Myocardial Stiffness in Patients With Heart Failure and a Preserved Ejection Fraction
Contributions of Collagen and Titin

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Background—The purpose of this study was to determine whether patients with heart failure and a preserved ejection fraction (HFpEF) have an increase in passive myocardial stiffness and the extent to which discovered changes depend on changes in extracellular matrix fibrillar collagen and cardiomyocyte titin.

Methods and Results—Seventy patients undergoing coronary artery bypass grafting underwent an echocardiogram, plasma biomarker determination, and intraoperative left ventricular epicardial anterior wall biopsy. Patients were divided into 3 groups: referent control (n=17, no hypertension or diabetes mellitus), hypertension (HTN) without (+) HFpEF (n=31), and HTN with (+) HFpEF (n=22). One or more of the following studies were performed on the biopsies: passive stiffness measurements to determine total, collagen-dependent and titin-dependent stiffness (differential extraction assay), collagen assays (biochemistry or histology), or titin isoform and phosphorylation assays. In comparison with controls, patients with HTN(−)HFpEF had no change in left ventricular end-diastolic pressure, myocardial passive stiffness, collagen, or titin phosphorylation but had an increase in biomarkers of inflammation (C-reactive protein, soluble ST2, tissue inhibitor of metalloproteinase 1). In comparison with both control and HTN(−)HFpEF, patients with HTN(+)HFpEF had increased left ventricular end-diastolic pressure, left atrial volume, N-terminal propeptide of brain natriuretic peptide, total, collagen-dependent, and titin-dependent stiffness, insoluble collagen, increased titin phosphorylation on PEVK S11878(S26), reduced phosphorylation on N2B S4185(S469), and increased biomarkers of inflammation.

Conclusions—Hypertension in the absence of HFpEF did not alter passive myocardial stiffness. Patients with HTN(+) HFpEF had a significant increase in passive myocardial stiffness; collagen-dependent and titin-dependent stiffness were increased. These data suggest that the development of HFpEF depends on changes in both collagen and titin homeostasis. (Circulation. 2015;131:1247–1259. DOI: 10.1161/CIRCULATIONAHA.114.013215.)

Key Words: collagen ◼ diastole ◼ heart failure ◼ hypertension ◼ hypertrophy

Clinical Perspective on p 1259

Patients with heart failure and a preserved ejection fraction (HFpEF) have been shown to have abnormalities of left ventricular (LV) diastolic function including slowed/incomplete relaxation, decreased suction/recoil, and increased passive chamber stiffness.1−5 However, the myocardial basis for these changes remains incompletely understood. Some recent clinical studies have suggested that development of HFpEF is accompanied by significant changes in the composition and structure of the extracellular matrix (ECM), especially fibrillar collagen.6,7 Other studies and editorials have emphasized the contribution of changes in titin (the giant molecular spring protein that is one important factor responsible for cardiomyocyte passive stiffness)9−21; specifically, changes in the phosphorylation of titin.22−32 However, in previous studies of patients with HFpEF, the role of titin was examined in isolated, chemically demembranated cardiomyocytes obtained from endocardial myocardial biopsies; these preparations did not include surrounding ECM structures. The aforementioned changes in collagen and titin are expected to increase passive myocardial stiffness; however, myocardial stiffness has never...
been measured directly in myocardium from HFP EF patients. Thus, the magnitude of the assumed increase in myocardial passive stiffness and therefore its importance as a determinant of diastolic dysfunction are unknown. In addition, the role of increased passive stiffness in the development and clinical course of patients with HFP EF and the relative contribution of changes in the ECM versus titin to passive stiffness in HFP EF are also unknown. In the present study, we hypothesized that the changes in both collagen and titin occur during the development of HFP EF and that these changes combine to cause a major increase in myocardial passive stiffness that contributes to the development of HFP EF.

To determine the extent to which ECM collagen and titin contribute to changes in myocardial passive stiffness in patients with HFP EF, certain experimental conditions must be met. First, because several potential determinants of passive stiffness could change concurrently during the development of HFP EF, the contribution of each must be determined in samples of myocardium that include an integrated, intact composite structure. To satisfy this condition, methods of differential extraction have been developed to determine the separate contributions of collagen and titin to stiffness in demembranated myocardial strips. In the current studies, these methods were applied to strips prepared from LV myocardial biopsies obtained during coronary artery bypass grafting (CABG). Second, the effects of comorbidities and antecedent diseases must be distinguished from changes associated with the clinical syndrome of HFP EF. Two of the most common antecedent disease processes that lead to HFP EF are arterial hypertension (HTN) and diabetes mellitus (DM). In the current study, patients with HTN (or DM combined with HTN) without HFP EF who were matched with patients with HTN (or DM combined with HTN) with HFP EF (HTN(+)/HFP EF). Third, an appropriate referent control group must be studied. For this purpose, CABG patients with no history of HTN or DM were chosen. There are limitations in using these patients as referent controls in that coronary artery disease (CAD) could have uncertain effects on myocardial stiffness that cannot be distinguished from those related to HTN. However, CAD is present in a majority of patients with HFP EF; thus, a CAD background is quite representative of the HFP EF population. With the use of these methods, the purpose of this study was to determine whether patients with HFP EF and an antecedent history of HTN or HTN/DM have an increase in passive myocardial stiffness and whether changes in stiffness depend on changes in collagen and titin. Additionally, we sought to determine the relationship between changes in myocardial passive stiffness and echocardiographic measures of LV structure and function and selected plasma biomarkers.

Methods

Study Population

Recruitment

The study cohort consisted of 70 males and females recruited to undergo intraoperative LV myocardial biopsy from among those scheduled for CABG at (1) Fletcher Allen Health Care in Burlington, VT, the clinical facility of the University of Vermont College of Medicine; (2) the Ralph H. Johnson Department of Veterans Administration Medical Center and the Medical University of South Carolina Hospital Authority (MUSC) in Charleston, SC; and (3) selected National Heart, Lung, and Blood Institute Heart Failure Research Network centers (University of Alberta [Alberta, Canada], Intermountain Medical Center [Murray, UT], the Mayo Clinic [Rochester, MN], Minnesota Heart Institute [Minneapolis, MN], University of Utah [Salt Lake City, UT] and the Utah VA Medical Center [Salt Lake City, UT]). Between October 1, 2008 and August 6, 2012 who satisfied the inclusion and exclusion criteria specified below. All patients signed consent forms approved by their respective institutional review boards.

