Although it appears too difficult to rank the three proposed model-selecting CRMs, our simulations provide some insight into their different characteristics. For example, when the true dose–toxicity relationship is quite flat up to the targeted MTD (in this situation some existing methods fail in the MTD estimation [20]), the model-selecting CRM based on the DIC works well. The model-selecting CRM based on the PPL appears to provide a conservative dose escalation, resulting in the number of patients allocated to the higher more toxic dose levels being relatively smaller than with the other model-selecting techniques. This may be a result of the PPL being based on the posterior predictive distribution allowing for larger variability than the posterior distribution. The model-selecting CRM based on the PMP could be quite similar in performance to the model averaging CRM because both use the PMP.

An interpretation of the working model in terms of Bayesian prior point estimates of the probabilities of toxicity is quite common in this area. Although such an interpretation is not only needed but may not correspond in practice to anything very concrete (since operating characteristics and final recommendations remain invariant to these prior point estimates being raised to any positive power), many clinicians and experimenters are reassured by the interpretation. When this is so, an additional reassurance can be obtained by using several working models as opposed to a single one, in particular in cases when there are differences in opinion among investigators. Rather than force the choice to any one particular working model it is possible to say that all the suggested potential working models can be employed and then, as the observations are accrued, the method itself will help select the most plausible given this information. In reality, this will still end up being equivalent to having worked with a particular simple model choice. However, for those investigators who may be concerned at the apparent arbitrariness of working model selection, and the worry that this may impact the relative performance of the method, the use of several models enables them to gain confidence in the resulting MTD estimate, based as it is on a number of rival models.

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Pulmonary hypertension predicts adverse cardiac events after restrictive mitral annuloplasty for severe functional mitral regurgitation

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Objectives: Pulmonary hypertension (PH) is an indicator of a poor prognosis in patients with dilated cardiomyopathy. Few studies have investigated the prognostic role of PH in patients undergoing restrictive mitral annuloplasty (RMA) for severe functional mitral regurgitation secondary to advanced cardiomyopathy.

Methods: A total of 46 patients undergoing RMA were classified into 3 groups on the basis of the Doppler-derived systolic pulmonary artery pressure (PAP) at baseline. Of the 46 patients, 19 had a systolic PAP less than 40 mm Hg (mild PH group), 17 had a systolic PAP of 40 to 60 mm Hg (moderate PH group), and 10 had a systolic PAP greater than 60 mm Hg (severe PH group).

Results: Postoperative cardiac catheterization showed that the RMA procedure resulted in a significant reduction of the left ventricular (LV) preload and improvements in LV systolic function in all 3 groups, along with the relief of symptoms. During the follow-up period (mean 36 ± 19 months), cardiac death occurred in 6 patients, readmission because of heart failure in 3, and fatal arrhythmia in 1. The rate of freedom from these cardiac events at 3 years was 93% ± 7%, 88% ± 8%, and 56% ± 17% in the mild, moderate, and severe PH groups ($P < .001$). Serial echocardiography showed that significant LV reverse remodeling occurred in 89%, 71%, and 25% of the mild, moderate, and severe PH groups, respectively. Multivariate Cox regression analysis identified severe PH (systolic PAP > 60 mm Hg) as a significant predictor of adverse cardiac events, as well as LV remodeling after RMA.

Conclusions: Noninvasive assessment of preoperative PH has a prognostic value in patients undergoing RMA for severe functional mitral regurgitation secondary to advanced cardiomyopathy. (J Thorac Cardiovasc Surg 2010;■:1-10)

It is well known that pulmonary hypertension (PH) is an indicator of a poor prognosis in patients with dilated cardiomyopathy and functional mitral regurgitation (MR),[1] as well as in heart transplant recipients.[2] However, whether PH has the same prognostic value in patients who have undergone surgery for functional MR complicated by advanced cardiomyopathy is unknown.

Since Bolling and colleagues[3] first reported the feasibility of surgery for uncontrollable severe MR in patients with end-stage cardiomyopathy, restrictive mitral annuloplasty (RMA) has become the preferred surgical treatment of this condition. Several factors have been shown to be predictors of poor outcome after RMA.[4-8] However, few investigations have assessed the influence of PH on symptomatic improvements, left ventricular (LV) function, or survival after RMA in patients with advanced cardiomyopathy.

In the present study, we investigated whether PH has a prognostic value in patients undergoing RMA for functional MR. In addition, we determined which parameters might function as predictors of postoperative outcome and reverse LV remodeling in those patients, with a focus on preoperative PH.

MATERIALS AND METHODS

Patients

We examined the records of 65 consecutive patients who had undergone RMA for functional MR at our institution from March 2004 to December 2009. Of those, 46 patients were chosen as study subjects, according to the following inclusion criteria: 1) chronic heart failure with New York Heart Association (NYHA) functional class III or IV and a history of at least 1 hospitalization; 2) advanced LV remodeling, defined as a LV ejection

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Abbreviations and Acronyms

BNP = brain natriuretic peptide
LV = left ventricular
LVEDD = left ventricular end-diastolic dimension
LVEF = left ventricular ejection fraction
MR = mitral regurgitation
NYHA = New York Heart Association
PAP = pulmonary artery pressure
PH = pulmonary hypertension
PVR = pulmonary vascular resistance
RMA = restrictive mitral annuloplasty
TR = tricuspid regurgitation

fraction (LVEF) of less than 40% and LV end-systolic volume index greater than 60 ml/m^2, as shown by left ventriculography; and 3) severe MR caused by restrictive leaflet motion secondary to global LV dilatation. Patients with a lesser degree of LV remodeling and ischemic MR secondary to a regional LV deformity due to inferior/posterior myocardial infarction were excluded from the present study. The patients with recent myocardial infarction (< 3 months), organic MR, rheumatic mitral disease, or a known noncardiac cause of PH and those who had undergone concomitant surgical ventricular reconstruction were not included in the present study.

To determine whether the degree of preoperative PH was associated with the postoperative outcome, the patients were classified into 3 groups according to the Doppler-derived systolic pulmonary artery pressure (PAP) at baseline. Of the 46 patients, 19 had a systolic PAP of less than 40 mm Hg (mild PH group), 17 had a systolic PAP of 40 to 60 mm Hg (moderate PH group), and 10 had a systolic PAP greater than 60 mm Hg (severe PH group).

The clinical characteristics and surgical data are listed in Table 1. No significant differences were found in gender, body surface area, NYHA functional class, or prevalence of complications among the 3 groups. The patients in the severe PH group had had a longer duration of heart failure than those in the other groups, although they were younger than the patients in the moderate PH group.

The related institutional ethics committees approved the present study and waived the need for individual consent for the retrospective analysis. Each patient provided written informed consent for the procedure before surgery.

Echocardiographic Measurements and Calculations

Two-dimensional and Doppler transthoracic echocardiography were performed at baseline, at 1 month after surgery (mean 28 ± 5 days), and annually thereafter. The preoperative (baseline) and postoperative echocardiographic examinations at 1 month after surgery were performed within 1 day of cardiac catheterization. Transesophageal echocardiography was also performed within 1 week before surgery to confirm the severity and precise mechanism of MR in all patients. All echocardiographic studies were performed using commercially available 3.75-MHz transducers (Toshiba, Tokyo, Japan, and Hewlett-Packard Sonos) by the same echocardiographic expert examiner (S.F.), who was unaware of the clinical status of the patients.

LV Function and Left Atrial Dimensions

The LV end-diastolic dimension (LVEDD), LV end-systolic dimension, and left atrial dimension was determined from 2-dimensional echocardiographic images in the parasternal long-axis views. The LVEF was calculated using Simpson's method with 2 apical views.

