Effects of therapy using the Celacade system on structural and functional cardiac remodelling in rats following myocardial infarction

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BACKGROUND: Immune modulation by the Celacade system (Vasogen Inc, Canada) decreases mortality and hospitalization in human heart failure.

OBJECTIVES: To study the effects of Celacade in rats on acute cytokine expression after coronary artery ligation, cardiac dimensions following myocardial infarction (MI), and systolic and diastolic function of cardiac muscle in MI.

METHODS: Celacade treatment was administered 14 days before coronary artery ligation and monthly after the surgery. Cytokine expression in cardiac tissue was measured on days 1 and 7 by ELISA in sham rats and in rats with MI (with or without Celacade treatment). Echocardiograms were obtained serially for 16 weeks. Force and sarcomere length (SL) were measured by strain gauge and laser diffraction in isolated right ventricle trabeculas at 16 weeks. The inotropic effect of pacing on force was quantified as $F_{5\text{ Hz}/0.5\text{ Hz}}$. Diastolic dysfunction was quantified as the root mean square of spontaneous SL fluctuations.

RESULTS: Celacade inhibited transforming growth factor beta-1 production in the infarct area on day 7 (191.6 ± 22.6 pg/mg versus 275.4 ± 30.1 pg/mg; $P < 0.05$), but did not attenuate cardiac dilation in MI. Celacade restored positive inotropism of pacing in MI ($F_{5\text{ Hz}/0.5\text{ Hz}}$ in Celacade, $219.1 \pm 46.7\%$; MI, $148.1 \pm 27.1\%$ [$P < 0.05$ compared with $211.4 \pm 37.9\%$ in sham]). Celacade reduced diastolic dysfunction in MI (root mean square of spontaneous SL fluctuations: $121 \pm 15\%$ and $143 \pm 19\%$ with Celacade versus $184 \pm 19\%$ and $190 \pm 26\%$ without Celacade at 26°C and 36°C , respectively) compared with sham (100% ; $P < 0.05$).

CONCLUSIONS: Celacade reduces the increase of transforming growth factor beta-1 expression during the acute stage of MI in rats, but does not prevent chronic cardiac dilation. Celacade restores the positive inotropic effect of increased pacing rate in trabeculas from rat right ventricles with large MIs and reduces diastolic dysfunction.

Key Words: Congestive heart failure; Contractility; Excitation-contraction coupling; Muscle; Myocardial infarction

Effets du traitement par Celacade sur la déformation structurale et fonctionnelle du cœur chez des rats ayant subi un infarctus du myocarde

CONTEXTE : La modulation immunitaire provoquée par Celacade (Vasogen Inc., Canada) diminue la mortalité et les hospitalisations dues à l'insuffisance cardiaque chez l'homme.

BUT : L'étude avait pour but d'examiner les effets de Celacade, chez des rats, sur l'expression des cytokines durant la phase aiguë après la ligature d'une artère coronaire, sur les dimensions du cœur après un infarctus du myocarde (IM) et sur le fonctionnement systolique et diastolique du muscle cardiaque après un IM.

MÉTHODE : Celacade a été administré 14 jours avant la ligature de l'artère coronaire et tous les mois après l'intervention chirurgicale. L'expression des cytokines a été mesurée dans le tissu cardiaque au 1^{er} et au 7^e jour selon la technique ELISA chez des rats témoins et des rats ayant subi un IM (avec ou sans traitement par Celacade). Des échocardiogrammes ont été produits en série pendant 16 semaines. La force et la longueur des sarcomères (LS) ont été mesurées au moyen d'une jauge de contrainte et d'un instrument de diffraction au laser dans des trabécules isolées de ventricule droit à la 16^e semaine. L'effet inotropique de la stimulation sur la force a été quantifié selon la formule suivante : $F_{5\text{ Hz}/0.5\text{ Hz}}$ et le dysfonctionnement diastolique, comme la racine carrée moyenne des fluctuations spontanées de la LS.

RÉSULTATS : Celacade a inhibé la production du facteur de croissance transformant bêta-1 dans la zone infarctée au 7^e jour ($191,6 \pm 22,6$ pg/mg contre [c.] $275,4 \pm 30,1$ pg/mg; $P < 0,05$), mais n'a pas diminué la dilatation du cœur après un IM. Celacade a également rétabli l'inotropisme positif de la stimulation après un IM ($F_{5\text{ Hz}/0.5\text{ Hz}}$ avec Celacade : $219,1 \pm 46,7\%$; IM : $148,1 \pm 27,1\%$ [$P < 0,05$ comparativement à $211,4 \pm 37,9\%$ chez les témoins]). Celacade a aussi atténué le dysfonctionnement diastolique après un IM (racine carrée moyenne des fluctuations spontanées de la LS : $121 \pm 15\%$ et $143 \pm 19\%$ avec Celacade c. $184 \pm 19\%$ et $190 \pm 26\%$ sans Celacade à 26°C et à 36°C , respectivement) par rapport aux témoins (100% ; $P < 0,05$).