Experimental Measurements

Demographic, medication, and laboratory data and cardiac catheterization results (coronary anatomy, LV end-diastolic pressure) were tabulated. The severity of CAD was graded based on the number of major vessels (left anterior descending, left circumflex, right coronary arteries) with a stenosis >70%, with left main coronary stenosis considered as 2 vessels. Patients recruited at the University of Vermont College of Medicine and MUSC underwent an echocardiographic-Doppler examination to assess LV chamber structure and function. In addition, in these patients a 10 mL plasma sample was obtained for measurement of biomarkers. Intraoperative LV anterior wall epicardial biopsies were obtained as previously described. Patients recruited to this protocol were part of an National Institutes of Health grant (RO1HL089944) with multiple specific aims; therefore, biopsy samples were allocated to several protocols, data from some of which have been published. Given the size of each biopsy, all protocols could not be performed on each biopsy. For this study, 25 biopsies were used to assess myocardial passive stiffness, 30 were used to measure tissue collagen content, and 14 were used for titin phosphorylation studies (Table 1).

General Inclusion Criteria

Patients scheduled to undergo CABG over 21 years of age, with a preserved LV ejection fraction (≥50%), normal wall motion and end-diastolic volume index (<75 mL/m²), and without evidence of previous myocardial infarction were eligible. Patients were categorized into 3 groups: control, HTN(–)/HFP EF, and HTN(+)HFP EF.

Specific Patient Group Inclusion Criteria

Control patients fulfilled the general inclusion criteria, but did not have a history of HTN or DM. HTN(–)/HFP EF patients fulfilled the general inclusion criteria and had a history of HTN documented in their records and had been told of this diagnosis by a physician, and were receiving medications for its treatment. These patients had no evidence of heart failure as defined below.

HTN(+)HFP EF patients fulfilled the general inclusion criteria and had HTN and HFP EF as specified by the European Society of Cardiology and Heart Failure Society of America criteria. These criteria require: (1) signs and symptoms of heart failure (Framingham or Boston criteria, exercise testing, quality of life questionnaire), (2) LV ejection fraction ≥50%, (3) LV end-diastolic volume index <75 mL/m²; (4) evidence of diastolic LV dysfunction obtained invasively

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<th>Table 1. Sample Size for Each End Point</th>
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<td>Echocardiogram</td>
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<td>Myocardial stiffness*</td>
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<td>Collagen volume fraction*</td>
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<td>Titin*</td>
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*Biopsy size limited number of studies/patient.

HFP EF indicates heart failure with a preserved ejection fraction.
cardiac catheterization) or noninvasively (transmitral or tissue Doppler or left atrial size), and (5) exclusion of noncardiac diseases that could cause symptoms commonly present in patients with heart failure.

**Exclusion Criteria**

Patients were excluded if they had a previous transmural myocardial infarction, LV ejection fraction <50%, LV end-diastolic volume index >75 ml/m2, significant valvular or other noncoronary heart disease, severe chronic pulmonary disease requiring oral steroids and oxygen therapy, any noncardiac disease or condition known to affect myocardial function, anemia (hemoglobin <13.0 g/dL), serum creatinine >2.0 mg/dL, poorly controlled HTN (blood pressure >140/90 mmHg), off-pump or emergency CABG, morbid obesity, history of substance abuse, inability to provide informed consent, poorly controlled DM (hemoglobin A1c >8.5% within the past 6 months), active malignancy, severe connective tissue disease, severe liver disease, hypertrophic cardiomyopathy, restrictive cardiomyopathy or constrictive pericarditis, human immunodeficiency virus or active infection.

**Myocardial Biopsy Procedure**

Anterior LV free wall epicardial biopsies weighing ≥25 to 50 mg were obtained during CABG soon after the patient was placed on cardiopulmonary bypass, as previously described.39,40 The biopsy was placed in oxygenated 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–based Krebs buffer containing 30 mmol/L 2,3-butanedione monoxime at room temperature.39,40 Small samples (<5 mg) were removed and frozen for collagen and titin studies or placed in formalin for histology. The remainder of the tissue was processed for stiffness studies. From the section of the biopsy that remained in buffer, tissue was dissected into pieces <2 mm in length and placed in skinning solution containing Triton-X100 at 4°C. For samples obtained at MUSC and the Heart Failure Research Network centers, the skinning period coincided with overnight transit to the University of Vermont College of Medicine at 4°C. After 18- to 24-hour skinning, strips were dissected to 150- to 200-µm diameter and 800- to 1200-µm length and then underwent measurements of myocardial stiffness as described below.

All patients were followed until discharge, with particular attention to ventricular arrhythmias and bleeding complications. No adverse effects or postoperative complications ascribable to the biopsy procedure were detected and all patients were discharged alive.

**Measurement of Passive Myocardial Stiffness**

At the time of the study, aluminum T clips were attached to the ends of each strip. The strip was mounted between a piezoelectric motor (Physik Instrumente, Auburn, MA) and a strain gauge (Kronex Technologies, Oakland, CA) and initially lowered into a 30-µL droplet of relaxing solution maintained at 37°C. The composition of relaxing solution is specified in previous reports.9,10 Sarcomere length (SL) was measured by Fourier Transform of digital images (IonOptix Corp, Milton, MA).6 Measurements of total, collagen-dependent, and titin-dependent stiffness were made by using a previously published differential extraction protocol.9,10 The extraction method removes the anchors of titin within the myofilament, leaving only ECM-based stiffness. This method, originally developed and validated by Granzier and colleagues,9,10 has been successfully used in other studies.41 In previous control studies in which titin’s stiffness was eliminated by protease treatment and the muscle strip was then treated with KCl/KI,3 ECM stiffness was shown to be unaffected by the KCl/KI treatment. Thus, although the differential extraction protocol causes irreversible myocardial damage, it does not affect the measurements that are central to the questions addressed in this study.