Doppler-Derived Systolic PAP

The systolic PAP was calculated by adding the systolic pressure gradient across the tricuspid valve derived from the tricuspid regurgitation (TR) to the estimated right atrial pressure.[9,10] The tricuspid regurgitant signal was recorded by continuous-wave Doppler echocardiography, and its maximal velocity was measured. Using the simplified Bernoulli equation, the pressure gradient across the tricuspid valve was calculated. The right atrial pressure was estimated using the dimension of the inferior vena cava and the response to changes in respiration.[11] In the present patients, a tricuspid regurgitant signal was detected in all patients during the baseline examination and in the great majority of patients during the follow-up examinations.

Mitral and Tricuspid Valve Measurements

The severity of MR and TR was graded semiquantitatively from the color flow Doppler data. In our routine assessment, MR severity was characterized as none (0), trivial (1+), mild (2+), moderate (3+), or severe (4+), depending on how far beyond the mitral valve the regurgitation extended into the left atrium. The tenting height was measured between the line connecting the annular hinge points and the leaflet coaptation point, and the coaptation length was measured directly. The area of the mitral valve orifice was determined by direct planimetry or using the pressure half-time method, and the mean transmitral diastolic gradient was calculated using the Bernoulli equation determined from continuous-wave Doppler echocardiography.

Cardiac Catheterization and Hemodynamic Measurements

Cardiac catheterization was performed before and 1 month after surgery to measure the following routine cardiac hemodynamic parameters: LV end-diastolic volume index, LV end-systolic volume index, LV systolic pressure, LV end-diastolic pressure, pulmonary capillary wedge pressure, systolic and mean PAP, transmitral diastolic pressure gradient (pulmonary capillary wedge pressure minus the LV end-diastolic pressure), right atrial pressure, systemic vascular resistance ([mean arterial pressure minus right atrial pressure] multiplied by 80 and divided by the cardiac output), and pulmonary vascular resistance (PVR) ([mean PAP minus pulmonary capillary wedge pressure] multiplied by 80 and divided by the cardiac output). Our catheterization technique has been previously described.[12]

Surgical Procedures

A median sternotomy was performed under a mild hypothermic cardiopulmonary bypass, with intermittent cold blood cardioplegia. Mitral valve surgery was performed through a trans-septal superior approach. Carpentier-Edwards physio rings (Carpentier Ring, Edwards Lifesciences, Irvine, Calif) were used for all RMA procedures. The ring size was determined after careful measurement of the intercommissural distance and the height of the anterior leaflet and then downsizing by 2 to 3 sizes. No other adjunct procedures were performed on the valve itself. No significant differences were found in regard to the surgical procedures among the groups, including the size of the mitral annulus ring implanted and frequency of the concomitant procedures (Table 1).

Clinical Follow-up

Clinical follow-up examinations were completed for the 43 operative survivors, with a mean duration of 36 ± 19 months (range 5–77 months). After surgery, the patients were treated with standard heart failure medications, including angiotensin-converting enzyme inhibitors or angiotensin-II receptor blockers, β-blockers, and diuretics. Every 6 to 12 months, they were assessed in our department and by their primary cardiologist. The functional status was assessed according to the NYHA criteria for

TABLE 1. Clinical characteristics and surgical data

Parameter	All (n = 46)	Mild (n = 19)	Moderate (n = 17)	Severe (n = 10)	*P* Value
Age (y)	64 ± 8	62 ± 9	68 ± 6*	60 ± 7	.02
Men (%)	35 (76)	13 (68)	14 (82)	8 (80)	NS
Body surface area (m^2)	1.7 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	1.6 ± 0.2	NS
NYHA functional class	3.2 ± 0.4	3.2 ± 0.4	3.1 ± 0.3	3.2 ± 0.4	NS
Duration of HF (mo)	25 ± 15	18 ± 9*	24 ± 14*	39 ± 15	< .01
Ischemic etiology (%)	32 (70)	14 (74)	12 (71)	6 (60)	NS
Hypertension	30 (65)	13 (68)	12 (71)	5 (50)	NS
Diabetes	17 (37)	8 (42)	6 (35)	3 (30)	NS
Hyperlipidemia	20 (43)	10 (0)	6 (35)	4 (40)	NS
COPD	7 (15)	1 (5)	3 (18)	3 (30)	NS
Chronic renal disease	16 (35)	7 (37)	3 (18)	6 (60)	NS
Peripheral vascular disease	6 (13)	0 (0)	3 (18)	3 (30)	NS
Cerebrovascular accident	14 (30)	4 (21)	6 (35)	4 (40)	NS
Atrial fibrillation	21 (46)	9 (47)	8 (47)	4 (40)	NS
Previous CABG	1 (2)	0 (0)	0 (0)	1 (10)	NS
Previous PCI	13 (28)	5 (26)	5 (29)	3 (30)	NS
β-Blockers	33 (72)	17 (89)	11 (65)	5 (50)	NS
ACE inhibitors	9 (20)	3 (16)	4 (24)	2 (20)	NS
ARB	17 (37)	9 (47)	7 (41)	1 (10)	NS
Nitrate	12 (26)	6 (32)	3 (18)	3 (30)	NS
Diuretics	34 (74)	11 (58)	15 (82)	8 (80)	NS
Surgical data					
CPB (min)	240 ± 71	222 ± 43	252 ± 69	252 ± 109	NS
ACC (min)	133 ± 43	130 ± 40	147 ± 42	114 ± 43	NS
Physio ring (mm)					
24	37 (80)	15 (79)	15 (82)	7 (70)	NS
26	9 (20)	4 (21)	2 (12)	3 (30)	NS
Concomitant procedure					
CABG	26 (57)	11 (58)	10 (59)	5 (50)	NS
TAP	43 (93)	17 (89)	16 (94)	10 (100)	NS
Modified maze	21 (46)	9 (47)	8 (47)	4 (40)	NS

NYHA, New York Heart Association; *HF,* heart failure; *COPD,* chronic obstructive pulmonary disease; *CABG,* coronary artery bypass grafting; *PCI,* percutaneous coronary intervention; *ACE,* angiotensin-converting enzyme; *ARB,* angiotensin-II receptor blocker; *CPB,* cardiopulmonary bypass; *ACC,* aortic crossclamp; *TAP,* tricuspid annuloplasty.
*$P < .05$ versus severe PH.

symptoms of heart failure and serum brain natriuretic peptide (BNP) level. A retrospective review of the medical records of these patients was performed for the preoperative and postoperative data, and the current information was obtained by interviewing the patient or the referring cardiologist. The postoperative adverse cardiac events included cardiac death, myocardial infarction, endocarditis, thromboembolism, reoperation for recurrent MR, readmission for heart failure, and fatal arrhythmia.

