (group B) than in those with a lower PIP/CITP ratio (group A) in the present study.

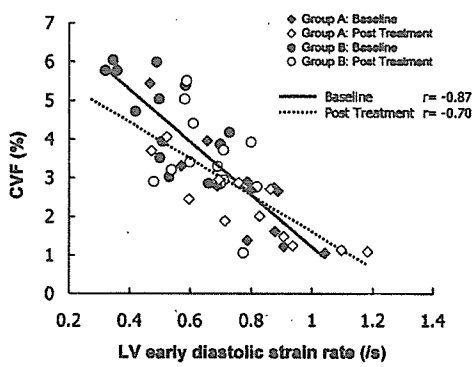


**Figure 4.** Correlation between CVF and LV early diastolic strain rate at baseline and after treatment with spironolactone for all patients.

The plasma concentration of aldosterone correlates with mortality in individuals with chronic heart failure.[24] We have now shown that treatment with the mineralocorticoid receptor antagonist spironolactone induced both a substantial regression of myocardial fibrosis and a significant amelioration of LV diastolic dysfunction in patients with an elevated serum marker of myocardial collagen accumulation. In addition, myocardial collagen content was strongly correlated with LV diastolic function in all patients before and after treatment with spironolactone. We therefore propose that the ability of spironolactone to improve LV diastolic function in mildly symptomatic patients with DCM is related to its capacity to reduce the extent of myocardial fibrosis through normalization of collagen metabolism. This scenario might explain the previous finding that the beneficial effect of spironolactone on survival and hospitalization in RALES was significant only in patients with higher levels of serum markers of collagen synthesis.[11]

LV diastolic dysfunction is an important prognostic marker in patients with chronic heart failure.[25] The efficacy of mineralocorticoid receptor antagonism with regard to mortality reduction in patients with heart failure might thus be due to the improvement in LV diastolic function induced by such treatment. Our present results and those of the previous study by Zannad et al[11] suggest that such an effect of spironolactone might be limited to patients with increased collagen accumulation in myocardial tissue. Indeed, the serum concentration of BNP, which is also an important prognostic marker in chronic heart failure,[26] was reduced by treatment with spironolactone to a greater extent in patients with an increased serum PIP/CITP ratio at baseline than in those with a lower PIP/CITP ratio.

Although a marked effect of spironolactone on myocardial fibrosis was evident in the present study, the number of patients in this pilot study was small. In addition, our study included only patients with a nonischemic origin, and none of the subjects had been treated with β-blockers. Further studies with larger numbers of patients, including those with an ischemic origin and those treated with β-blockers, are needed before our results can be applied more generally to mildly symptomatic patients with heart failure.

In summary, we have shown that treatment with the mineralocorticoid receptor antagonist spironolactone resulted in an improvement in LV diastolic function that was associated with regression of myocardial fibrosis in mildly symptomatic patients with DCM. This effect of spironolactone, however, was limited to patients with increased myocardial collagen accumulation. Our data suggest that collagen accumulation that results from mineralocorticoid receptor stimulation might play a critical role in the impairment of LV diastolic function in patients with DCM.

## References

1. Fuster V, Gersh BJ, Giuliani ER, Tajik AJ, Brandenburg RO, Frye RL. The natural history of idiopathic dilated cardiomyopathy. *Am J Cardiol.* 1981;47:525–531.