**Echocardiography**

Echocardiographic studies performed at the University of Vermont College of Medicine and MUSC were interpreted by a core laboratory at MUSC. Studies were deidentified, coded, and interpreted in a blinded fashion. Measurements were made by using American Society of Echocardiography criteria.44

**Collagen**

Collagen was assessed by using both biochemical and histological methods. Soluble, insoluble, and total collagen content were determined by using tissue samples sequentially extracted and assayed directly using the microplate picrosirius red assay.45-47 Collagen volume fraction (CVF) was measured using light microscopy with samples stained with picrosirius red to detect collagen and viewed with polarized light under dark field optics to detect birefringence of the fibers.45

**Titin Studies**

Titin isoform analysis was performed with 1% agarose gels using a vertical sodium dodecyl sulfate-agarose gel system as previously described.23,48 LV myocardium coexpresses compliant N2B titin and stiffer N2B titin isoforms; their expression ratio was determined. We also measured titin degradation as the ratio of T2 (+2 MDA degrada-

**Plasma Biomarkers**

Biomarkers were chosen that reflect changes in ECM homeosta-
sis, specifically matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Four classes of MMPs, gelatinases (MMP-2 and MMP-9), collagenase (MMP-1 and MMP-8), stromelysin (MMP-3), and matrilysin (MMP-7), and all 4 tissue inhibitors of MMPs (TIMP-1, -2, -3, and -4) were assayed. In addition, blots were probed with phospho-specific rabbit polyclonal antibodies against phosphorylated S11878(S26) and S12022(S170) of the PEVK element.33 These sites are known to be phosphorylated by protein kinase C (PKC). In addition, blots were probed with phospho-specific rabbit polyclonal antibodies against phosphorylated S1485(S469) of titin’s N2B element. This site is known to be phosphorylated by protein kinase A (PKA) and protein kinase G (PKG).32 Membranes were labeled with secondary antibodies conjugated with fluorescent dyes with infrared excitation spectra (CF680, goat anti-rabbit, Biotium Company, Hayward CA). Blots were scanned by using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln NE), and images were analyzed using Li-Cor software. Ponceau S scans were analyzed in One-D scan to normalize phosphorylation signal to protein loading.

**Statistical Analysis**

Data are reported as mean±standard deviation in tables and text and mean±standard error in figures. A t test was used to detect differences in continuous variables, Pearson χ2 test was used to detect differences in categorical variables for demographics, collagen-dependent and titin-dependent tension, collagen content, and titin phosphorylation and isoforms and plasma biomarkers among referent controls, HTN(–)HFpEF and HTN(+)HFpEF groups. A general linear mixed model was also used to compare the relationship between stress and sarcomere length in the 3 groups (Figure 1); this type of model is ideal for handling repeated measures obtained within the same study subjects.49 Within the general linear mixed model, linear and quadratic relationships between stress and sarcomere length were considered, and an unstructured covariance structure was selected after comparing the model’s Akaike information criterion value with those from models incorporating other covariance structures (eg, autoregressive, compound symmetry).49 In addition, stress was compared between the 3 groups at selected common values of SL by using analysis of variance. Linear correlations were used to examine the
The structural and functional data obtained from the echocardiographic and plasma biomarker studies also confirm the category definitions for each group (Table 3). By definition, LV volumes and ejection fractions were normal in each group. Both HTN groups had increased LV mass and relative wall thickness. The number in each HTN group with concentric LV hypertrophy (LVH) was comparable (39% in HTN(–)HFpEF and 41% in HTN(+)HFpEF); the number with concentric remodeling was also comparable (32% in HTN(–)HFpEF and 30% in HTN(+)HFpEF). Thus, the percentage with either concentric LVH or concentric remodeling (ie, increased relative wall thickness without increased LV mass) was ≈70% in both groups. Measurements that reflect diastolic function and filling pressure (E, E’; pulmonary capillary wedge pressure, LV end-diastolic pressure, left atrial volume) were increased in HTN(+HFpEF). With the exception of a small increase in left atrial volume, these measurements were normal in HTN(–)HFpEF patients. Left atrial enlargement was also more frequent in the HTN(+)HFpEF group (41%) versus HTN(–)HFpEF (20%).

### Myocardial Stiffness

Relationships between myocardial stress and SL between 2.0 and 2.6 μm for the 3 groups, which reflect total passive myocardial stiffness, are shown in Figure 1. Results of the general linear mixed model indicated that a model assuming a quadratic relationship between stress and SL provided a superior fit in comparison with a linear model. The general linear mixed model indicated that as SL increased, the slope increased most rapidly in the HTN(+)HFpEF group (P<0.0001 in comparison with both control and HTN(–)HFpEF), and that the control and HTN(–)HFpEF curves were not significantly different from 1 another. Significant (P<0.01) differences were noted between HTN(+)HFpEF vs HTN(–)HFpEF groups. Overall, the curves for the control and HTN(–)HFpEF groups were not significantly different from 1 another. Significant (P<0.01) differences were noted between HTN(–)HFpEF vs referent control patients. There were no significant differences in each HTN group with concentric LV hypertrophy (LVH) was comparable (39% in HTN(–)HFpEF and 41% in HTN(+)HFpEF); the number with concentric remodeling was also comparable (32% in HTN(–)HFpEF and 30% in HTN(+)HFpEF). Thus, the percentage with either concentric LVH or concentric remodeling (ie, increased relative wall thickness without increased LV mass) was ≈70% in both groups. Measurements that reflect diastolic function and filling pressure (E, E’; pulmonary capillary wedge pressure, LV end-diastolic pressure, left atrial volume) were increased in HTN(+HFpEF). With the exception of a small increase in left atrial volume, these measurements were normal in HTN(–)HFpEF patients. Left atrial enlargement was also more frequent in the HTN(+)HFpEF group (41%) versus HTN(–)HFpEF (20%).

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between the HTN(+)-HFpEF group and the other groups at 2.1 μm and at each SL assessed up to and including 2.6 μm. Passive myocardial tension was also examined at SL 2.6 μm before and after differential extraction to estimate collagen-dependent and titin-dependent stiffness (Figure 2). There were no significant differences in collagen- and titin-dependent tension between control and HTN(−)-HFpEF. However, both collagen- and titin-dependent tension was significantly increased in HTN(+)-HFpEF in comparison with the other groups. Collagen-dependent stiffness was increased by 220%, and titin-dependent stiffness was increased by 92% in the HTN(+)-HFpEF group.