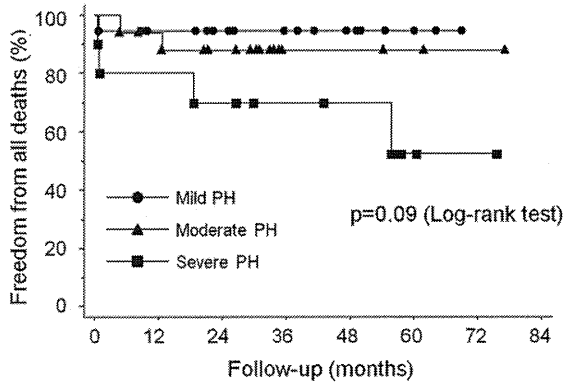
Statistical Analysis

The values for continuous variables are expressed as the mean ± standard deviation. The Kruskal-Wallis and chi-square tests were used to compare the pre- and postoperative values among the 3 groups. The functional and echocardiographic variables over time were compared using repeated measures analysis of variance, followed by a Bonferroni test for individual significant differences. Univariate analysis of the predictors of adverse cardiac events was performed using a Cox proportional hazard model (see Appendix). The factors with $P < .1$ were then entered appropriately into a multivariate model. The results are summarized as hazard ratios and 95% confidence intervals. Stepwise logistic regression analysis was performed to identify predictors for failure of reverse LV remodeling (see Appendix). Kaplan-Meier and log-rank analyses were performed to compare survival and the freedom from adverse cardiac events. Correlations between the Doppler-derived and catheter-measured systolic PAP were tested using linear correlation analysis. A Bland-Altman analysis was used to further determine the agreement between the 2 modalities by calculating the bias (mean difference) and 95% limits of agreement (± 2 standard deviation).[13] $P < .05$ was considered statistically significant. The statistical analyses were performed using JMP, version 7.0 (SAS Institute, Cary, NC).

RESULTS

Survival

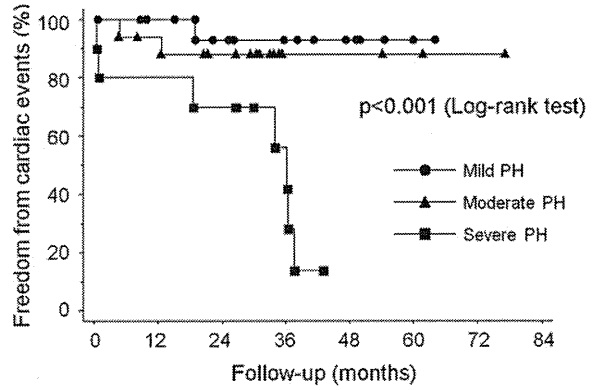
In the mild PH group, 1 early noncardiac death and 1 late fatal arrhythmia occurred. In the moderate PH group, 2 late cardiac deaths occurred. In the severe PH group, 2 early cardiac deaths, 2 late cardiac deaths, and 3 late readmissions because of heart failure occurred. None of the patients required reoperation for MR recurrence or endocarditis or presented with myocardial infarction or cerebrovascular or thromboembolic events during the follow-up period. The actuarial survival rate free from all deaths at 3 years was 95% ± 5%, 88% ± 8%, and 70% ± 15% in the mild, moderate, and severe PH groups, respectively ($P = .09$;



Patients at risk

	0	12	24	36	48	60	72 (month)
Mild PH	19	16	13	10	6	3	0
Moderate PH	17	15	12	3	3	2	1
Severe PH	10	8	7	5	4	2	1
Total	46	39	32	18	13	7	2

FIGURE 1. Actuarial survival rates. *Circles*, *triangles*, and *squares* indicate mild, moderate and severe groups, respectively. *PH,* Pulmonary hypertension. Numbers at bottom indicate patients at risk at each interval.



Patients at risk

	0	12	24	36	48	60	72 (month)
Mild PH	19	16	12	9	5	2	0
Moderate PH	17	15	12	3	3	2	1
Severe PH	10	8	7	4	0	0	0
Total	46	39	31	16	8	4	1

FIGURE 2. Freedom from adverse cardiac events. *Circles*, *triangles*, and *squares* indicate mild, moderate, and severe groups, respectively. *PH,* Pulmonary hypertension. Numbers at bottom indicate patients at risk at each interval.

Figure 1). The corresponding rates of freedom from these cardiac events at 3 years were 93% ± 7%, 88% ± 8%, and 56% ± 17% ($P < .001$; Figure 2). The severity of preoperative PH was significantly associated with overall survival and the freedom from adverse cardiac events.

Acute Hemodynamic Changes After RMA

Preoperatively, all patients showed impairment of systemic and pulmonary hemodynamic conditions, the severity of which increased with the severity of the PH (Table 2, Figure 3). The systolic and mean PAP and PVR in the severe PH group were significantly greater than those in the other 2 groups.

From baseline to 1 month after surgery, the patients in the mild PH group showed good functional improvement, but those in the severe PH group showed less improvement. The patients in the moderate PH group showed improvement that was intermediate between that of the mild and severe PH groups. The LV volumes significantly decreased, and the LVEF improved in all 3 groups, although patients in the severe PH group showed less improvement in those parameters than in the other 2 groups. The LV systolic pressures did not change; however, the LV end-diastolic pressure decreased significantly in all groups.

In the patients with mild and moderate PH, the cardiac index increased significantly, the systemic vascular resistance decreased, and the systolic and mean PAP and PVR remained unchanged. In contrast, in the severe PH group, the cardiac index and systemic vascular resistance remained unchanged, and the systolic and mean PAP and the PVR significantly decreased. In the severe PH group, both systolic and mean PAP and PVR significantly decreased; Importantly, in patients with severe PH, the systolic PAP and PVR at 1 month after surgery were significantly greater than those in the mild PH group and remained substantially abnormal, suggesting the persistence of abnormal pulmonary hemodynamics.

Serial Echocardiographic Examinations

LV dimensions and function and left atrial dimensions. In the mild and moderate groups, the LVEDD, left ventricular end-systolic dimension, and left atrial dimension had decreased significantly and the LVEF had improved at 1 month after surgery (Table 3, Figure 4). Also, these improvements (reverse remodeling) persisted during the follow-up period. In the severe PH group, the LV dimensions remained unchanged at 1 month after surgery and were significantly greater than the other 2 groups. In addition, the severe PH group had lower LVEF values than the mild and moderate PH groups at all follow-up examinations.

Doppler-derived systolic PAP. In the mild and moderate PH groups, the mean systolic PAP at 1 month after surgery had remained or returned to a normal range in most patients. Those values had stabilized within the normal range during the follow-up period, along with an improvement in MR. In contrast, the mean systolic PAP in the severe PH group had decreased at 1 month after surgery; however, the values had never returned to a normal range, regardless of significant improvement in MR. The systolic PAP in the severe PH group had gradually worsened for a period of years, in contrast to the LV systolic function and LV dimension.

Mitral valve performance and measurements. Serial examinations showed significant improvements in MR in all groups, with optimal mitral valve geometry in terms of

TABLE 2. Acute hemodynamic changes

Variable	All		Mild		Moderate		Severe	
	Baseline (n = 46)	Discharge (n = 33)	Baseline (n = 19)	Discharge (n = 13)	Baseline (n = 17)	Discharge (n = 14)	Baseline (n = 10)	Discharge (n = 6)
LVEDVI (mL/m^2)	140 ± 38	110 ± 35*	129 ± 29	105 ± 22*	144 ± 45	106 ± 42*	155 ± 35	133 ± 41*
LVESVI (mL/m^2)	104 ± 34	59 ± 29*	94 ± 25	53 ± 17*	105 ± 40	54 ± 28*	120 ± 33	85 ± 40*
LVEF (%)	26 ± 7	37 ± 12*	27 ± 8	39 ± 10*	28 ± 7	38 ± 13*	23 ± 6	32 ± 16*
LVSP (mm Hg)	116 ± 22	120 ± 15	114 ± 19	120 ± 14	120 ± 20	121 ± 15	111 ± 29	118 ± 20
LVEDP (mm Hg)	20 ± 8	12 ± 4*	17 ± 8†	10 ± 3*	20 ± 5	14 ± 4*	26 ± 8	14 ± 5*
PCWP (mm Hg)	20 ± 8	15 ± 5*	16 ± 8†	13 ± 4	20 ± 6	16 ± 4	27 ± 8	17 ± 7
Systolic PAP (mm Hg)	44 ± 15	35 ± 8*	34 ± 10†	31 ± 7†	43 ± 10†	38 ± 8	59 ± 16	39 ± 10*
Mean PAP (mm Hg)	29 ± 10	23 ± 5*	22 ± 7†	20 ± 4†	29 ± 7†	25 ± 5	40 ± 10	26 ± 5*
RAP (mm Hg)	8.2 ± 4.5	8.9 ± 3.0	6.7 ± 3.8	8.0 ± 2.9	7.9 ± 4.8	9.7 ± 2.7	10.0 ± 4.2	9.0 ± 4.0
CI (L/min/m^2)	2.6 ± 0.6	3.0 ± 0.7*	2.6 ± 0.5	3.1 ± 0.7*†	2.7 ± 0.6	3.0 ± 0.7*†	2.2 ± 0.6	2.2 ± 0.4
TMPG (mm Hg)	0.4 ± 6.4	2.5 ± 3.1	0.1 ± 6.6	2.8 ± 3.1	0.2 ± 7.0	2.1 ± 2.9	1.4 ± 5.5	2.7 ± 3.7
SVR (dynes · s · cm^{-5})	1520 ± 380	1320 ± 420*	1590 ± 340	1310 ± 310*	1490 ± 470	1240 ± 290*	1470 ± 310	1520 ± 740
PVR (dynes · s · cm^{-5})	171 ± 90	137 ± 54	127 ± 62†	123 ± 44†	162 ± 48†	143 ± 63	243 ± 122	147 ± 53*