CONCLUSIONS : Celacade amoindrit la surexpression du facteur de croissance transformant bêta-1 durant la phase aiguë de l'IM chez des rats, mais ne prévient pas la dilatation chronique du cœur. Celacade rétablit l'effet inotropique positif de la fréquence accrue de stimulation dans des trabécules de ventricule droit de rats ayant subi un IM étendu et atténue également le dysfonctionnement diastolique.

Myocardial infarction (MI) induces remodelling of cardiac architecture, which may lead to progressive contractile dysfunction. In previous studies of trabeculas from the right ventricles (RVs) of rats with MIs, we reported that the force generation of myofilament as a function of stimulation frequency is dramatically reduced during congestive heart failure (1). Spontaneous sarcomere length (SL) fluctuation in the diastolic interval is often visible from these muscles at lower extracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]$). The occurrence of SL fluctuation appears

to be proportional to the frequency of contractile Ca^{2+} waves in the trabeculas, suggesting a sequence of spontaneous diastolic Ca^{2+} release events. Nevertheless, at the same $[\text{Ca}^{2+}]$, normal trabeculas are distinctly quiescent (1,2). The heterogeneous SL fluctuation during diastole is believed to play a critical role in the force deficit measured after frequency potentiation in heart failure.

The Celacade system (Vasogen Inc, Canada) is designed to expose a sample of whole blood briefly to oxidative stress by exposing the

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blood to ultraviolet light and a gaseous oxidizing agent at an elevated temperature, after which, the treated blood is readministered to the same individual by intramuscular injection at regular intervals. The results of a number of experimental studies suggest that the administration of treated blood using the Celacade system reduces cell apoptosis caused by ischemia-reperfusion injury (3) and inhibits atherosclerotic progression by reducing local inflammation (4). These biological responses induced by Celacade treatment may be beneficial in the treatment of patients with heart failure. Preliminary clinical data on Celacade suggest that this approach reduces the mortality and hospitalization of heart failure patients (5,6). An improved quality of life has been reported in clinical trials on patients with disabling intermittent claudication (7) and patients with severe Raynaud's syndrome (8).

In the present study, the effects of immune modulation therapy using Celacade in a rat model of MI were investigated. The animals were pretreated with Celacade for 14 days, then monthly after experimental MI. The objective was to investigate the therapeutic potential of using the Celacade system to prevent cardiac contractile dysfunction in isolated rat RV trabeculas four months after experimental MI. In addition, the effects of treatment on cytokine expression during the acute phase of MI were examined.

METHODS

Experimental MI

Ninety-three male Lewis-Brown-Norway rats supplied by Harlan Laboratories Inc (USA), weighing 250 g to 300 g, were housed in the Animal Resource Centre at the University of Calgary (Calgary, Alberta) following standard guidelines. MI was induced during open-chest surgery by ligation of the left coronary artery as previously described (9). Briefly, after being anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg), rats were ventilated via a nose mask connected to a rodent ventilator (Harvard Apparatus, USA). A left thoracotomy was performed and the pericardium was opened using scissors. The coronary artery was ligated 1 mm to 2 mm from its origin with a 6-0 silk suture. The chest was then closed after removing air from the thoracic cavity. Torbugesic (Wyeth, Canada), an analgesic agent, was administered by subcutaneous injection (3 mg/kg) before and 3 h after the surgery. Penicillin and Duplocillin LA (Intervet Canada Corp, Canada) were administered subcutaneously to prevent infection. The acute mortality rate due to coronary artery ligation was 22%. Age-matched sham rats were subjected to the same procedure, leaving the ligation suture untied. The surgical procedure was approved by the Animal Care Committee at the University of Calgary.

All animals showed left ventricle (LV) dilation in response to coronary artery ligation, reflecting LV dysfunction. For trabecula studies, experimental animals were divided into three groups: sham (n=5), MI rats without Celacade treatment (n=16) and MI rats with Celacade treatment (n=23). Four rats died during anesthesia for echocardiography and were not included in the study. Separate groups of animals were used for the cytokine analysis during the acute stage of MI (n=5 or n=6 for each group).