2. Keogh A, Freund J, Baron D, Hickie J. Timing of cardiac transplantation in idiopathic dilated cardiomyopathy. *Am J Cardiol.* 1988;61:418–422.
3. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J, Wittes J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure: Randomized Aldactone Evaluation Study Investigators. *N Engl J Med.* 1999;341:709–717.
4. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, Bittman R, Hurley S, Kleiman J, Gatlin M. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. *N Engl J Med.* 2003;348:1309–1321.
5. Brilla CG, Pick R, Tan LB, Janicki JS, Weber KT. Remodeling of the rat right and left ventricles in experimental hypertension. *Circ Res.* 1990;67: 1355–1364.
6. Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium: fibrosis and renin-angiotensin-aldosterone system. *Circulation.* 1991;83: 1849–1865.
7. Brilla CG, Matsubara LS, Weber KT. Anti-aldosterone treatment and the prevention of myocardial fibrosis in primary and secondary hyperaldosteronism. *J Mol Cell Cardiol.* 1993;25:563–575.
8. Slight SH, Joseph J, Ganjam VK, Weber KT. Extra-adrenal mineralocorticoids and cardiovascular tissue. *J Mol Cell Cardiol.* 1999;31:1175–84.
9. Weber KT. Aldosterone and spironolactone in heart failure. *N Engl J Med.* 1999;341:753–755.
10. Zannad F, Dousset B, Alla F. Treatment of congestive heart failure: interfering the aldosterone-cardiac extracellular matrix relationship. *Hypertension.* 2001;38:1227–1232.
11. Zannad F, Alla F, Dousset B, Perez A, Pitt B. Limitation of excessive extracellular matrix turnover may contribute to survival benefit of spironolactone therapy in patients with congestive heart failure: insights from the Randomized Aldactone Evaluation Study (RALES): Rales Investigators. *Circulation.* 2000;102:2700–2706.
12. Tsutamoto T, Wada A, Maeda K, Mabuchi N, Hayashi M, Tsutsui T, Ohnishi M, Sawaki M, Fujii M, Matsumoto T, Matsui T, Kinoshita M. Effect of spironolactone on plasma brain natriuretic peptide and left ventricular remodeling in patients with congestive heart failure. *J Am Coll Cardiol.* 2001;37:1228–1233.
13. Hansen A, Haass M, Zugck C, Krueger C, Unnebrink K, Zimmermann R, Kuebler W, Kuecherer H. Prognostic value of Doppler echocardiographic mitral inflow patterns: implications for risk stratification in patients with chronic congestive heart failure. *J Am Coll Cardiol.* 2001;37:1049–1055.
14. Shimizu Y, Uematsu M, Shimizu H, Nakamura K, Yamagishi M, Miyatake K. Peak negative myocardial velocity gradient in early diastole as a noninvasive indicator of left ventricular diastolic function: comparison with transmitral flow velocity indices. *J Am Coll Cardiol.* 1998; 32:1418–1425.
15. Richardson P, McKenna W, Bristow M, Maisch B, Mautner B, O'Connell J, Olsen E, Thiene G, Goodwin J, Gyarfas I, Martin I, Nordet P. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of Cardiomyopathies. *Circulation.* 1996;93:841–842.
16. Lopez B, Gonzalez A, Varo N, Laviades C, Querejeta R, Diez J. Biochemical assessment of myocardial fibrosis in hypertensive heart disease. *Hypertension.* 2001;38:1222–1226.
17. Abraham TP, Belohlavek M, Thomson HL, Pislaru C, Khandheria B, Seward JB, Pellikka PA. Time to onset of regional relaxation: feasibility, variability and utility of a novel index of regional myocardial function by strain rate imaging. *J Am Coll Cardiol.* 2002;39:1531–1537.
18. Garcia MJ, Thomas JD, Klein AL. New Doppler echocardiographic applications for the study of diastolic function. *J Am Coll Cardiol.* 1998;32:865–875.
19. Izawa H, Yokota M, Takeichi Y, Inagaki M, Nagata K, Iwase M, Sobue T. Adrenergic control of the force-frequency and relaxation-frequency relations in patients with hypertrophic cardiomyopathy. *Circulation.* 1997;96:2959–2968.
20. Diez J, Querejeta R, Lopez B, Gonzalez A, Larman M, Martinez Ubago JL. Losartan-dependent regression of myocardial fibrosis is associated with reduction of left ventricular chamber stiffness in hypertensive patients. *Circulation.* 2002;105:2512–2517.
21. Somura F, Izawa H, Iwase M, Takeichi Y, Ishiki R, Nishizawa T, Noda A, Nagata K, Yamada Y, Yokota M. Reduced myocardial sarcoplasmic reticulum $Ca^{2+}$-ATPase mRNA expression and biphasic force-frequency relations in patients with hypertrophic cardiomyopathy. *Circulation.* 2001;104:658–663.
22. Querejeta R, Varo N, Lopez B, Larman M, Artinano E, Etayo JC, Martinez Ubago JL, Gutierrez-Stampa M, Emparanza JI, Gil MJ, Monreal I, Mindan JP, Diez J. Serum carboxy-terminal propeptide of procollagen type I is a marker of myocardial fibrosis in hypertensive heart disease. *Circulation.* 2000;101:1729–1735.
23. Laviades C, Varo N, Fernandez J, Mayor G, Gil MJ, Monreal I, Diez J. Abnormalities of the extracellular degradation of collagen type I in essential hypertension. *Circulation.* 1998;98:535–540.
24. Swedberg K, Eneroth P, Kjekshus J, Wilhelmsen L. Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality: CONSENSUS Trial Study Group. *Circulation.* 1990;82:1730–1736.
25. Kim IS, Izawa H, Sobue T, Ishihara H, Somura F, Nishizawa T, Nagata K, Iwase M, Yokota M. Prognostic value of mechanical efficiency in ambulatory patients with idiopathic dilated cardiomyopathy in sinus rhythm. *J Am Coll Cardiol.* 2002;39:1264–1268.
26. Tsutamoto T, Wada A, Maeda K, Hisanaga T, Maeda Y, Fukai D, Ohnishi M, Sugimoto Y, Kinoshita M. Attenuation of compensation of endogenous cardiac natriuretic peptide system in chronic heart failure: prognostic role of plasma brain natriuretic peptide concentration in patients with chronic symptomatic left ventricular dysfunction. *Circulation.* 1997;96:509–516.