There were significant correlations between in vitro measurements of passive myocardial tension and in vivo echocardiographic measurements of diastolic function (Figure 3). Thus, there was a statistically significant direct relationship between collagen-dependent tension and left atrial diameter ($r^2=0.42$, $P=0.006$) and pulmonary capillary wedge pressure ($r^2=0.46$, $P=0.002$) and between titin-dependent tension and left atrial diameter ($r^2=0.43$, $P=0.006$), but not pulmonary capillary wedge pressure ($r^2=0.16$, $P=0.11$).

Collagen

Myocardial collagen content was measured by using both biochemical and histological methods. Biochemical studies assessed soluble, insoluble, and total collagen (Figure 4A). CVF was estimated by light microscopy of picrosirius red-stained sections (Figure 4B through 4F). There were no differences in soluble collagen across the 3 groups. There was a significant increase in insoluble collagen and total collagen (by ≈100%) and CVF (by ≈130%) in the HTN(+)-HFpEF group in comparison with the controls and HTN(−)HFpEF groups.

Table 3. Echocardiography

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<th>Referent Control</th>
<th>(−) HFpEF</th>
<th>(+) HFpEF</th>
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<tbody>
<tr>
<td>LV LVEDVi, mL/m²</td>
<td>58±11</td>
<td>60±10</td>
<td>58±14</td>
</tr>
<tr>
<td>LV LVESVi, mL/m²</td>
<td>20±7</td>
<td>21±4</td>
<td>21±11</td>
</tr>
<tr>
<td>LV LVEF, %</td>
<td>65±7</td>
<td>65±6</td>
<td>65±14</td>
</tr>
<tr>
<td>LV LV mass, g/m²</td>
<td>88±15</td>
<td>107±20*</td>
<td>116±20*</td>
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<tr>
<td>LV wall thickness, cm</td>
<td>0.9±0.1</td>
<td>1.1±0.1*</td>
<td>1.2±0.1*</td>
</tr>
<tr>
<td>RWT</td>
<td>0.38±0.04</td>
<td>0.45±0.06*</td>
<td>0.48±0.10*</td>
</tr>
<tr>
<td>E, cm/s</td>
<td>80.1±16.9</td>
<td>76.7±19.8</td>
<td>83.5±22.2</td>
</tr>
<tr>
<td>E', cm/s</td>
<td>10.8±1.8</td>
<td>10.1±3.3</td>
<td>7.3±2.0†</td>
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<tr>
<td>PCWP, mmHg</td>
<td>12.1±2.8</td>
<td>12.5±3.3</td>
<td>17.6±5.4†</td>
</tr>
<tr>
<td>LA Vol, mL/m²</td>
<td>20.8±3.6</td>
<td>25.7±8.0*</td>
<td>37.0±11.9†</td>
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Data are mean±standard deviation. BSA indicates body surface area; E, transmitral early peak-filling velocity; E', tissue Doppler mitral annular early peak diastolic velocity; EDV, end-diastolic volume indexed (normalized) to BSA; ESV, end-systolic volume indexed (normalized) to BSA; HFpEF, heart failure with a preserved ejection fraction; LV, left ventricle; LV mass i, LV mass indexed (normalized) to BSA; LV, left atrium; LA Vol, left atrial volume indexed (normalized) to BSA; PCWP, pulmonary capillary wedge pressure as estimated from E/E′ ratio; and RWT, relative wall thickness.

Figure 2. Collagen-dependent and titin-dependent myocardial stress at a sarcomere length (SL) of 2.6 μm for referent control patients (white column), patients with hypertension but without heart failure and a preserved ejection fraction (HTN(−)-HFpEF, gray column), and patients with hypertension and HFpEF (HTN(+)-HFpEF, black column). Total stress is the numeric sum of collagen- and titin-specific data. Patients with HTN(+)-HFpEF had an increase in collagen-dependent and titin-dependent myocardial stress. There were no significant differences between HTN(−)-HFpEF or referent control patients. *P<0.01 vs referent control; #P<0.01 vs HTN(−)-HFpEF.

Titin

Titin N2B and N2BA isoforms and phosphorylation were examined in the 3 patient groups. Three phosphorylation sites in titin’s spring region were examined: S11878(S26) and S12022(S170) of the PEVK element, sites phosphorylated by PKC and calcium/calmodulin-dependent protein kinase II (CaMKII), and S4185(S469) of the N2B element, a site phosphorylated by PKA and PKG (Figure 5). There were no differences in the N2BA/N2B titin ratio between the 3 groups (0.53±0.14 in control, 0.50±0.13 in HTN(−)-HFpEF, and 0.59±0.14 in HTN(+)-HFpEF) and no changes in T2 (titin degradation product): T1 (full-length titin) ratio among the groups. Patients with HTN(+)-HFpEF had a 31% higher phosphorylation value at the S11878(S26) PKC/CaMKII site, no change at the S12022(S170) PKC CaMKII site and a 28% lower value at the S4185(S469) PKA/PKG site in comparison with HTN(−) HFpEF. There were no significant differences between HTN(−)-HFpEF and control patients at any of the phosphorylation sites.

Plasma Biomarkers

Of the 16 biomarkers measured, 4 were significantly altered in the HTN patients in comparison with the controls (Table 4). C-reactive protein, sST2, TIMP-1, and NT-proBNP were higher in the HTN(−)-HFpEF patients compared with the controls. Levels of sST2, TIMP-1 and NT-proBNP increased further in the HTN(+)HFpEF group. There were no differences in MMP-1, 2, 3, 7, 9 or TIMP-2, 3, 4 or IL-6, IL-8 or tumor necrosis factor-α between groups.