Celacade treatment

Syngeneic donor rats were anesthetized by halothane inhalation and 10 mL of blood was collected by cardiac puncture and mixed with 2 mL of 4% sodium citrate (Baxter Corporation, Canada). The blood was immediately transferred into a sterile polyethylene container (model VC7002, Vasogen Inc) and treated using the Celacade device (model VC7001A, Vasogen Inc). The treatment involved exposure of citrated whole blood to an elevated temperature (42°C), ultraviolet light and a gaseous oxidizing agent (ozone/oxygen gas). An aliquot (150 µL) of the treated blood was injected into the gluteus muscle of each experimental rat. Control MI animals received the same volume of physiological saline. Intramuscular injections were administered 14 days, 13 days and one day before coronary artery ligation, and monthly after the surgery. The experiment was terminated four months after coronary artery ligation.

Scar size measurement

Rats were anesthetized with ether and hearts were rapidly removed. The blotted scar tissue, and noninfarcted LV, RV and lung tissue were weighed immediately after removing the trabeculas. The scar size was duplicated on paper by tracing the edge carefully and the scar area was determined by planimetry using NIH Image software (National Institutes of Health, USA). For cytokine measurement, the heart tissue was crushed with metal clamps prechilled in liquid nitrogen and stored at -80°C until analysis.

Cardiac tissue cytokine measurements

Interleukin (IL)-6, IL-10, tumour necrosis factor-alpha (TNF-α) and transforming growth factor beta-1 (TGF-β1) levels in the rat myocardium were measured using ELISA (Biosource International Inc, USA). Cytokines in the infarcted and noninfarcted areas of the LV were tested separately on day 1 and day 7 after coronary artery ligation with and without treatment using the Celacade system (n=5 or n=6). Sham animals (n=5) were used as controls. Tissue cytokines were extracted using protocols described previously (10), with modifications. The tissue was homogenized in denaturing buffer containing phosphate-buffered saline, protease inhibitor cocktail with 4-(2-aminoethyl) benzenesulfonyl fluoride (0.5 mM), aprotinin (0.4 µM), leupeptin (10 µM), bestatin (20 µM), pepstatin A (7 µM), E-64 (7 µM) (Sigma-Aldrich Inc, USA) and 1% Triton X-100 (pH 7.4). After incubation on ice for 15 min, the samples were centrifuged at 120,000 g for 15 min. The supernatants were collected and stored at -80°C until the assay was performed. All protein extraction procedures were performed at 4°C. The protein concentration of the samples was determined with a protein assay kit (Bio-Rad Laboratories, USA).

Echocardiography

Cardiac systolic function and chamber dimensions were monitored under light anesthesia (pentobarbital 30 mg/kg) with an Agilent SONOS 5500 (Agilent Technologies, USA) and a 12 MHz transducer at two, six, 12 and 16 weeks after the surgery. Three separate short- and long-axis views, along with M-mode at the level of the tips of the papillary muscles, were recorded for each animal and averaged. The measured dimensions of the LV (volume, mass and ellipticity [E_{LV}]; E_{LV} is the ratio of the long axis to short axis dimensions) accurately correlated with the predictions of a model consisting of a three-quarter truncated ellipsoid (long axis 6.8 mm) when it was assumed that ellipticity, wall thickness (Θ) and end-diastolic LV filling pressure (P_{LVED}) ($E_{LV}=2.7$, $\Theta=2.8$ mm and P_{LVED} is approximately 8 mmHg, in control rats) vary in linear proportion to the area of the noncompliant scar caused by coronary artery ligation (changes [Δ] per cm² scar: change in ellipticity=-0.13; $\Delta\Theta=0.113$ mm; $\Delta P_{LVED}=3$ mmHg [11]). The correlations of LV volume, mass and E_{LV} with the model predictions ($P<0.0001$ for each of the parameters) were obtained without a change of the long-axis dimension of the LV. Hence, in the current study, use of the short-axis area was believed to be justified. The endocardium was delineated at end diastole and end systole. LV end-diastolic area (LVEDA) and LV end-systolic area (LVESA) were calculated from the pixel count in the delineated area, averaged from three short-axis views. The percentage fractional area change (FAC%) was calculated using:

$$FAC\% = \frac{LVEDA - LVESA}{LVEDA} \times 100\%$$

LV end-diastolic and end-systolic dimensions (LVEDD and LVESD, respectively) were also measured on M-mode recordings of two short-axis views, and used to estimate short-axis area and avoid sampling errors (less than 3%) of LVEDA and LVESA based on the echocardiographic frame rate (55 Hz).