LVEDVI, Left ventricular end-diastolic volume index; *LVESVI,* left ventricular end-systolic volume index; *LVEF,* left ventricular ejection fraction; *PCWP,* pulmonary capillary wedge pressure; *PAP,* pulmonary artery pressure; *RAP,* right atrial pressure; *CI,* cardiac index; *TMPG,* transmitral pressure gradient; *SVR,* systemic vascular resistance; *PVR,* pulmonary vascular resistance. *$P < .05$ versus variables at baseline in each group. †$P < .05$ versus severe PH group at each point.

a decreased tenting height and adequate coaptation length. No significant differences were found postoperatively in the changes in MR grade, TR grade, coaptation length, tenting height, mitral valve orifice area, or mean transmitral pressure gradient among the 3 study groups.

Symptoms and Serum BNP Levels

The NYHA functional improvements and changes in serum BNP levels were not significantly associated with the preoperative severity of PH (Figure 5). The patients in the severe PH group tended to show less improvement. In

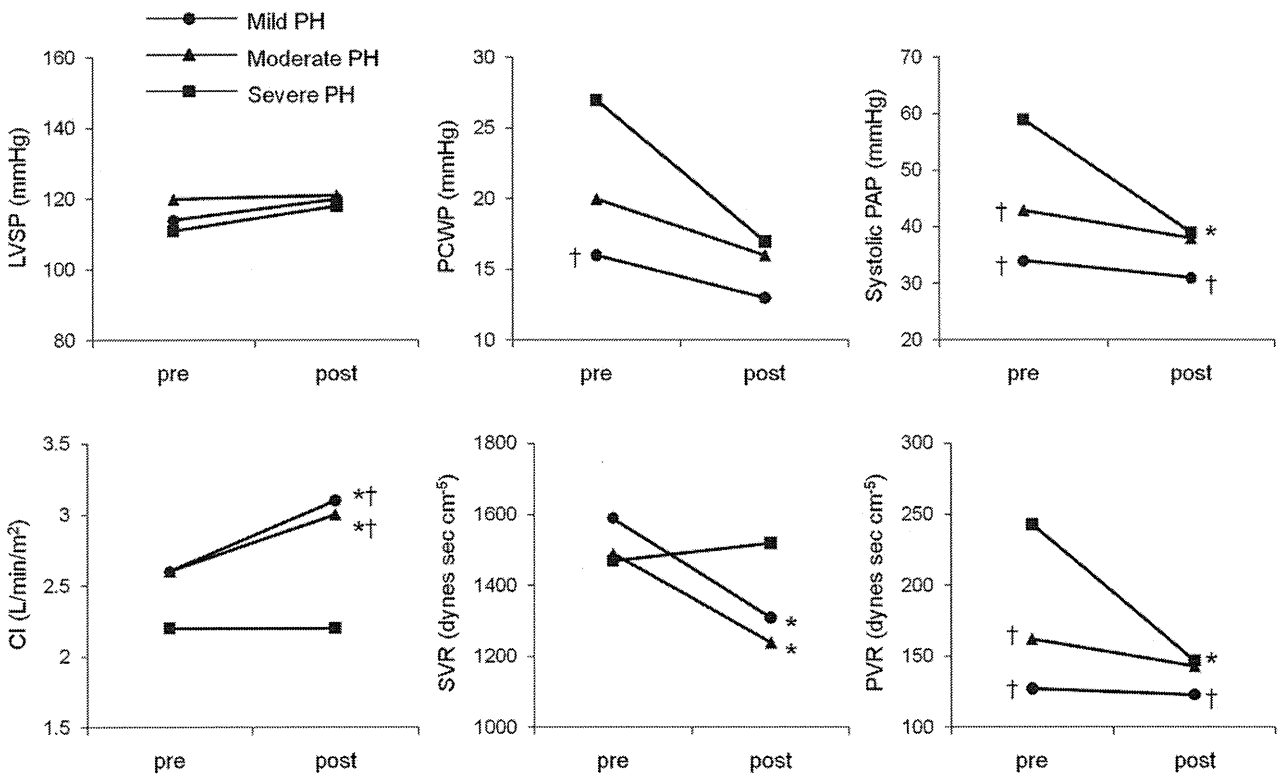


FIGURE 3. Acute hemodynamic changes after restrictive mitral annuloplasty. *$P < .05$ versus value at baseline, †$P < .05$ versus value for severe pulmonary hypertension *(PH)* group. *LVSP,* Left ventricular systolic pressure; *PCWP,* pulmonary capillary wedge pressure; *PAP,* pulmonary artery pressure; *CI,* cardiac index; *SVR,* systemic vascular resistance; *PVR,* pulmonary vascular resistance. *Circles, triangles,* and *squares* indicate mild, moderate, and severe groups, respectively.