There were significant correlations between plasma biomarkers and echocardiographic measurements of diastolic function (Figure 6); there was a statistically significant direct relationship between pulmonary capillary wedge pressure and NT-proBNP ($r^2=0.32$, $P=0.001$) sST2 ($r^2=0.26$, $P=0.005$), and TIMP-1 ($r^2=0.36$, $P<0.001$).
Correlations between NT-proBNP and titin phosphorylation at S4185(S469), S11878(S26), and S12022 (S170), and NT-proBNP and titin-dependent stiffness were tested. Although the sample size was limited, there was no clear relationship between NT-proBNP and S4185(S469; $r^2=0.009$) or S12022 (S170) phosphorylation ($r^2=0.04$). However, there was a trend with S11878(S26; $r^2=0.32$, $P=0.2$). There was also a trend between NT-proBNP and titin-dependent stiffness ($r^2=0.22$, $P=0.08$).

Finally, we also tested for correlations between sST2 and CVF and collagen-dependent stiffness. There were trends for both relationships, with sST2 increasing with increased CVF ($r^2=0.58$, $P=0.047$) and increased collagen-dependent stiffness ($r^2=0.40$, $P=0.011$).

**Effects of Diabetes Mellitus**

In the 2 HTN groups, the presence of DM did not affect echocardiographic parameters of hypertrophy or diastolic function, in vitro measures of stiffness, collagen or titin assays or biomarkers. DM also did not alter the differences in any of these parameters between HTN without or with HFpEF.
Discussion

The results of this study support several novel conclusions. The current study showed for the first time that patients with HTN(+)HFpEF have a significant increase in passive myocardial stiffness measured directly in LV myocardial strips. Although consistent with previous studies in animal models of HFpEF, this has never previously been documented in patients with HFpEF. Previous studies in HFpEF patients have examined the passive properties of the composite LV chamber or the passive properties of isolated cardiomyocytes, but not the passive properties of the intact myocardium. Making direct measurements in intact myocardium was a pivotal step for several reasons, including defining the relative mechanistic contributions of changes in titin and collagen to the increased passive myocardial stiffness seen in patients with HFpEF and the ability to differentiate the effects of comorbid conditions on myocardial properties from the effects of HFpEF itself. The techniques used to make direct measurements of passive myocardial stiffness in LV myocardial strips were developed by using samples from patients with heart failure and a preserved ejection fraction (HFpEF), but these techniques have not previously been applied to patients with HFpEF.

Previous studies in patients with HFpEF examined the contribution of changes in titin to the resting tension (passive stiffness) of isolated cardiomyocytes obtained from LV endomyocardial biopsies; these studies also showed that collagen volume fraction was increased. However, no previous study in patients with HFpEF has shown the relative contributions of changes in both collagen and titin to passive myocardial stiffness. The current study showed for the first time that the increase in myocardial stiffness in HFpEF patients was caused by changes in both ECM fibrillar collagen and cardiomyocyte titin. Specifically, HFpEF patients had increased collagen-dependent stiffness in association with increased fibrillar collagen content. HFpEF patients also had increased titin-dependent stiffness in association with significant changes in the phosphorylation state of titin, with decreased phosphorylation of a PKA/PKG site in the N2B element and increased phosphorylation of one of the PKC sites in the PEVK element.

Although all of the HFpEF patients examined in the current study had antecedent HTN and =50% had DM, the presence of these comorbid antecedent disease processes alone, in the absence of heart failure, did not alter total myocardial stiffness or its titin- and collagen-dependent components. This is the first clinical study in patients with HFpEF to examine and differentiate the effects of a major antecedent disease substrate, such as HTN, on myocardial structure and function before the clinical syndrome of HFpEF has actually developed. There has been significant controversy regarding the ability to differentiate or distinguish patients with HTN from those with HFpEF. It has been proposed that HFpEF is simply the aggregate of a number of comorbid factors such as HTN, DM, CAD, obesity, etc. The current study clearly demonstrates that there are specific structural and functional differences between patients with HTN without and with HFpEF. Similar conclusions are likely to be applicable to other comorbid or antecedent disease processes.

The current study also shows for the first time that the development of collagen- and titin-based changes in diastolic function...
in patients with HFpEF occur in association with proinflammatory and profibrotic stimuli as measured by plasma biomarkers. These data provide additional support for the overall schematic hypotheses proposed by Paulus and Tschape (ie, that proinflammatory and profibrotic signaling play a significant mechanistic role in the development of HFpEF) and constitute new mechanistic insights into the pathophysiology underlying HFpEF.13

Hypertensive Heart Disease

HTN results in LV pressure overload and causes a spectrum of structural, functional, and clinical outcomes that have collectively been called hypertensive heart disease (HHD). Data from animal models of pressure overload suggest that the myocardium responds to increased load by undergoing cardiomyocyte hypertrophy that results in an increase in myofibrillar content.9,10,45,51 In addition to the myofibrils, other structures and processes within both the cardiomyocyte and the ECM are altered in response to increased load. In animal models these events have a temporal sequence in which the additional changes in the cardiomyocyte and ECM occur only after myofibrillar hypertrophy is well under way or even complete.45,51 For example, in murine and feline models, during the first 1 to 2 weeks of pressure overload, there are no significant changes in CVF or diastolic filling pressures and no evidence of heart failure; however, after 4 to 8 weeks CVF and filling pressures are increased and evidence of heart failure develops. Data from the current study appear to parallel these temporal changes. Patients with HHD constitute a spectrum: HTN with LVH, HTN with LVH/concentric remodeling but no heart failure; however, after 4 to 8 weeks CVF and filling pressures are increased and evidence of heart failure develops. Data from the current study significantly advance our understanding of how these mechanisms contribute to changes in myocardial stiffness by examining intact myocardial samples that allowed integrated physiological studies to selectively examine the individual contributions of collagen and titin. The current study showed hypophosphorylation of PKG/PKA sites on titin in HFpEF patients, consistent with the reduction in passive tension detected when cardiomyocytes from HFpEF patients are treated with PKA/PKG19,20 and consistent with phosphorylation studies in animal models.43,54 In contrast to Van Heerebeck et al19 and Borbely et al,20 we did not detect changes in the N2BA/N2B ratio in HFpEF patients. In some but not all of the studies from these investigators, the N2BA/N2B ratio was increased in HFpEF, which would be expected to result in a potentially compensatory decrease in titin stiffness.16–20,26,28 It is worth noting that the HFpEF patients studied by these investigators had overtly decompensated heart failure and were therefore very likely at a more advanced stage of the

Overall, there is good concordance between the current study and previous clinical studies in pressure overload and HFpEF.16,19,20,26,28 Van Heerebeck et al19 and Borbely et al20 showed that patients with pressure-overload–induced HFpEF had increased cardiomyocyte stiffness; however, because these mechanical studies were performed in isolated cardiomyocytes with the ECM removed, the relative contribution of these changes in collagen and titin to myocardial stiffness could not be determined.