Studies of trabeculas

Hearts were rapidly removed from ether-anesthetized rats and perfused in a modified HEPES buffer solution: sodium chloride (140 mM), potassium chloride (5 mM), magnesium chloride (1.2 mM), acetate (2.8 mM),

Figure 1 Interleukin-6 (IL-6) (A) and transforming growth factor beta-1 (TGF- β 1) (B) levels in cardiac tissue on day 1 and day 7, respectively following coronary artery ligation. The left ventricular tissue was separated into infarct (white bars) and noninfarct (black bars) areas. The corresponding anterior left ventricular free wall was also isolated from the septum of the sham hearts; $n=5$ or $n=6$ for each group. Celacade manufactured by Vasogen Inc, Canada. *Significant ($P<0.05$) versus sham; †Significant ($P<0.05$) versus myocardial infarction (MI)

HEPES (10 mM), glucose (10 mM) and taurine (10 mM); pH 7.4. The buffer contained 0.4 mM Ca^{2+} and was pre-equilibrated with O_2 for longer than 40 min. Unbranched thin trabeculas, running between the atrioventricular ring and the free wall of the RV, were dissected. Each trabecula was then transferred to a perfused chamber on the stage of an inverted microscope (Nikon Inc, Canada). With the help of a dissection microscope, the tissue end of the trabecula was mounted in a platinum basket connected to a force transducer and the valve end was mounted to a hook connected to a servo-controlled motor. The trabeculas were equilibrated in HEPES buffer for approximately 1 h at 25°C until the force at 0.2 Hz and sarcomere shortening reached a steady state. The cross-sectional area of individual trabeculas was calculated from an elliptical assumption of the width and thickness of the trabeculas.

Force measurement

Force development of the muscle was measured with a strain gauge (AE 801, Sensor One Technologies Corp, USA) at an SL of approximately 2.1 μm . Twitch force (F_{tw} , mN/mm²) was normalized to the cross-sectional area of the trabeculas. The muscle was field-stimulated by two silver electrodes along the perfusion chamber. The effects of increasing stimulation frequency on force development were assessed at 26°C and at the physiologically more relevant 36°C. The muscles were stimulated at 0.2 Hz, 0.3 Hz, 0.5 Hz, 1 Hz and 2 Hz at 26°C; at 36°C, they were stimulated at 5 Hz as well. At each frequency, forces were allowed to reach the steady state before data were recorded.

Diastolic SL fluctuation

SL was measured by the laser diffraction technique described previously (1,12). A laser beam was projected onto a region of the muscle with minimal translocation during contraction. The position of the median of the first-order diffraction pattern was converted electronically to a voltage proportional to SL by a nonlinear amplifier, which was calibrated with a testing grating. In quiescent muscle, the resolution of SL was approximately 3 nm (13). RV trabeculas from rats with heart failure showed microscopically visible spontaneous diastolic contractions accompanied by SL fluctuations in the first-order diffraction pattern, which lasted 200 ms to 300 ms. The SL fluctuations reflected the frequency of spontaneous diastolic contractions in the myocytes (as quantified from video microscopy) (2). To quantify the SL fluctuation, the root mean square of SL fluctuation (RMS_{SL}) around the average SL during diastole was calculated as follows:

$$\text{RMS}_{\text{SL}} = \frac{1}{12} \sum_{i=1}^{12} \sqrt{(SL_{\text{mean}} - SL)^2}$$

The RMS_{SL} in sham trabeculas at the initial $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_0$) of

0.4 mM (0.93 ± 0.01 nm) was defined as the reference (100%).

Statistical analysis

Data are presented as mean $\pm$ SEM. Multiple group comparison was performed with ANOVA followed by the Student-Newman-Keuls test or least significant differences. Events in two groups were compared using Student's t test. Differences were considered to be significant at $P<0.05$.

RESULTS

Effects of Celacade on cytokine expression in the acute stage of MI

LV tissue from MI rats was separated into infarct and noninfarct areas. The corresponding anterior LV free wall was also isolated from the septum in the hearts of sham rats. In a preliminary study ($n=2$ or $n=3$), IL-10 and TNF- α in the infarct tissue of MI rats did not show significant differences compared with the values from sham controls, respectively, at day 1 (IL-10: 31.79 ± 8.37 pg/mg versus 34.19 ± 3.38 pg/mg and TNF- α : 16.49 ± 0.48 pg/mg versus 20.65 ± 3.81 pg/mg) and day 7 (IL-10: 34.7 ± 7.9 pg/mg versus 30.1 ± 4.24 pg/mg and TNF- α : 15.19 ± 1.44 pg/mg versus 15.18 ± 3.62 pg/mg) following coronary artery ligation, while IL-6 was elevated on day 1 and TGF- β 1 was elevated in the infarct LV on day 7 following coronary artery ligation. Therefore, the effects of Celacade on LV IL-6 and TGF- β 1 following MI surgery were determined.