TABLE 3. Serial echocardiographic and functional variables

Variable	Baseline	1 mo	1 y	2 y	*P* Value
LVEDD (mm)					
All	66 ± 6	60 ± 7*	60 ± 8*	59 ± 8*	< .01
Mild PH group	66 ± 6	58 ± 6*†	58 ± 9*	57 ± 9*	< .01
Moderate PH group	65 ± 6	58 ± 7*†	58 ± 5*	56 ± 4*	< .01
Severe PH group	69 ± 6	67 ± 6	66 ± 8	64 ± 10	NS
LVESD (mm)					
All	55 ± 7	48 ± 10*	45 ± 10*	44 ± 12*	< .01
Mild PH group	54 ± 7†	45 ± 8*†	42 ± 9*	40 ± 11*	< .01
Moderate PH group	53 ± 7†	46 ± 10*†	43 ± 10*	41 ± 6*	< .01
Severe PH group	61 ± 6	58 ± 7	52 ± 11	52 ± 13	NS
LVEF (%)					
All	32 ± 5	39 ± 8*	44 ± 7*	45 ± 7*	< .01
Mild PH group	34 ± 4†	42 ± 5*†	46 ± 7*†	49 ± 6*†	< .01
Moderate PH group	33 ± 6†	40 ± 10*†	45 ± 5*†	45 ± 5*†	< .01
Severe PH group	29 ± 4	31 ± 6	37 ± 9*	39 ± 8*	.02
LA dimension (mm)					
All	51 ± 7	45 ± 6*	48 ± 5*	48 ± 5*	< .01
Mild PH group	49 ± 6	44 ± 6*†	48 ± 5	47 ± 5	.03
Moderate PH group	51 ± 6	45 ± 3*	48 ± 5	49 ± 4	< .01
Severe PH group	52 ± 9	50 ± 7	50 ± 6	49 ± 8	NS
Systolic PAP (mm Hg)					
All	47 ± 15	36 ± 8*	38 ± 9*	37 ± 13*	< .01
Mild PH group	33 ± 4†	33 ± 6	32 ± 9	29 ± 9	NS
Moderate PH group	48 ± 6†	36 ± 7*	36 ± 8*	36 ± 10*	.02
Severe PH group	70 ± 9	42 ± 8*	50 ± 11*	54 ± 17	.01
MR grade					
All	3.6 ± 0.5	0.9 ± 0.7*	0.9 ± 0.7*	1.0 ± 0.7*	< .01
Mild PH group	3.6 ± 0.5	0.8 ± 0.4*	0.8 ± 0.7*	0.8 ± 0.6*	< .01
Moderate PH group	3.6 ± 0.5	0.9 ± 0.7*	0.8 ± 0.6*	1.0 ± 0.0*	< .01
Severe PH group	3.8 ± 0.5	1.3 ± 1.2*	1.0 ± 0.9*	1.3 ± 1.0*	.02
TR grade					
All	2.3 ± 1.1	0.9 ± 0.6*	0.9 ± 0.6*	1.0 ± 0.6*	< .01
Mild PH group	2.3 ± 1.2	0.8 ± 0.7*	0.8 ± 0.6*	1.0 ± 0.0*	< .01
Moderate PH group	2.2 ± 1.0	0.8 ± 0.4*	0.8 ± 0.4*	1.2 ± 0.8*	.02
Severe PH group	2.5 ± 1.3	1.1 ± 0.6*	1.3 ± 0.7*	1.2 ± 0.4*	.03
Tenting height (mm)					
All	7.9 ± 2.4	4.2 ± 1.9*	3.8 ± 0.9*	4.0 ± 1.4*	< .01
Mild PH group	6.7 ± 2.3	4.7 ± 2.2*	4.0 ± 1.1*	3.6 ± 0.4*	< .01
Moderate PH group	8.0 ± 2.0	3.7 ± 1.2*	3.6 ± 0.7*	4.3 ± 2.0*	< .01
Severe PH group	10.1 ± 1.7	4.1 ± 2.3*	3.8 ± 0.8*	4.2 ± 0.3*	< .01
Coaptation length (mm)					
All	4.1 ± 1.5	8.0 ± 2.4*	8.2 ± 1.1*	7.9 ± 1.1*	< .01
Mild PH group	4.4 ± 1.7	7.4 ± 1.8*	8.1 ± 1.4*	8.0 ± 1.3*	< .01
Moderate PH group	4.2 ± 1.5	8.7 ± 3.0*	8.2 ± 1.0*	7.9 ± 1.0*	< .01
Severe PH group	3.0 ± 0.7	7.9 ± 2.0*	8.3 ± 1.3*	7.7 ± 1.3*	< .01
Effective orifice area (cm^2)					
All		2.6 ± 0.3	2.6 ± 0.4	2.5 ± 0.4	NS
Mild PH group		2.6 ± 0.3	2.6 ± 0.3	2.5 ± 0.3	NS
Moderate PH group		2.6 ± 0.4	2.6 ± 0.3	2.5 ± 0.3	NS
Severe PH group		2.6 ± 0.4	2.7 ± 0.4	2.6 ± 0.4	NS

(Continued)

contrast, in the mild and moderate PH groups, the NYHA functional class improved significantly, and the improvement persisted during the follow-up period, along with reduced serum BNP levels.

Prediction for Reverse LV Remodeling

When substantial reverse LV remodeling was defined as a 10% reduction in the LVEDD, LV reverse remodeling was seen in 16 (89%) of 18 patients with mild PH, 12

TABLE 3. Continued

Variable	Baseline	1 mo	1 y	2 y	*P* Value
TMPG (mm Hg)					
All		4.2 ± 1.5	4.3 ± 1.6	4.7 ± 2.3	NS
Mild PH group		4.7 ± 1.6	4.6 ± 1.3	4.9 ± 2.0	NS
Moderate PH group		3.7 ± 1.3	3.9 ± 1.3	4.7 ± 2.1	NS
Severe PH group		4.4 ± 1.4	4.5 ± 2.6	4.5 ± 3.4	NS

LVEDD, left ventricular end-diastolic dimension; *PH*, pulmonary hypertension; *LVESD*, left ventricular end-systolic dimension; *LVEF*, left ventricular ejection fraction; *LA*, left atrial; *PAP*, pulmonary artery pressure; *MR*, mitral regurgitation; *TR*, tricuspid regurgitation; *TMPG*, transmitral pressure gradient. **P* < .05 versus values at baseline in each group. †*P* < .05 versus severe PH group at each point.

(71%) of 17 patients with moderate PH, and 2 (25%) of 8 with severe PH (*P* = NS) at their most recent examination (Table 4). Univariate analysis identified LV systolic dysfunction (*P* = .022), a longer duration of heart failure (*P* = .01), and severe PH (systolic PAP > 60 mm Hg; *P* = .004) as important predictors of failure of reverse LV remodeling. In addition, multivariate analysis identified severe PH as a significant predictor.

Prediction of Postoperative Adverse Cardiac Events

Finally, the potential predictors of postoperative adverse cardiac events were examined using a Cox proportional hazard model (Table 5). Univariate analysis identified preoperative LV systolic dysfunction (*P* = .04), a longer duration of heart failure (*P* = .021), and severe PH (systolic PAP > 60 mm Hg; *P* = .002) as important predictors. In addition, multivariate analysis identified severe PH as a significant predictor.

DISCUSSION

The results of the present study suggest that PH in patients with advanced cardiomyopathy undergoing RMA is significantly associated with adverse short-term clinical outcome in terms of overall survival, adverse cardiac events (including cardiac death, readmission for heart failure, and fatal arrhythmia), improvements in NYHA functional class and serum BNP levels, acute hemodynamic changes, and serial echocardiographic changes in LV dimensions and function. Our findings have also demonstrated that severe PH (systolic PAP > 60 mm Hg) was an important hemodynamic predictor of adverse cardiac events, as well as failure of LV reverse remodeling after surgical treatment for functional MR and LV dysfunction.

In previous studies, clinical variables such as advanced age, preoperative hemodialysis and diabetes,[4] larger LV dimensions,[5,6] and nonischemic etiology and a longer duration of heart failure[7] were shown to be significantly associated with poor outcomes after RMA. In addition, the more sophisticated echocardiographic parameters of LV systolic and diastolic dysfunction such as LVEDD (> 65 mm) and left ventricular end-systolic dimension (> 51 mm),[8] myocardial performance index, systolic sphericity, wall motion score index,[14,15] restrictive LV filling