# Eplerenone Attenuates Myocardial Fibrosis in the Angiotensin II Induced Hypertensive Mouse: Involvement of Tenascin-C Induced by Aldosterone-Mediated Inflammation.

Tomohiro Nishioka MS(c), Maiko Suzuki MS(c), Katsuya Onishi MD*, Nobuyuki Takakura MD**, Hiroyasu Inada MD, Toshimichi Yoshida MD, Michiaki Hiroe MD#, and Kyoko Imanaka-Yoshida MD

Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, Tsu, Japan
*Department of Laboratory Medicine, Mie University Graduate School of Medicine, Tsu, Japan
** Department for Signal Transduction Research Institute for Microbial Diseases,Osaka University, Suita, Japan
# Department of Nephrology and Cardiology, International Medical Center of Japan, Tokyo, Japan

**Address for correspondence**:
Kyoko Imanaka-Yoshida, MD., PhD.
Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507 JAPAN.
Tel: +81-59-231-5009, Fax: +81-59-231-5210
e-mail: Imanaka@doc.medic.mie-u.ac.jp

**Short title:** Tenascin-C in mouse cardiac fibrosis

**Sources of support:**
A Grant-in-aid for scientific research (No. 17590725) from the Ministry of Education, Culture Sports, Science and Technology of Japan to K.I.-Y. A grant for Intractable Disease from the Ministry of Health, Labor and Welfare of Japan to K.I.-Y and M. H.

**Abstract**
Tenascin-C is an extracellular matrix glycoprotein supposed to be a profibrotic molecule in various fibrogenic processes. To elucidate its significance for myocardial fibrosis in hypertensive heart, we employed a mouse model with infusion of angiotensin II, and examined results by histology, immunohistochemistry, *in situ* hybridization and quantitative real-time RT-PCR. Angiotensin II treatment elevated blood pressure and expression of tenascin-C by interstitial fibroblasts in perivascular fibrotic lesions. Angiotensin II infusion also caused accumulation of macrophages, upregulated expression of collagen Iα2, IIIα1, proinflammatory/profibrotic mediators including TGFβ, PDGF-A, PDGF-B, and PDGF-receptor α, but not IL-1β and PDGF-receptor β, in the myocardium. Treatment with an aldosterone receptor antagonist, eplerenone, significantly attenuated angiotensin II induced fibrosis, expression of tenascin-C and inflammatory changes without affecting the blood pressure level. *In vitro*, neither eplerenone nor aldosterone exerted any influence on tenascin-C expression of cardiac fibroblasts, while angiotensin II, TGF-β1 and PDGF significantly upregulated expression of tenascin-C. These results suggest that, in the angiotensin II induced hypertensive mouse heart: 1) tenascin-C may be involved in the progression of cardiac fibrosis; 2) aldosterone may elicit inflammatory reactions in myocardium, which might, in turn, induce tenascin-C synthesis of fibroblasts through at least two pathways mediated by TGF-β and PDGF-A-B/PDGF-receptor α.