Our results indicate that, at SL 2.6 μm, increases in ECM collagen account for more than two-thirds of the increase in resting tension in HFpEF. However, the relative contributions of titin and collagen to resting tension are SL dependent; collagen accounts for a larger proportion at longer SLs and titin for a larger proportion at short SLs. Their relative contributions are therefore ultimately determined by the actual operating SL range in our patients, which is unknown. Based on studies performed in pigs,51 we do know that in large mammals the upper end of the operating range extends to 2.5 to 2.6 μm. To the extent that the actual operating range is lower, the increase in total resting tension in HFpEF will be more evenly divided between the 2.

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syndrome. Perhaps more advanced disease is required to elicit such a compensatory change. The current study also showed for the first time that patients with HFpEF have hyperphosphorylation of one of the PKC/CaMKII sites on titin. Previous animal studies have demonstrated that hyperphosphorylation at these sites results in increased titin stiffness\(^\text{33}\); however, the current study is the first clinical study to show that this contributes to increased myocardial stiffness in patients with HFpEF. In addition, although not yet studied in HFpEF, studies in HFrEF have suggested that CaMKII-dependent phosphorylation of S12022 (S170) may reduce passive stiffness.\(^\text{34}\)

The finding that both changes in collagen and titin may play a pivotal role in the development of HFpEF in HHD suggests the possibility that there may be common upstream mechanisms resulting in both changes.\(^\text{13}\) The presence of a HTN-induced proinflammatory, profibrotic, and redox stress state is supported by the plasma biomarker profiles found in the current study. The increase in C-reactive protein, sST2, and TIMP-1 in the HFpEF patients supports this hypothesis. Clearly, more investigation is needed in this area.

**Diabetes Mellitus**

DM has been shown in both animal studies and clinical disease to cause abnormal diastolic function that may contribute to the development of HFpEF.\(^\text{14,16,18}\) DM is reported to cause changes in collagen homeostasis (by altering cross-linking) and titin phosphorylation. Approximately 50% of the patients in the current study had DM, and HTN, as well. The presence of DM in addition to HTN did not appear to alter myocardial stiffness, collagen, or titin to an extent greater than that of HTN alone. Because of their rarity in the CABG population, we were unable to identify sufficient numbers of patients with DM without HTN for analysis. This lack of effect differs from previous studies of pressure overload (caused by aortic stenosis) in which the presence of DM caused a significant increase in cardiomyocyte stiffness over and above that caused by pressure overload alone. This may be explained by patient selection differences. Thus, previous studies did not specifically identify whether patients with pressure overload or DM had HFpEF or selectively examine patients with LVH without HFpEF.\(^\text{14,16,18}\)

Additionally, the current study may not have been powered sufficiently to examine the separate effects of DM; there may be differences between the pressure overload caused by HTN versus aortic stenosis, and the duration, severity, and management of DM may differ between studies. The current study does not necessarily bring into question the importance of DM in HFpEF, but it does raise the question of whether HTN and DM together have a combinatorial effect on passive stiffness. In addition, we did not examine the effects of DM on other determinants of diastolic function that could affect passive stiffness such as calcium homeostasis and contractile proteins, key determinants of relaxation rate and extent.

**Effects of Changes in Collagen and Titin Homeostasis on Diastolic Function**

Changes in collagen content, geometry, and composition are associated with abnormal diastolic function in HHD and other comorbidities common in patients with HFpEF.\(^\text{55}\) Collagen content is the product of the balance between collagen synthesis, post-synthetic processing, post-translational modification, and degradation. The current study was not designed to examine the determinants of collagen homeostasis or mechanisms that alter it. However, the changes in plasma biomarkers detected in the HFpEF patients may provide some insights.\(^\text{28}\) Increases in sST-2 and TIMP-1 suggest that a profibrotic stimulus was present that would be expected to increase collagen synthesis and decrease collagen degradation. TIMP-1 has been shown to inhibit MMPs, the major degradation enzymes present in the ECM. ST2 is a member of the interleukin 1 receptor family. Its functional ligand is IL-33, a cardiac fibroblast protein. Binding of IL-33 to membrane ST2 elicits an antihypertrophic and antifibrotic response. This cardioprotective effect is negated by sST2 which prevents the binding of IL-33 to membrane-bound ST2. Thus, increased TIMP-1 and sST-2 provide 2 potential mechanisms for the profibrotic state in HHD.

Titin’s I-band segment serves as a molecular spring that develops passive force when extended.\(^\text{27}\) Alternative splicing results in either the shorter and stiffer N2B or the longer and more compliant N2BA isoforms, which are coexpressed within the sarcomere. When the N2BA/N2B isoform ratio increases, cardiomyocyte and myocardial stiffness decrease as reported in heart failure with reduced ejection fraction.\(^\text{58,59}\) In contrast, Zile et al\(^\text{28}\) reported an increase in the N2BA/N2B isoform ratio in HFpEF patients but cardiomyocyte stiffness was increased, implying that changes in phosphorylation outweigh the isoform shift. The N2BA/N2B ratio did not change in the current study. As discussed above, it is possible that the isoform switch occurs at a later stage of HFpEF. In addition, the N2BA:N2B ratio did not change in at least 1 study of human hypertrophic cardiomyopathy and dilated cardiomyopathy.\(^\text{60}\)