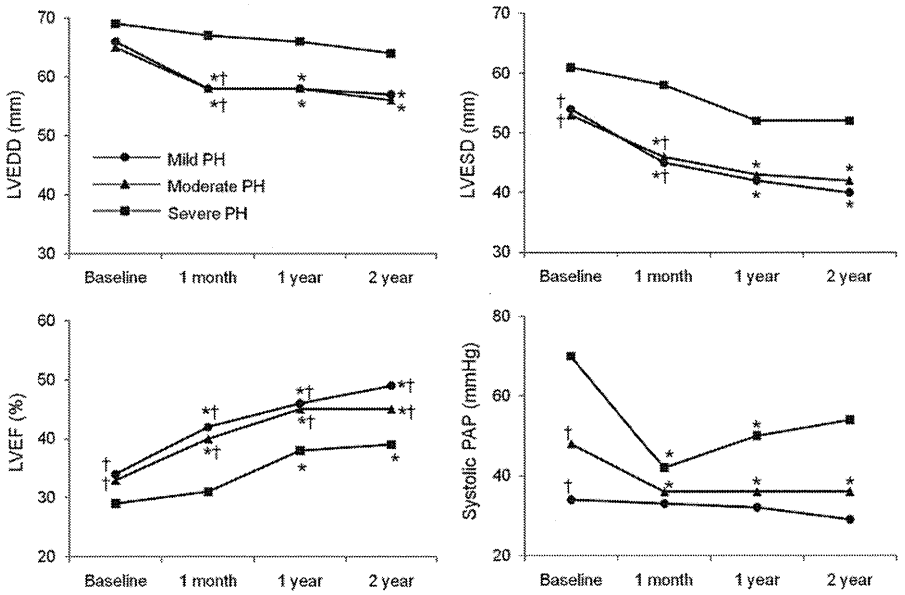


FIGURE 4. Serial echocardiographic changes after restrictive mitral annuloplasty. **P* < .05 versus value at baseline, †*P* < .05 versus value for severe PH group. *LVEDD*, Left ventricular end-diastolic dimension; *LVEF*, left ventricular ejection fraction; *LVESD*, left ventricular end-systolic dimension; *PAP*, pulmonary artery pressure; *PH*, pulmonary hypertension.

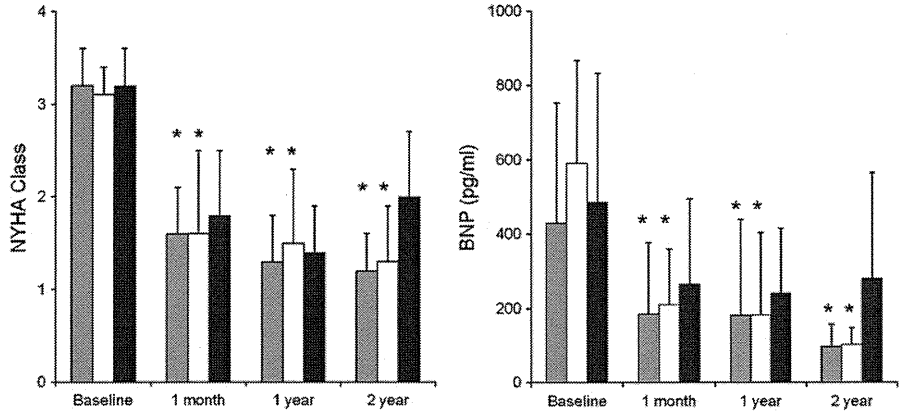


FIGURE 5. Serial changes in New York Heart Association *(NYHA)* functional class and serum brain natriuretic peptide *(BNP)* level. *$P < .05$ versus value at baseline. *Gray*, *white*, and *black* bars indicate mild, moderate, and severe groups, respectively.

pattern and mitral deceleration time (< 140 ms)[16] have been reported to be strong predictors of poor outcome or a lack of LV reverse remodeling after RMA. These parameters have also been shown to predict late MR recurrence after mitral annuloplasty. The present results have suggested that the PH level is a strong predictor of outcome after RMA and also provided additional important prognostic information that is complementary to the classic echocardiographic parameters of LV systolic and diastolic dysfunction.

The etiologies of PH in patients with cardiomyopathy are heterogeneous, and the determinants of its reversibility after an annuloplasty procedure have not been fully clarified. Classically, PH in patients with cardiomyopathy has been attributed to elevated LV filling pressures, reactive pulmonary arterial vasoconstriction, pulmonary vascular remodeling, or all three[17]; it has also been associated with functional MR.[18] The increased PAP associated with early-stage cardiomyopathy principally results from elevated LV filling pressures.[18] In addition, longstanding elevation of LV filling pressures can lead to several histologic changes in pulmonary circulation, including medial hypertrophy of arterioles, intimal fibroproliferation, and arterialization of the pulmonary veins.[17] These changes have also been associated with neurohumoral activation, in particular of endothelin-1,[19,20] a potent vasoconstrictor that is markedly increased in patients with advanced heart failure. These pathophysiologic changes are dependent on the chronicity and severity of PH and can initially be reversible, although the conditions can eventually become irreversible.[17]

The changes in pulmonary hemodynamics seen in the present severe PH group were consistent with this sequence of PH progression. The patients in the severe PH group had had a longer duration of heart failure and greater PVR values before surgery. Furthermore, the mean PVR value in this group remained considerably elevated at 1 month

TABLE 4. Predictors of failure in left ventricular reverse remodeling

	Univariate			Multivariate		
Variable	*P* Value	Odds Ratio	95% CI	*P* Value	Odds Ratio	95% CI
Age	NS					
Ischemic etiology	NS					
Duration of HF	.01	1.1	1.02–1.15	NS		
History of VT	NS					
LVEDD (continuous)	NS					
LVEDD (> 65 mm)	NS					
LVESD (continuous)	NS					
LVESD (> 50 mm)	NS					
LA dimension (mm)	NS					
LVEF (%)	.02	0.8	0.70–0.97	NS		
Severe PH*	.004	25.7	4.3–155	.03	10.0	1.3–75
MR grade	NS					
TR grade	NS					

CI, Confidence interval; *HF*, heart failure; *VT*, ventricular tachycardia; *LVEDD*, left ventricular end-diastolic dimension; *LVESD*, left ventricular end-systolic dimension; *LA* , left atrial; *LVEF*, left ventricular ejection fraction; *PH*, pulmonary hypertension; *MR*, mitral regurgitation; *TR*, tricuspid regurgitation. *Systolic PAP > 60 mm Hg.

TABLE 5. Predictors of adverse cardiac events

	Univariate			Multivariate		
Variable	*P* Value	Hazard Ratio	95% CI	*P* Value	Hazard Ratio	95% CI
Age	NS					
Ischemic etiology	NS					
Duration of HF	.02	1.05	1.01–1.10	NS		
History of VT	NS					
LVEDD (continuous)	NS					
LVEDD (> 65 mm)	NS					
LVESD (continuous)	NS					
LVESD (> 50 mm)	NS					
LA dimension (mm)	NS					
LVEF (%)	.04	0.89	0.79–0.96	NS		
Severe PH*	.002	9.1	2.3–35	.04	6.9	1.1–44
MR grade	NS					
TR grade	NS					

CI, Confidence interval; *HF*, heart failure; *VT*, ventricular tachycardia; *LVEDD*, left ventricular end-diastolic dimension; *LVESD*, left ventricular end-systolic dimension; *LA*, left atrial; *LVEF*, left ventricular ejection fraction; *PH*, pulmonary hypertension; *MR*, mitral regurgitation; *TR*, tricuspid regurgitation. *Systolic PAP > 60 mm Hg.

after surgery. The elevated PAP levels (> 40 mm Hg) persisted at all follow-up examinations, suggesting the presence of irreversible pulmonary vascular disease. In contrast, the moderate PH group showed a decline in the systolic PAP without a change in the lowered PVR at discharge. Also, the lower PAP values were maintained until the latest follow-up examination, suggesting that PH was reversible in these patients. Thus, our findings emphasize the importance of assessing PVR and the degree of pulmonary vascular remodeling. In addition, our results might support the use of surgery for patients with medically uncontrollable severe functional MR before the pulmonary vascular disease becomes irreversible.