**Key words**
Myocardial fibrosis, Tenascin, Aldosterone, Angiotensin II, Hypertension, Inflammation

## Introduction

Diastolic dysfunction in hypertensive heart is an important clinical problem, partly due to the rigidity caused by myocardial fibrosis. Such fibrosis has been classified into replacement (=secondary) and reactive (=primary) types [1,2]. In replacement fibrosis, necrosis of myocytes elicits acute inflammation and myocardial dropout is subsequently replaced by collagen fibers. In contrast, reactive fibrosis is characteristically observed in pressure overloaded hearts, in which collagen fibers increase in perivascular regions without loss of cells and eventually extend among individual cardiomyocytes[3]. Recently, inflammation mediated by the renin-angiotensin II-aldosterone system, especially involvement of aldosterone, has received much attention as a trigger of reactive fibrosis [4-7], however, the molecular pathways remain to be detailed.

Fibrotic lesions do not form by abrupt deposition of collagen molecules, but through multiple steps of synthesis and degradation of various matrix proteins, including tenascin-C. Tenascin-C is an extracellualr glycoprotein with strong bioactivity, transiently expressed during embryonic development, wound healing, and cancer invasion [8-10]. Accumulating evidences suggest that tenascin-C may be a key regulator in early step of fibrotic process in various tissue [11-13]. In the heart, tenascin-C is sparsely detected in the normal adults, but becomes expressed in the pathological

myocardium closely associated with inflammation and tissue remodeling [14-21]. Based on this specific expression, we recently reported that tenascin-C can be a clinical marker for active inflammation[18, 19] and ventricular remodeling [21, 22].

The aim of the present study was clarify the involvement of tenascin-C in the progression of reactive fibrosis in the hypertensive heart and the regulatory mechanism of tenascin-C expression, focusing on angiotensin II-aldosterone system. We employed a mouse model of hypertensive cardiac fibrosis with infusion of angiotensin II (Ang II) in which reactive fibrosis develops in perivascular regions of myocardium without necrosis of cardiomyocytes or scar formation. First, we examined histological changes and gene expression of collagen and tenascin-C in the mouse myocardium with immunohistochemistry, *in situ* hybridization and quantitative real-time RT-PCR. Next, to study the involvement of aldosterone and inflammation in regulation of tenascin-C synthesis, the effect of an aldosterone receptor blocker eplerenone, and expression of proinflammatory /fibrotic mediators, TGF-β, IL-1β, and PDGF in the model mouse were examined. Furthermore, the direct effects of these factors on tenascin-C synthesis were studied using cultured cells.

## Materials and Methods

### *Animal Model*

Female 7-week-old Balb/c mice were used. Ang II (n=95) were given 1% NaCl drinking water and assigned to one of the following five groups: 1)vehicle control mice (n=25); 2) Ang II-treated mice (n=20); 3) Ang II/eplerenone-treated mice (n=20); 4) eplerenone-treated mice (n=19); 5) aldosterone-treated mice (n=11). A micro-osmotic pump (model 1002;Durect Co, Cuperino, CA) containing 0.1 mL of the vehicle (0.9% NaCl: 99.7 % acetic acid=15:1), 2.83 mg/mL of Ang II (SIGMA, St. Louis, MO) or 1 mg/mL of aldosterone (ACROS ORGANICS, NJ) was subcutaneously inserted under the back skin of each mouse for treatment for 4 weeks. The approximate doses of Ang II and aldosterone administered were 560 and 200 ng/kg body weight/min, respectively. The micro-osmotic pumps were replaced every two weeks. Eplerenone (Pfizer, New York, USA), an aldosterone receptor blocker, was orally administered in the diet at 1.67 g per kg chow (the estimated dose of eplerenone was 250 mg/kg body weight /day). Body weights and blood pressure were examined every week. Blood pressure measurements were performed with the BP-98A (Softron, Tokyo, Japan) tail cuff system while the animals were conscious. All experimental protocols conformed to international guidelines and were approved by the Mie University Animal Experiment and Care Committee.

### *Tissue Preparation*

After the 4-week infusion treatment period, the hearts were excised, fixed in 4% paraformaldehyde and embedded in paraffin. For histopathological analysis, sections were cut at 3 μm.