The expression of IL-6 on day 1 was significantly ($P<0.05$) increased in the infarct area (707.1 ± 38.8 pg/mg) compared with the corresponding area of sham hearts (484.8 ± 43.8 pg/mg) (Figure 1A). Although not statistically significant, a slight increase of IL-6 in the noninfarct area following MI was also observed compared with that in sham animals (644.1 ± 72.4 pg/mg versus 468.8 ± 28.2 pg/mg, respectively). In animals that had been treated with the Celacade system, IL-6 expression in both infarct (609.3 ± 67 pg/mg) and noninfarct areas (517.6 ± 60.1 pg/mg) were slightly but not significantly lower than untreated animals. A twofold increase of TGF- β 1 in the infarct area (275.4 ± 30.1 pg/mg, $P<0.05$) of LV following MI (day 7) was observed compared with the corresponding area of sham hearts (118.1 ± 9.7 pg/mg) (Figure 1B). The level of TGF- β 1 was also slightly elevated in the noninfarct area of MI hearts (170.6 ± 22.5 pg/mg versus 136.3 ± 14.9 pg/mg; $P>0.05$). Celacade prevented the increase of TGF- β 1 (191.6 ± 22.5 pg/mg) in the infarct area ($P<0.05$).

Heart weight and echocardiographic LV dimensions after MI

Table 1 shows heart weight results four months after MI. LV weights were significantly decreased ($P<0.05$) in both Celacade-treated and untreated MI rats compared with sham rats. Celacade treatment did not affect body weight (BW), the ratio of lung weight to BW, the ratio of RV to BW or scar area in rats after MI surgery.

TABLE 1
Heart weight, lung weight (LW) and scar area four months after myocardial infarction (MI)

	Sham (n=5)	MI (n=16)	Celacade-MI (n=23)
BW, g	418.8±14.7	419.8±6.3	414.3±4.8
LW/BW, mg/g	3.8±0.08	5.0±0.46	5.2±0.48
LV/BW, mg/g	1.9±0.05	1.7±0.04*	1.7±0.02*
RV/BW, mg/g	0.44±0.02	0.6±0.03	0.6±0.04
Scar area, cm ²	—	1.37±0.08	1.34±0.06
Heart rate, beats/min	288.3±54.1	328.7±34.3	321±37.3

Data presented as mean ± SEM. Celacade manufactured by Vasogen Inc, Canada. *Significant ($P<0.05$) versus sham group. BW Body weight; LV Left ventricle; RV Right ventricle

As shown in Figure 2, both LVEDA and LVESA significantly increased ($P<0.01$) following coronary artery ligation, while FAC% significantly decreased ($P<0.01$). Celacade treatment appeared to have no influence on the remodelling process of LV as determined with both echocardiography and the ratio of LV to BW determined at the moment of sacrifice of the animals (Table 1 and Figure 3B).

The changes of LVEDA (Figures 2A and 3A) and the small reduction of residual LV muscle mass (LV to BW ratio in Figure 3B) were in close agreement with the model predictions (see the Methods section and Figure 3A) and were not prevented by Celacade (Figures 2 and 3). Increased lung weight, which is characteristic of manifest heart failure in this model, occurred both in control and in Celacade-treated animals when the scar area exceeded approximately 140 mm² or 30% of the LV surface (Figure 3C), as has been reported previously (14).

F_{tw} and spontaneous contractions in RV trabeculas after MI

Effects of Celacade on SL fluctuations: The behaviour of the sarcomeres in normal cardiac muscle is characterized by monotonic sarcomere shortening during the twitch, rapid lengthening during relaxation and quiescence during the diastolic interval (13). The first order of the diffraction pattern of quiescent trabeculas always consists of a stationary bell-shaped light distribution with dispersion around the median equivalent to $\pm 0.1 \mu\text{m}$. The SL in sham at $[\text{Ca}^{2+}]_o = 1.2 \text{ mM}$ fluctuated less than the detection limit of the diffraction technique (approximately 3 nm). Even in normal trabeculas, $[\text{Ca}^{2+}]_o$ greater than 2.5 mM causes microscopically visible focal spontaneous diastolic contractions in individual cells, which are accompanied by fluctuations of the first-order intensity and consequently, fluctuations of the calculated SL. When spontaneous contractions occurred, the reproducibility of the twitch SL transient deteriorated.