The degree of PH is not only related to the severity of LV systolic dysfunction, but is also strongly associated with the LV diastolic dysfunction (i.e., a greater level of PH has been associated with a shorter mitral deceleration time).[18] Gelsomino and colleagues[21] reported that the Doppler-derived mitral deceleration time was prognostic for LV reverse remodeling after undersized mitral annuloplasty. Our results are not necessarily inconsistent with those of previous studies, because a strong correlation between the Doppler-derived systolic PAP and mitral deceleration time has been reported.[18] Also, the prevalence of a restrictive filling pattern has been inversely related to the LVEF. PH has also been significantly associated with right ventricular function and right heart hemodynamics. In contrast, the right ventricular EF will correlate with the systolic PAP and is an independent predictor of survival in patients with moderate heart failure.[22] This association between PH and right ventricular dysfunction could explain why the level of PH provides additional prognostic information beyond the LV systolic and diastolic dysfunction variables.

It remains controversial whether patients with end-stage heart failure and functional MR can benefit from RMA.[23-25] In our study, patients with mild and moderate PH (Doppler-derived PAP < 60 mm Hg) showed functional improvement and satisfactory long-term survival. In contrast, those with severe PH (systolic PAP > 60 mm Hg) had relatively poor outcomes. Our results have shown that RMA was able to improve hemodynamics and symptoms in these patients. However, the lack of an untreated control group did not allow us to investigate the survival benefit conferred by RMA for patients with significant MR and severe LV dysfunction. Additional studies of late mortality after RMA in similar patient populations are needed.

Study Limitations

The main limitations of the present study were its retrospective nature and the small number of subjects. The inclusion of patients with ischemic and nonischemic cardiomyopathy and those who had undergone concomitant coronary artery bypass grafting, tricuspid annuloplasty, and a maze procedure for atrial fibrillation might have influenced the results. However, these concomitant procedures are usually required in a population of very sick patients who present with similar clinical and pathophysiologic status despite the etiology of LV dysfunction. To minimize the potential bias related to patient selection, our study population consisted only of patients with advanced nonischemic or ischemic cardiomyopathy owing to anterior infarction and functional MR. Patients with less LV remodeling and ischemic MR secondary to predominant inferior/posterior infarction, who have often been included in previous studies,[6-8,15,16,21,24] were excluded from our study. Therefore, our results would not be applicable to patients with previous inferior or lateral infarction.

During the late follow-up examinations, systolic PAP was determined noninvasively using Doppler echocardiography and not measured by catheterization. This noninvasive method has been fully validated and currently represents a standard approach for PH determination. In the present study, we confirmed that systolic PAP estimated using Doppler echocardiography correlated significantly with the nonsimultaneously catheter-derived systolic PAP ($r = .833$, $P < .001$). Moreover, Bland-Altman analysis showed that the 2 modalities had good agreement in the measurements of systolic PAP, although it was slightly overestimated using Doppler echocardiography (mean bias 1.5 ± 8.4 mm Hg).

The medical treatments administered could have also affected our findings. However, the preoperative medical therapies were continued without significant modifications after surgical intervention. In particular, the use and dosage of angiotensin-converting enzyme inhibitors and angiotensin-II receptor blockers were not changed; thus, their influence on LV remodeling and pulmonary vascular remodeling in our patients was considered to be negligible.

CONCLUSIONS

Noninvasive assessment of systolic PAP was found to be an excellent prognostic tool for patients who underwent RMA for functional MR secondary to advanced cardiomyopathy. Additional studies are needed to define the mechanism of PH and its postoperative reversibility in patients with cardiomyopathy to establish new treatment strategies.

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APPENDIX

The following variables were tested: age, gender, body surface area, NYHA functional class, ischemic etiology, hypertension, diabetes, hyperlipidemia, chronic obstructive pulmonary disease, chronic renal failure, peripheral vascular disease, cerebral vascular disease, atrial fibrillation, history of ventricular arrhythmia, duration of heart failure (in months), multivessel coronary artery disease, previous coronary artery bypass grafting, previous percutaneous coronary intervention, β-blockers, angiotensin-converting enzyme inhibitors, angiotensin-II receptor blocker, diuretics, LVEDD (continuous), LVEDD (>65 mm), left ventricular end-systolic dimension (continuous), left ventricular end-systolic dimension (> 50 mm), left atrial dimension, LVEF, tenting height, coaptation length, MR grade, TR grade, systolic PAP (continuous), and severe PH (systolic PAP > 60 mm Hg).

000 Pulmonary hypertension predicts adverse cardiac events after restrictive mitral annuloplasty for severe functional mitral regurgitation

Satoshi Kainuma, MD, Kazuhiro Taniguchi, MD, PhD, Koichi Toda, MD, PhD, Toshihiro Funatsu, MD, PhD, Haruhiko Kondoh, MD, PhD, Masami Nishino, MD, PhD, Takashi Daimon, PhD, and Yoshiki Sawa, MD, PhD, Osaka and Hyogo, Japan

We investigated the prognostic role of pulmonary hypertension in patients undergoing restrictive mitral annuloplasty for severe functional mitral regurgitation and found that pulmonary hypertension is an excellent prognostic tool for such patients. In particular, severe pulmonary hypertension (systolic pulmonary artery pressure > 60 mm Hg) was shown to be a predictor of adverse cardiac events and cardiac remodeling.

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Title: Induction of enamel matrix protein expression in an ameloblast cell line co-cultured with a mesenchymal cell line in vitro

Article Type: Articles (full research papers)

Keywords: ameloblastin; tooth differentiation; co-culture

Corresponding Author: Prof.Dr. Akiyoshi Taniguchi, Ph.D.

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Abstract: Interactions between epithelium and mesenchyme are important for organ and tissue development. In this study, in order to mimic interactions between epithelium and mesenchyme during native tooth development, we constructed three-dimensional culture systems in vitro using a collagen membrane. Two types of collagen membrane-based in vitro culture systems were constructed in which dental epithelial and dental follicle cell lines were cultured. One co-culture method involved inoculation of one cell line into one side of the collagen membrane, and the other cell line into the opposite side of the membrane (sandwich co-culture; SW). As a control, the second method involved culture of one of the cell lines on a culture dish and the second cell line on a collagen membrane, facing away from the first cell line (separate co-culture; SC). The HAT-7 cells were also grown as a monolayer culture on collagen. Ameloblast differentiation in these cultures was investigated by analysis of the mRNA and/or protein expression of ameloblastin and amelogenin. Our results suggest that interaction of epithelial and mesenchymal cells via the extracellular matrix is important for tooth differentiation in vitro. Our culture system should be a useful method for investigation of epithelial-mesenchymal interactions.

Induction of enamel matrix protein expression in an ameloblast cell line co-cultured with a mesenchymal cell line *in vitro*.

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Keywords: ameloblastin, amelogenin, tooth differentiation, co-culture, ECM

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Summary

Interactions between epithelium and mesenchyme are important for organ and tissue development. In this study, in order to mimic interactions between epithelium and mesenchyme during native tooth development, we constructed three-dimensional culture systems *in vitro* using a collagen membrane. Two types of collagen membrane-based *in vitro* culture systems were constructed in which dental epithelial and dental follicle cell lines were cultured. One co-culture method involved inoculation of one cell line into one side of the collagen membrane, and the other cell line into the opposite side of the membrane (sandwich co-culture; SW). As a control, the second method involved culture of one of the cell lines on a culture dish and the second cell line on a collagen membrane, facing away from the first cell line (separate co-culture; SC). The HAT-7 cells were also grown as a monolayer culture on collagen. Ameloblast differentiation in these cultures was investigated by analysis of the mRNA and/or protein expression of ameloblastin and amelogenin. Our results suggest that interaction of epithelial and mesenchymal cells via the extracellular matrix is important for tooth differentiation *in vitro*. Our culture system should be a useful method for investigation of epithelial-mesenchymal interactions.