### *Immunohistochemistry*

Immunostaining of tissue sections was performed as previously described [16]. In brief, after treatment with pepsin for 10 minutes or heating in an autoclave for antigen retrieval, sections were incubated with either an anti-tenascin-C polyclonal rabbit antibody [16], an anti-Mac-3 rat monoclonal antibody (Pharmingen, San Diego, CA ; working dilution 1:10) for identification of macrophages, an anti-PDGF-A polyclonal rabbit antibody (Santa Cruz Biotechnology, Inc; working dilution 1:20), an anti-PDGF-B polyclonal rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA; working dilution 1:20), a rat monoclonal anti-murine PDGFR-α antibody(Clone APA5) [23], or a rat monoclonal anti-murine PDGFR-β antibody(Clone APAB) [24]. Three independent fields in perivascular regions of myocardium from each mouse were examined under a 20x objective lens, and Mac-3-positive cells, PDGF-A, and PDGF-B positive cells were counted.

### *Image Analysis*

Sirius red-stained slides were used to quantify myocardial collagen with an optical microscope (BH2, Olympus, Tokyo, Japan). Three independent perivascular fields from each mouse were visualized under a 20x objective lens and photographed with a FUJIX DIGITAL CAMERA HC 300Z/OL (Olympus). The images were analyzed using NIH Image, and percentage areas of perivascular fibrosis were calculated.

### *Quantitative real-time RT-PCR*

Total RNA was extracted from fresh mouse left ventricular tissues using ISOGEN (NipponGene, Toyama, Japan) and single-strand cDNA synthesis was performed by oligo $(dT)_{15}$ priming from 1 μg aliquots in a final volume of 20 μl with a single-strand cDNA synthesis kit for RT-PCR (Roche Diagnostics, Germany) according to the manufacturer's instructions. Quantitative analysis of target mRNA expression was performed with the TaqMan real-time reverse transcription-polymerase chain reaction and a relative standard curve method using Light Cycler Software Ver.3.5 (Roche). The GAPDH mRNA level was quantified as an internal control. The primers and probes for mice are listed in the Table.

***In situ* hybridization**

Preparation of digoxigenin (DIG)-labeled mouse tenascin-C cRNA probes and *in situ* hybridization were performed as previously described [16].

***Cell Cultures***

Cardiac fibroblasts were obtained from ventricles of Balb/c mice and grown in IMDM with 10 % FBS as previously described [20]. Experiments were performed on secondary cultures. Cells ($3x10^5$ cells/well) were plated in MULTIWELL™ 6-well plates (Becton Dickinson, Franklin Lakes, NJ) for 48 hours in serum-free IMDM media, then treated with Ang II (0 to $10^{-5}$ mol/L), aldosterone (0 to $10^{-6}$ mol/L), IL-1β (R&D Systems, Oxon, UK ; 0 to 30 ng/mL), TGF-β1 (Roche Diagnostics; 0 to 10 ng/mL) or PDGF-BB (R&D Systems; 0 to 100 ng/mL) for 6 hours. Some cells were pretreated with eplerenone ($10^{-8}$, $10^{-7}$, $10^{-6}$ mol/L) for 1 hour and then stimulated with Ang II ($10^{-7}$ mol/L). To assess the combined effects of co-treatment with Ang II and aldosterone, cardiac fibroblasts were pretreated with aldosterone ($10^{-6}$ mol/L) for 6 hours and then incubated with Ang II ($10^{-7}$ mol/L) for 6 hours. Total RNA was isolated using ISOGEN, and the relative tenascin-C mRNA levels were determined by quantitative real-time RT-PCR.

***Statistical analysis***

All data are expressed as means ± standard deviations (SDs). Numeric data were statistically evaluated by one-way analysis of variance, followed by the Tukey-Kramer method for multiple comparisons. A $p$ value less than 0.05 was considered to be statistically significant.

**Results**

***Systolic blood pressure and body weight.***

Blood pressure was elevated within 1 week after the onset of Ang II treatment, and remained significantly increased compared with that of the control mice for up to 4 weeks (Figure 1A). No significant difference was observed between Ang II-treated and Ang II/eplerenone-treated groups. Blood pressure remained normal in vehicle-control, aldosterone-receiving, and eplerenone-alone mice. Increase in body weight was similar in all experimental groups during the study and no statistically significant inter-group differences were observed at any time point (Figure 1B).

***Myocardial Fibrosis and expression of tenascin-C***

In Ang II-treated and aldosterone-treated mice, the volume of perivascular collagen fibers in myocardium was clearly increased, extending into spaces between individual myocytes. Eplerenone treatment almost completely abolished Ang II-induced perivascular fibrosis, and aldosterone treatment induced fibrotic changes (Figure 2A). Necrosis of
cardiomyocytes and scar formation were not found in our models.