The central regions of MI with heart failure showed spontaneous contractions at all $[\text{Ca}^{2+}]_o$. In contrast, sham rats were quiescent (ie, spontaneous contractions were absent in the interval between twitches) at $[\text{Ca}^{2+}]_o$ less than 1.0 mM (Figure 4). At higher $[\text{Ca}^{2+}]_o$, spontaneous contractions became visible in small areas of the muscles (less than $10 \times 10 \mu\text{m}^2$). These microcontractions usually started several seconds after the twitch, appeared to propagate over short distances (usually less than 100 μm), and increased in spatial extent and number with time. Their propagation velocities (100 $\mu\text{m/s}$ to 150 $\mu\text{m/s}$; data not shown) were similar to those that have been described for Ca^{2+} waves in single myocytes. At high $[\text{Ca}^{2+}]_o$, spontaneous contractions started earlier in all groups and persisted during the inter-stimulus interval, while their extent increased and they frequently propagated over distances greater than 100 μm . These contractions were readily detected by the diffraction methods, and their speed and frequency were proportional to the $[\text{Ca}^{2+}]_o$. When spontaneous contractions occurred, F_{tw} did not increase further with increasing $[\text{Ca}^{2+}]_o$ or declined.

RMS_{SL} was calculated using LabVIEW (National Instruments, USA) software. Figure 5 shows RMS_{SL} at all stimulation frequencies in MI trabeculas. An approximately 1.8-fold increase ($P<0.05$) of RMS_{SL} was observed in trabeculas from MI animals at both 26°C and

36°C compared with sham rats. Celacade treatment prevented this increase in SL fluctuation at 26°C ($121 \pm 15\%$) and significantly ($P<0.05$) lowered RMS_{SL} at 36°C ($143 \pm 19\%$).

Effects of Celacade on the force-frequency relationship: In isolated trabeculas, the effects of varied stimulation frequency on F_{tw} were studied at 26°C and 36°C and normalized to the F_{tw} of 0.5 Hz. Resting SL was maintained at 2.1 μm during the experiment. Figure 5 shows the force-frequency relationship (FFR) in trabeculas from the RV of sham, MI and Celacade-treated MI. At 26°C, a positive FFR was observed in all three groups and was not significantly different among the groups (Figure 6A). Under physiological conditions, developed F_{tw} increased approximately twofold from 0.5 Hz to 5.0 Hz in sham trabeculas showing a positive FFR curve (Figure 6B). After MI, the positive FFR curve was significantly repressed, particularly at high stimulation rates. The $F_{5 \text{ Hz}/0.5 \text{ Hz}}$ in MI trabeculas was $148.1 \pm 27.1\%$, which was substantially lower than the $F_{5 \text{ Hz}/0.5 \text{ Hz}}$ in sham muscle ($211.4 \pm 37.9\%$, $P<0.05$). Muscle from Celacade-treated MI hearts had a similar FFR to that in sham hearts ($F_{5 \text{ Hz}/0.5 \text{ Hz}}$: $219.1 \pm 46.7\%$; $P<0.05$ compared with MI trabeculas). Figure 5C shows F_{tw} expressed as percentages after being normalized to the F_{tw} of sham trabeculas. At 36°C, $F_{5 \text{ Hz}/0.5 \text{ Hz}}$ in trabeculas from MI hearts was significantly depressed (85.6% of sham, $P<0.05$), while Celacade treatment increased the F_{tw} to 96.7% of that in sham hearts.

DISCUSSION

The present study confirms that induction of MI by left coronary artery ligation causes gross LV dysfunction, eliminates the positive inotropic effect of increased heart rate and causes spontaneous subcellular contractions during diastole in trabeculas from the RV (2). The administration of whole blood treated with the Celacade system partially prevented this effect on muscle function, which may explain its beneficial effects observed in clinical trials. In addition, Celacade inhibited production of TGF- β 1 during the acute stage of MI, suggesting new mechanisms for the use of the Celacade system in the treatment of heart failure.

Effects of Celacade on SL fluctuations

Previous studies have indicated that trabeculas from rat MI hearts exhibit microscopically visible spontaneous contractions during diastole (1). The intensity of the spontaneous SL fluctuation appeared to correlate with cardiac damage induced by the ligation of the coronary arteries (ie, muscle from failing hearts showed more spontaneous diastolic SL fluctuations than muscle from nonfailing hearts; the diastolic SL fluctuations increased in response to increased stimulus rate and $[\text{Ca}^{2+}]_o$). Ryanodine, an inhibitor of the calcium release channel (RyR) of the sarcoplasmic reticulum, reduced the SL fluctuation in MI trabeculas during diastole, suggesting that Ca^{2+} leaks from the sarcoplasmic reticulum via the RyR have a critical role in the occurrence of SL fluctuation in MI hearts in congestive failure (1).