Introduction

Epithelial cells communicate with epidermal cells through the extracellular matrix (ECM) to maintain and regulate highly differentiated functions. These interactions are thought to play an important role in the control of various characteristics of cells such as proliferation and differentiation. In order to mimic these interactions that occur in native tissue, three-dimensional culture systems have been created using various cell populations (Kurosawa et al. 2005; Ohno et al. 2008; Takayama et al. 2007) and reconstituted ECM (Takezawa et al. 2007). Co-culture models *in vitro* between two different cell types via reconstituted ECM have been developed for clarification of cell behavior associated with development, differentiation, regeneration, and pathogenesis *in vitro* (Gingras et al. 2003).

Tooth development is a classic instance of the process of epithelium-mesenchyme interactions and provides a useful experimental system for understanding the molecular mechanisms of organogenesis (Jernvall et al. 2000; Thesleff and Sharpe 1997). The early stage of tooth development is regulated by reciprocal interactions between epithelial and mesenchymal cells via cytokines such as transforming growth factor beta (TGFβ), fibroblast growth factor (FGF), and Leucine-Rich Amelogenin Protein (LRAP) [reviewed by (Thesleff and Mikkola 2002)], (Aberg et al. 1997; Thesleff and Mikkola 2002). Mesenchyme differentiates into odentoblasts which then form dentin. Epithelium differentiates into ameloblasts which secrete enamel matrix and form enamel. In the presecretory stage of tooth development, the dental epithelium and mesenchymal preodontoblasts are separated by the basement membrane matrix. [reviewed by (Thesleff and Hurmerinta 1981)].

Enamel matrix secreted by ameloblasts has been classified into two major

categories: amelogenin which makes up about 90% of the enamel extracellular matrix, and nonamelogenin including ameloblastin, enamelin, and tuftelin [reviewed by (Smith 1998)]. Ameloblastin, also known as amelin (Cerny et al. 1996) or sheathlin (Hu et al. 1997), and amelogenin are tooth-specific genes which play a critical role in proper tooth enamel formation (Fong et al. 1996; Fukumoto et al. 2004; Krebsbach et al. 1996; Xu et al. 2006a; Xu et al. 2006b). *In vitro* culture models that facilitate the study of epithelium-mesenchyme interactions are important for understanding tooth development at a molecular level. However, there is no useful model available that permits investigation of the molecular mechanism by which the ECM at sites of epithelial-mesenchymal interactions modulates tooth formation *in vitro*.

Collagen gels and matrigel often play important roles as scaffolds for reconstructing co-culture models. For example, endothelial cells form a network of branching tubular capillary-like structures into an overlaid collagen gel with embedded fibroblasts (Velazquez et al. 2002). The use of transparent collagen membranes has a number of advantages. It allows observation of the cells using a phase-contrast microscope, it can store cytokines and it is a simple culture method for three-dimensional culture systems (Orisaka et al. 2006).

In this study, we constructed three-dimensional culture systems *in vitro* using a collagen membrane for the co-culture of dental epithelial cells and dental follicle cells and gene and protein expression levels in these co-cultures were examined. Our results suggest that the interaction of epithelial and mesenchymal cells via the extracellular matrix is important for tooth differentiation *in vitro*.

Material and methods

Cells and Cell Cultures

The HAT-7 cells used in this study were derived from a dental epithelial cell line, which originated from the apical bud of a rat incisor (Kawano et al. 2002). The culture medium consisted of Dulbecco's modified Eagle's medium/F-12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/ml)/streptomycin (100 μg/ml). The BCPb8 cells used were derived from a dental follicle cell line (a cementoblast progenitor), which originated from the follicle tissue of a bovine incisor (Saito et al. 2005). The culture medium consisted of α-minimum essential medium (α-MEM) supplemented with 10% FBS, penicillin (100 units/ml)/streptomycin (100 μg/ml), 50 mg/ml ascorbic acid and 2 mM L-glutamine (Invitrogen). All cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

Co-culture using a transparent collagen membrane

Two different co-culture models were set up, both of which used the two dental cell lines and a transparent collagen membrane (Fig. 1). In the sandwich co-culture (SW) system one cell line was inoculated into one side of the collagen film in a culture dish and the second cell line was inoculated into the opposite side of the collagen film. (Orisaka et al. 2006). In the separate co-culture (SC) system, the BCPb8 cells were first seeded onto a 60 mm dish. After confirming microscopically that the BCPb8 cells had adhered to the dish, the dish and the transparent collagen membranes were washed with PBS. The HAT-7 cells (1×10^6 cells) were then seeded onto the collagen film inserted into the dish, on the side of the film facing away from the BCPb8 cells. The cells were

cultured in DMEM/F12 using 6.5 ml of medium per dish. The media were replaced with fresh media every two days. As a further control, HAT-7 cells were also grown in monolayer culture on a collagen film.

RNA Extraction and Real Time PCR Analysis

The mRNA levels of differentiation-related marker genes were determined using quantitative real-time PCR and species-specific primers (Kurosawa et al. 2005). Briefly, total RNA was extracted at various time points using ISOGEN (Nippon Gene, Tokyo, Japan). Three micrograms of total RNA was reverse-transcribed into cDNA using the SuperScript first-strand synthesis system (Invitrogen) according to the manufacturer's protocol.

Real-time PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A standard reaction was performed in a 96-well plate. This reaction was composed of 10 μl of SYBR Premix Ex TaqTM II (Takara, Siga, Japan), 10 pmol each of the forward and reverse primers, 1 μl of HAT-7 cDNA and distilled water to a final volume of 20 μl. The thermocycling conditions were 95 °C for 30 s, following by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Species-specific primers corresponding to a region of low-homology between rat and bovine cDNA were designed using Primer Express Software version 2.0 (Applied Biosystems) based on the sequence of the target gene. The data were normalized using expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control in the same reaction as the gene of interest. The primers used in this study were designated as follows: rat ameloblastin forward primer 5'-TTCACCCAAGGGAGGAGACTT-3, rat ameloblastin reverse primer

5'-CTCTCCTTTCTCAGGGCCTTTAGT-3', rat amelogenin forward primer 5'-TGGGAGCCCTGGTTATATCAA-3', rat amelogenin reverse primer 5'-GCTGCCTTATCATGCTCTGGT-A-3', rat GAPDH forward primer 5'-GCCCCCAACACTGAGCAT-3', rat GAPDH reverse primer, 5'-CCAGGCCCCTCCTGTTGT-3'.

Immunocytochemistry and immunohistochemistry

The surfaces of the cultured cells were washed three times with phosphate buffered saline (PBS) and the cells were fixed in 10% formalin for 10 min. After permeabilization with 0.5% Triton X-100 in PBS for 10 min, ameloblastin was stained using a polyclonal goat anti-ameloblastin antibody (1:50, Santa Cruz, Santa Cruz, CA, USA) for 1 h. After several washes with PBS containing 0.1% Tween 20 (Sigma, St. Louis, MO, USA), the cells were incubated with the secondary antibody, Alex-488-conjugated anti-goat IgG serum (1:200), for 1 h at room temperature. Nuclei were visualized by Hoechst 33258 (Wako, Osaka, Japan) staining. Confocal microscopy was performed using a Zeiss LSM 510 microscope (Carl Zeiss, Oberkochen, Germany).

Statistical Analysis

Result were presented as means ± standard deviation (n=3).SD. Date were statistically analyzed by Student t tests. $P < 0.05$ was regarded as significant.