Cardiac expression of tenascin-C was immunohistochemically detected in perivascular regions in Ang II - treated mice myocardium where collagen fibers had accumulated, whereas no immunostaining was observed in the control mice. Eplerenone reduced the tenascin-C expression induced by Ang II treatment, and aldosterone treatment induced tenascin-C expression (Figure 2B).

*In situ* hybridization analysis for tenascin-C mRNA in Ang II-treated mice demonstrated signals in interstitial fibroblasts residing around the vascular tunica adventitia (Figure 2C, D). Vascular endothelial cells, vascular smooth muscle cells and cardiomyocytes were negative for tenascin-C mRNA.

Quantitative analysis of percentage fibrotic areas (Figure 2E) confirmed significant increased in Ang II-treated (12.9 ± 2.5%, $p<0.001$) and aldosterone-treated (7.2 ± 2.0%, $p<0.001$) mice compared with control mice (3.2 ± 0.9%). Fibrotic areas in eplerenone/Ang II mice were significantly attenuated (4.3 ± 1.8%, $p<0.001$) as compared with Ang II-alone. Real-time RT-PCR analysis (Figure 2F) showed that both Ang II and aldosterone significantly upregulated the mRNA levels for collagen type Iα2 (2.6 ± 1.0 and 1.8 ± 0.3 fold, $p<0.01$, respectively), and for IIIα1 (3.2 ± 2.1 fold, $p<0.05$ and 2.8 ± 0.4 fold, $p<0.001$, respectively) when compared with the control mice (1.0 ± 0.4 and 1.0 ± 0.3 fold, respectively). Eplerenone treatment significantly reduced this upregulation of collagen Iα2 and IIIα1 (gene expression (to 1.3 ± 0.3 fold, $p<0.05$ and 1.0 ± 0.3 fold, $p<0.05$,) when compared to Ang II-alone mice (2.6 ± 1.0 fold, 3.2 ± 2.0 fold, respectively). Parallel to the change in collagen gene expression, tenascin-C mRNA levels in Ang II-treated and aldosterone-treated groups was significantly upregulated (11.2 ± 5.8 fold increase for Ang II, $p<0.05$ and 2.6 ± 0.6 fold for aldosterone, $p<0.001$) compared with the control mice (1.0 ± 0.2). Eplerenone treatment significantly abrogated the induction of tenascin-C expression by Ang II (2.3 ± 1.3 fold, $p<0.05$).

***Macrophage infiltration***

Immunohistological analysis demonstrated many Mac 3 positive macrophages to have accumulated in the perivascular spaces in Ang II-treated mice, whereas only a few macrophages were observed in control mice (14.3 ± 3.4 vs. 1.8 ± 0.9 cells/optic field, $p<0.05$, Figure 3A). In Ang II/eplerenone-treated mice, the number of macrophages

was significantly reduced (4.5 ± 2.9 cells/optic field, $p<0.05$) as compared with the Ang II-alone case. Aldosterone treatment also caused increase of the number of macrophages (6.7 ± 1.25 cells/optic field, $p<0.01$)

***Expression of cytokines and growth factors***

Immunostaining demonstrated that Ang II treatment significantly increased the numbers of PDGF- A (Figure 3B) and PDGF- B (Figure 3C) positive cells in the perivascular region as compared with the controls (17.83 ± 3.37 vs. 7.11 ± 1.67 cells/optic field, $p<0.01$, 32.3 ± 7.2 vs. 8.23 ± 3.35 cells/optic field, $p<0.001$, respectively). Aldosterone treatment also significantly increased the number of PDGF-A, -B positive cells (12.72 ± 0.97 vs. 7.11 ± 1.67 cells/ optic field, $p<0.01$, 17.7 ± 3.86 vs. 8.23 ± 3.35 cells/optic field, $p<0.001$, respectively). In AngII/eplerenone-treated mice, the numbers of PDGF- A and PDGF-B positive cells were significantly reduced (8.39 ± 1.46 cells/optic field, $p<0.01$, 10.7 ± 1.63 cells/optic field, $p<0.001$, respectively) as compared with the Ang II-alone case. Perivascular fibroblasts in Ang II or aldosterone treated mouse heart upregulated the expression of PDGF receptor α (Figure 3D) but did not express PDGF receptor β (Figure 3E). No expression of either receptor was detected in the control group.