We confirm here that RMS_{SL} – which quantifies the spontaneous activity in the muscle (see Methods) – in the trabeculas of MI rat hearts was significantly increased compared with sham rats at both 26°C and 36°C. Treatment using the Celacade system prevented this increase of RMS_{SL} in MI trabeculas at 26°C and significantly lowered this increase at 36°C. The molecular mechanisms behind this reduction of diastolic cardiac muscle fluctuation were not revealed by the present study. Considering the important role of RyR function in SL fluctuation, the effect of Celacade on SL fluctuation in MI trabeculas might involve a regulatory step of the RyR functional pathway, possibly including RyR phosphorylation by either protein kinase A or Ca^{2+} -calmodulin-dependent protein kinase II (2,15-17), or alteration of stoichiometry of the FK506 binding protein 12.6 to the channel (18), and increased oxidative stress in the MI heart (19).

Effects of Celacade on the FFR

The FFR and the Frank-Starling law are the essential mechanisms by which cardiac tissue adjusts mechanical performance in response to

Figure 2 Echocardiographic assessment of left ventricular (LV) dimension and function following coronary artery ligation or sham operation. Graphs show the time course of LV end-diastolic area (LVEDA) (A), LV end-systolic area (LVESA) (B) and fractional area change (FAC%) (C) in sham (○, n=5), untreated (□, n=11) and Celacade-treated (■, n=11) MI rats. *Significant ($P<0.01$) versus all other groups

Figure 3 Left ventricular (LV) end-diastolic area (LVEDA) (n=27) and the ratio of LV to body weight (BW) increased with scar area. This linear increase was predicted by an ellipsoid model of the LV in which the ellipticity of the LV decreased in proportion to the scar area, while thickness of the surviving LV wall increased in proportion to the scar area (see Methods). **A** Linear regression of LV end-systolic area (LVESA) (○) and LVEDA (●) to scar area. The model-predicted LVEDA (thick line) overlapped completely with the linear regression line (thin line) of LVEDA to scar area. **B** BW-corrected LV mass slightly declined with increasing scar size (sham [○, n=5], untreated [□, n=16] and Celacade-treated [■, n=23] MI rats). The model-predicted ratio of LV to BW (thick line) overlapped with the linear regression line (thin line) of the ratio of the LV to BW in proportion to scar area. **C** Correlation of scar area with the ratio of lung weight (LW) to BW in sham (○, n=5), untreated (□, n=16) and Celacade-treated (■, n=23) MI rats. The solid line shows the normal range for the ratio of LW to BW. The dashed line indicates the extent of the scar area (approximately 30% of LV surface area) at which LW increased

Figure 4 Spontaneous sarcomere contraction during the diastolic interval in rat cardiac trabeculas. Sarcomere length (SL) changes in sham (A) and myocardial infarction (MI) trabeculas (B) are shown. No SL fluctuations between twitches were detected in sham trabeculas during the diastolic period at a low external calcium concentration ($[Ca^{2+}]$). In contrast, SL spontaneous activation is often visible in MI trabeculas during late diastole at both 26°C and 36°C ($[Ca^{2+}]_0=0.4$ mM, SL calibration is as indicated)

hemodynamic needs (20,21). Loss of positive FFR has been reported in human and animal models of heart failure (1,22). The results of the present study showed that a positive FFR, measured at the stimulating frequency range of 0.5 Hz to 5 Hz and at the physiological temperature, was dramatically reduced in the rat heart with a large-area MI (ie, more than 30% of the LV surface; Table 1). As shown previously, reduced force at a high stimulus rate may be caused by limitation of

Figure 5 Effects of Celacade (Vasogen Inc, Canada) on sarcomere length (SL) fluctuation in trabeculas from myocardial infarction (MI) hearts. The root mean square of SL fluctuation (RMS_{SL}) around the average SL in diastole interval was used to calculate SL fluctuation (initial $[Ca^{2+}]=0.4$ mM; muscle stimulation: 0.2 Hz to 2 Hz at 26°C and 0.2 Hz to 5 Hz at 36°C). RMS_{SL} in MI (n=9, grey bar) and Celacade (Vasogen Inc, Canada)-MI trabeculas (n=9, black bar) was normalized to RMS_{SL} in sham trabeculas (n=5, white bar). * $P<0.05$ versus sham

oxygen diffusion in trabeculas with a thickness of more than 200 μm (23). This condition is unlikely to affect the present study because the thickness of the trabeculas was less than 150 μm in both MI and

Figure 6 Effects of Celacade (Vasogen Inc, Canada) on the force-frequency relationship (FFR) in trabeculas from myocardial infarction (MI) hearts. Twitch force (F_{tw}) was normalized to force at 0.5 Hz. **A** FFR at a stimulating frequency from 0.5 Hz to 2 Hz in sham (○, n=5), MI (□, n=9) and Celacade-MI (■, n=9) rats at 26°C. **B** FFR at a stimulating frequency from 0.5 Hz to 5 Hz in sham (○, n=5), MI (□, n=9) and Celacade-MI (■, n=9) rats at 36°C. **C** F_{tw} of MI (n=9, grey bar) and Celacade-MI trabeculas (n=9, black bar) was normalized to F_{tw} of the sham group (n=5, white bar). * $P < 0.05$ versus sham