The phosphorylation state of 2 elements within titin’s spring segment also modulates cardiomyocyte stiffness. Sites within the N2B element (eg, S4185[S469]) are phosphorylated by PKA and PKG, which decreases passive force.\(^\text{25,26}\) Sites within the PEVK element (S1178[S26] and S12022 [S170]) are phosphorylated by PKC\(\alpha\) (other PKC isoforms have not yet been studied), which increases passive force. Additionally our data and the data of other investigators suggest that these sites in titin are also a target of CaMKII.\(^\text{54,61,62}\) In both the current and previous studies in HFpEF patients,\(^\text{28}\) hypophosphorylation of the N2B element has been observed. Moreover, the treatment of skinned cardiomyocytes from HFpEF patients with PKG decreased cardiomyocyte stiffness,\(^\text{24}\) indicating that hypophosphorylation of PKA/PKG sites contributes to elevated passive force. Importantly, PKG treatment did not lower stiffness to the level present in control cardiomyocytes. It has been speculated\(^\text{22}\) that this residual elevation in tension is due to hyperphosphorylation of PKC sites. In the current study we demonstrate that hyperphosphorylation of the S11878(S26) PKC/CaMKII site in the PEVK segment is associated with increased myocardial stiffness in HFpEF patients. This constitutes a novel mechanism of increased stiffness in HFpEF.

The increased PKC\(\alpha\) activity that is suggested by the increased S26 phosphorylation did not result in phosphorylation of S12022 (S170). Previous studies indicate that PKC\(\alpha\) is preferably active at sites with N-terminal and C-terminal basic residues.\(^\text{63}\) These studies also indicate that PKC preferentially phosphorylates serines with basic residues within
3 amino acids of both the C and N termini. Based on these results, PKC should have a stronger affinity for S11878(S26) than S170 because of the closer proximity of the neighboring basic amino acids (lysine [K] and arginine [R]). Finally, increased PKCoT activity has been shown to directly increase protein phosphatase inhibitor-1 activity,\(^6\) an effect that should facilitate hypophosphorylation of PKA/PKG sites in the N2B element (its effect on PKCoT sites might be negated by the increased PKC activity). Thus, increased PKCoT levels might increase stiffness directly through phosphorylation of PEVK S11878(S26) and indirectly through protein phosphatase inhibitor-1 activation, causing hypophosphorylation of PKA/PKG sites.

**Limitations**

The demographic characteristics of the current study population were typical of a CABG population. However, our HFrEF patients were slightly younger and more often male than typical patients in epidemiological studies or randomized clinical trials. From the viewpoint of LV structure and function, however, they were typical of previous HFrEF studies.

What constitutes an appropriate control group is a challenging issue in all research using human myocardium. The degree of angiographic CAD was similar in all 3 study groups; therefore, CAD was not likely to be a confounding variable across groups. Alternative choices for controls are limited and less than ideal. Unused explanted donor hearts are usually from young subjects who have been subjected to high levels of stress for varying durations. Open heart procedures in patients without CAD or LV remodeling (eg, atrial septal defect closure) are now rare and usually performed in younger patients. Samples from endomyocardial biopsies cannot be used for mechanical tissue studies because of tissue trauma. On the other hand, CAD, much of which is likely subclinical, is very common in HHD and HFpEF patients. Indeed, a recent report indicates that a majority of HFpEF patients have epicardial coronary stenosis.\(^3\) Therefore, the presence of CAD can be considered a real-world background in HTN patients and controls. The limitation imposed by the presence of CAD, however, is that we cannot exclude possible CAD effects on myocardial stiffness and distinguish them from the effects of HTN or DM.

The current study focused specifically on the contribution of collagen and titin to the development of HFpEF. As in HFrEF, in HFpEF other mechanisms also contribute to the development of heart failure. Thus, changes in calcium homeostasis, energetics, and actin-myosin cross-bridge dynamics, which govern the speed and completeness of relaxation, all may play a role in HFpEF in addition to changes in passive stiffness.

Titin is a large and complex molecule with many phosphorylation sites outside the mechanically relevant spring region; however, assessing all of them may not be required in the context of the current study. The current study focused on 3 sites in the N2B and PEVK elements that have been documented in several studies for which we have made and characterized phospho-site–specific antibodies. Two additional PKA/PKG sites in the N2B element were published recently by Köttet et al\(^\text{40}\) (S4010 and S4099). In their recent study, Köttet et al showed that all 3 sites on the N2B element are hypophosphorylated in heart failure, indicating that they change in concert. Thus, assessing the established Ser4185/485 site is likely to reveal the general phosphorylation status of the N2B element.

It is important to acknowledge other limitations of the current study. We did not systematically study all titin phosphorylation sites. The protein has at least 70 phosphorylation sites, as shown by in vivo phosphoproteomics with the use of a SILAC approach.\(^4\) Moreover, recent work has suggested that the N2B domain contains several more conserved serines that are differentially phosphorylated in human HFrEF and in animal models of HFpEF.\(^32,43,55\) Phospho-specific antibodies against the respective N2Bus sites were used in human hypertrophic cardiomyopathy and dilated cardiomyopathy hearts.\(^54,60\) These studies underscore the complexity of titin phosphorylation schemes and the need for further research in patients with HFpEF, and other forms of heart failure, as well, that will allow a more complete understanding of the contribution of titin to passive stiffness.

The current study also did not focus on upstream regulatory mechanisms affecting titin phosphorylation such as changes in the expression, abundance, and activity of relevant protein kinases (eg, PKA, PKG, CaMKII, PKC) and phosphatases (protein phosphatase inhibitor-1, protein phosphatase inhibitor-2a). Changes in kinase and phosphatase activity have been shown to be important in other forms of heart failure.\(^26,43\), changes in HFpEF may represent important targets for novel therapies and important areas for future research.

Several other important mechanisms influencing the ECM were not examined in the current study, eg, collagen isoform expression and content, troponin I and T, and galec-tin-3. Unfortunately, the size of the LV biopsy limited the total number of experimental measurements obtainable from each sample. Each of these additional mechanisms represents important directions for future research.

Finally, the effects of medications taken by the patients before surgery on titin phosphorylation and ECM homeostasis could not be assessed.