Real-time RT-PCR analysis (Figure 4) showed Ang II and aldosterone treatment significantly upregulated the mRNA levels for TGF-β1 (1.74 ± 0.20 fold, $p<0.05$ and 1.49 ± 0.18 fold, $p<0.05$, respectively, Figure 4A) in the mouse myocardium when compared with the control mice (1.0 ± 0.35 fold). Eplerenone treatment significantly reduced TGF-β1 gene upregulation induced by Ang II treatment (0.67 ± 0.11 fold, $p<0.05$). In contrast, no significant change of mRNA level of IL-1β, another major inflammatory mediator from macrophages, was observed in any of the groups (Figure 4B).

***The effects of Ang II, aldosterone and inflammatory/fibrotic cytokines on Tenascin-C synthesis by cardiac fibroblasts in culture.***

The direct effects of Ang II and aldosterone on tenascin-C gene expression of cardiac fibroblasts was examined in culture by quantitative real-time RT-PCR (Figure 5). Ang II (0 to $10^{-5}$ mol/L) significantly increased tenascin-C mRNA levels in a dose-dependent manner and the expression level reached a peak at the concentration of $10^{-7}$ mol/ L (2.1 ± 0.7 fold increase, Figure 5A). In contrast, addition of aldosterone (0 to $10^{-6}$ mol/L) did not significantly affect tenascin-C expression levels at any of the concentrations examined (Figure 5B). Eplerenone did not significantly influence Ang II-induced tenascin-C expression, and no synergism was evident with Ang II ($10^{-7}$ mol/L) in the presence of aldosterone ($10^{-8}$ mol/L, Figure 5C).

To identify possible mediators of upregulation of tenascin-C expression in cardiac fibroblasts, cells were treated with PDGF-BB (Figure 5D) and TGF-β (Figure 5E). These factors caused significant increase of tenascin-C expression in a dose-dependent manner.

## Discussion

### Possible involvement of tenascin-C reactive fibrosis of hypertensive heart

Tenascin-C has been proposed to promote fibrosis because its expression is upregulated in various fibrogenic process such as liver fibrosis [25], lung fibrosis [12], skin wound healing[11], and scar formation after myocarditis [17, 18]. Directly supporting this possibility, we have recently reported that locally applied tenascin-C accelerates collagen fiber formation in aneurismal cavities in a rat model [26], and that deficiency of tenascin-C significantly attenuates liver fibrosis in immune-mediated chronic hepatitis mouse model [13]. In the present study, we demonstrated that tenascin-C is not detected in the normal myocardium but becomes markedly upregulated in perivascular fibrosing areas in the Ang II induced hypertensive mouse heart, and that the expression level parallels the extent of fibrosis. These findings suggest that tenascin-C may be involved in the progression of reactive fibrosis, and its elevated expression might be a marker for active progression of the fibrosis in the hypertensive heart.

***Mechanism of upregulation of tenascin-C gene expression***

Previous studies demonstrated that expression of tenascin-C is upregulated with vascular remodeling in pulmonary hypertension [27, 28] and spontaneous hypertensive rats [29], and that mechanical stress is an important tenascin-C inducing factor (reviewed in [8]). In this study, we found that up-regulated expression of tenascin-C in Ang II induced hypertensive mice was blocked by an aldosterone blocker, eplerenone, without affecting the blood pressure level, which suggests that Ang II may induce tenascin-C expression in myocardium through an aldosterone-dependent pathway but independent of blood pressure. Based on our *in situ* hybridization analysis demonstrating that the source of tenascin-C was cardiac fibroblasts in perivascular region, we speculated that aldosterone might stimulate interstitial fibroblasts to synthesize tenascin-C. However, aldosterone did not induce tenascin-C synthesis in cardiac fibroblasts in culture, although Ang II enhanced tenascin-C expression as reported for other types of cells [29, 30]. While it remains controversial whether cardiac fibroblasts express mineral corticoid receptors [31-33], several reports have suggested synergism between Ang II and aldosterone [34, 35] because of induction of Ang II receptor levels [36] or receptor binding [37] by the latter. However, neither addition of aldosterone nor blocking with eplerenone exerted any influence on tenascin-C expression induced by Ang II in the present