Celacade-MI groups ($110 \pm 20 \mu\text{m}$ and $120 \pm 10 \mu\text{m}$, respectively). Various factors that affect intracellular Ca^{2+} homeostasis have been postulated to contribute to altered cardiac muscle contraction. The frequency-dependent sarcoplasmic reticulum Ca^{2+} load determined by SERCA2a, $\text{Na}^+/\text{Ca}^{2+}$ exchanger and L-type calcium channels plays a central role in characterizing the FFR (24). We previously showed that spontaneous diastolic contraction resulting from Ca^{2+} leakage from the sarcoplasmic reticulum contributes to the force deficit in MI cardiac muscle (2). There is increasing evidence that altered calmodulin-dependent protein kinase II activity in a diseased heart may contribute to loss of the positive inotropic effect of increased heart rate (25).

Treatment using the Celacade system greatly reduced the loss of the inotropic effect of increased stimulus rate in MI cardiac muscle. Because of our observation that pretreatment with Celacade led to a reduction in the spontaneous diastolic contraction of MI muscle, we suggest that improvement of force development in cardiac muscle from Celacade-treated MI rats may have resulted from a reduction of the spontaneous diastolic contractions. However, we cannot rule out alternative mechanisms through which Celacade may have enhanced force at high stimulus rates. Celacade may have had an effect on second-messenger mechanisms involved in the positive FFR, such as calmodulin-dependent protein kinase II. Alternatively, improved cardiac muscle function may have involved inhibitory effects of Celacade on TGF- β 1 expression in the myocardium.

The present study shows that Celacade treatment restores, at least partially, the positive FFR that is typical for cardiac muscle. Nevertheless, cardiac dilation and fraction shortening are not ameliorated by Celacade, as was shown by echocardiography. During development of the MI in the first days and weeks after coronary artery ligation, cardiac tissue in the infarcted area is replaced by scar tissue and the noninfarcted tissue hypertrophies. Although Celacade enhanced force development of MI trabeculas, improved muscle function was apparently not sufficient to compensate for the loss of cardiac structure.

Cytokine response

Myocardial injury induces an inflammatory response as well as cytokine production that is involved in tissue repair and adaptation processes. The upregulation of cytokine expression after MI is believed to have favourable and unfavourable effects on myocyte survival, later ventricular dilation and heart failure. In rodent MI models, proinflammatory cytokines, such as TNF- α , IL-6 and IL-1 beta are elevated in the early phase (first 24 h) of MI. The elevation of TGF- β 1 is followed and maintained at high levels for up to four weeks (26-28). TGF- β 1 is a fibrogenic cytokine that locally stimulates collagen and extracellular matrix production (29), and is thought to contribute to maladaptive remodelling of the myocardium owing to myocardial fibrosis, with depressed cardiac systolic function and diastolic function. Suppression

of the TGF- β pathway by introducing the extracellular domain of TGF- β type II receptor has recently been reported to attenuate LV remodelling and dysfunction (30,31).

We observed a 50% increase of IL-6 protein at day 1 and a 100% increase in TGF- β 1 protein at day 7 in the infarcted myocardium, consistent with the reported increase of IL-6 and TGF- β 1 messenger RNA (mRNA) (26-28). A slight but not significant increase of IL-6 and TGF- β 1 was also detected in remote noninfarcted myocardium. Absence of a change of TNF- α in myocardium postinfarction may seem unexpected, but may be consistent with the reported smaller increase of TNF- α mRNA (threefold) compared with the increase of IL-6 mRNA (30- to 40-fold) in this model (26,28). Pretreatment of the animals using the Celacade system significantly inhibited TGF- β 1 at day 7 and reduced the IL-6 response (day 1) following coronary artery ligation in the present study. This finding suggests that Celacade treatment modified cytokine expression in the heart following MI. Detailed studies are now required to address the significance of inhibiting TGF- β 1 signalling by Celacade in attenuation of cardiac muscle dysfunction.

CONCLUSION

Treatment of rats with the Celacade system before coronary artery ligation inhibited the increase of TGF- β 1 in the infarcted region during the acute phase of MI. A novel finding is that Celacade ameliorates diastolic dysfunction in cardiac muscle from the RV of rats with a large MI. This reduction of spontaneous diastolic SL fluctuation contributed to increased systolic force at increased stimulus rate in trabeculas from rats with MI.

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