**Clinical Implications**

HTN is commonly a progressive process that leads to adverse cardiovascular remodeling, abnormal diastolic function, and the development of heart failure, particularly HFpEF.\(^65,67\) Once HFpEF has developed, subsequent morbidity and mortality rates are very high.\(^67–76\) Treatment of HHD is thus a critical unmet challenge. One clear answer is the prevention of HHD by early treatment of high blood pressure, but prevention alone is insufficient. LVH, concentric remodeling, and diastolic dysfunction commonly develop without concomitant symptoms, often before HTN has been detected. Furthermore, once LV remodeling and HFpEF develop, even guideline-prescribed blood pressure control does not reduce morbidity and mortality. Randomized clinical trials in HFpEF of β-blockers, angiotension-converting enzyme inhibitors, angiotension II receptor blockers, aldosterone blockers, and phosphodiesters-ase type 5 inhibitors, in which >90% of the patients had HTN, have failed to show improvement in morbidity or mortality.\(^70–76\) Therefore, there is a need for novel treatments beyond blood pressure control.

The development of novel therapies must overcome several critical barriers. Most importantly, the cellular, extracellular, and...
molecular mechanisms that cause the progression from HHD to HFpEF must be defined in humans. Although animal models are useful, they do not capture all of the key elements of complex and chronic human disease processes. Once proven operative in patients, each mechanism can serve as a target for successful therapy. The current study identifies 2 of these mechanisms; changes in collagen content and titin phosphorylation, each of which is a potential target. In addition, plasma biomarkers that reflect these changes in collagen, titin, and the profibrotic milieu could be used to improve diagnostic criteria for HFpEF and prognostic assessment. Thus, changes in biomarkers such as TIMP-1 and sST-2 may detect the earliest transition from HTN to HFpEF. Finally, changes in these biomarkers could possibly be used to monitor treatment efficacy before changes in myocardial structure and function or clinical status are evident.

Conclusions

HTN in the absence of HFpEF was not associated with increased passive myocardial stiffness. However, we show for the first time that patients with HTN and HFpEF have markedly increased passive myocardial stiffness due to increases in the contribution of both collagen and titin. These results suggest that the development of HFpEF is linked to a major increase in passive stiffness caused by changes in both collagen and titin homeostasis.

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Once heart failure with a preserved ejection fraction (HFpEF) has developed, subsequent morbidity and mortality rates are very high; the guideline-prescribed treatment of blood pressure and other comorbid conditions does not reduce these adverse outcomes. Randomized clinical trials in HFpEF of β-blockers, angiotension-converting enzyme inhibitors, angiotension II receptor blockers, aldosterone blockers, and phosphodiesterase type 5 inhibitors have failed to show improvement in morbidity or mortality. Therefore, there is a need for novel treatments that target the cellular, extracellular, and molecular mechanisms that cause the development of HFpEF. The current study identified 2 such mechanisms: changes in collagen content and titin phosphorylation, each of which could serve as potential therapeutic targets. Patients with HFpEF had increased total, collagen-dependent, and titin-dependent stiffness, increased insoluble collagen, increased titin phosphorylation on PEVK S11878(S267), and reduced phosphorylation on N2B S4185(S469). In addition, plasma biomarkers that reflect these changes in collagen, titin, and the profibrotic milieu were found to correlate with the presence and severity of HFpEF. Plasma tissue inhibitor of matrix metalloproteinase 1 and soluble ST-2 increased during the transition from hypertension to HFpEF. In addition, these biomarkers might be useful in monitoring treatment efficacy before changes in myocardial structure and function or clinical status are evident. Thus, the current studies suggest that the development of HFpEF depends on changes in collagen and titin homeostasis and the presence of a proinflammatory/profibrotic milieu. Each of these cellular, extracellular, and molecular mechanisms constitute important targets for the development of novel management strategies for HFpEF patients.

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Myocardial Stiffness in Patients With Heart Failure and a Preserved Ejection Fraction: Contributions of Collagen and Titin
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좌심실 수축기능이 보존된 심부전 환자에서 보이는 심근 경직도는 콜라겐과 틈이의 향상성 변화 때문이다.

박 혼 준 교수 가톨릭대학교 서울성모병원 순환기내과

초록

배경
본 연구의 목적은 좌심실 수축기능이 보존된 심부전(heart failure with preserved ejection fraction, HFpEF) 환자에서 수동 심근 경직도가 증가하는지 여부와 이러한 현상이 세포외기질의 섬유성 틈이과 심근세포 내 틈이의 변화와 상관관계가 있는 지 알아보고자 하였다.

방법 및 결과
관상동맥 우회술을 시행 받은 70명의 환자에서 심초음파, 혈중 비아모커 분석 및 수술 중 좌심실 심외막 전벽에서 생검을 시행하였다. 환자들을 고혈압이나 당뇨병이 없는 대조군(17명), HFpEF가 없는 고혈압군[HTN(-)HFpEF, 31명], HFpEF가 있는 고혈압군[HTN(+)HFpEF, 22명]의 세 개 군으로 분류하고, 경계를 이용하여 수동 경직도 측정(differential extraction assay), 콜라겐 분석(biochemistry or histology) 및 틈이의 아형 및 인산화 분석 등을 시행하였다. 대조군과 비교했을 때 HTN(-)HFpEF군의 좌심실 확장기말 압력, 심근의 수동 경직도, 콜라겐이나 틈이의 인산화 분석에서는 차이가 없으나, 염증과 관련된 바이오마커(C-reactive protein, soluble ST2, tissue inhibitor of metalloproteinase 1)는 증가되어 있었다. HTN(+)HFpEF군에서는 대조군이나 HTN(-)HFpEF군과 비교하여 좌심실 확장 기말 압력, 좌심방 용적, NTproBNP(N-terminal propeptide of brain natriuretic peptide), 수동 경직도, 불용성 콜라겐 등이 증가되었으며, 틈이의 아형분석에서 PeVK S11878(S26)의 인산화 증가와 N2B S4185(S469) 인산화 감소 및 염증과 관련된 바이오마커의 증가가 관찰되었다.

결론
HTN(-)HFpEF는 수동 심근 경직도를 변화시키지 않았으나, HTN(+)HFpEF 환자에서는 콜라겐과 틈이에 의존적으로 수동 심근 경직도가 유의하게 증가한다. 이는 HFpEF의 발병 과정에서 콜라겐과 틈이의 향상성 변화가 연관이 있을음을 시사한다.