Involvement of Mast Cells in the Development of Fibrosis in Rats with Postmyocarditis Dilated Cardiomyopathy

Suresh PALANIYANDI SELVARAJ,^a Kenichi WATANABE,^{*,a} Meilei MA,^a Hitoshi TACHIKAWA,^b Makoto KODAMA,^b and Yoshifusa AIZAWA^b

^a Department of Clinical Pharmacology, Niigata University of Pharmacy and Applied Life Sciences; 5–13–2 Kamishineicho, Niigata 950–2081, Japan; and ^b First Department of Medicine, Niigata University School of Medicine; Niigata 951–8510, Japan. Received June 28, 2005; accepted August 9, 2005; published online August 23, 2005

Dilated cardiomyopathy (DCM) is a major cause of morbidity and mortality. Occurrence of myocardial fibrosis is an important event in the ventricular remodeling process, which takes place during DCM. Mast cells are well known inflammatory cells implicated in various biological phenomena. The involvement of mast cells in the development of myocardial fibrosis of DCM in rats after autoimmune myocarditis remains unknown. Nine-week-old male Lewis rats were immunized with cardiac myosin and divided into vehicle treated (group V) and disodium cromoglycate (DSCG), a mast cell stabilizer (24 mg/kg i.p.) treated (group DSCG) groups. The animals were sacrificed after 60 d of immunization. The myocardium was excised and preserved for histopathology and protein analysis. Myocardial levels of transforming growth factor (TGF) β 1 and collagen-III were quantified. Staining of mast cells was performed by toluidine blue. A significant correlation was obtained between myocardial fibrosis and cardiac mast cell density. DSCG reduced myocardial fibrosis besides preventing infiltration and degranulation of mast cells. Our findings confirm the active participation of mast cells in the progression of myocardial fibrosis in rats with postmyocarditis DCM.

Key words cardiomyopathy; fibrosis; mast cell; myocarditis

Mast cells have a variety of physiological and pathological functions through the production of several cytokines, chemokines and chemical mediators. The fibrogenic role of mast cells in idiopathic pulmonary fibrosis,¹⁾ neurofibroma²⁾ and cutaneous fibrosis³⁾ has been demonstrated. Recently, mast cells have been implicated in myocardial fibrosis of hypertensive hypertrophy,⁴⁾ ischemia-reperfusion⁵⁾ and postcardiac transplantation.⁶⁾ Progressive interstitial fibrosis is an essential component of left ventricular remodeling in patients with chronic heart failure.

Experimental autoimmune myocarditis (EAM) of rats mimics human idiopathic giant cell myocarditis.^{7,8)} Giant cell myocarditis is a fatal disorder, often leads to heart failure or arrhythmias.⁹⁾ Dilated cardiomyopathy (DCM) is recognized as a significant cause of morbidity and mortality. Treatment strategies of DCM vary due to its diverse etiopathology ranging from myocardial infarction, myocarditis (bacterial, viral, parasitic and autoimmune) and pressure overload *etc.* Our animal model, a non-viral autoimmune postmyocarditis DCM with diffusely distributed fibrosis, besides being a cellular immunity and inflammation mediated disease^{8,10)} offers suitable setting to investigate the role of mast cells in the development of fibrosis of DCM. To verify this issue we have used disodium cromoglycate (DSCG), a mast cell stabilizer in rats with postmyocarditis DCM, which decreased the myocardial fibrosis.

MATERIALS AND METHODS

Experimental Protocol Nine-week-old male Lewis rats (Charles River Japan Inc., Kanagawa, Japan) were injected in the footpads with antigen-adjuvant emulsion according to the procedure described previously.^{8,10)} In brief, porcine cardiac myosin was dissolved in phosphate buffered saline (PBS) at 5 mg/ml and emulsified with an equal volume of complete

freund's adjuvant with 11 mg/ml *Mycobacterium tuberculosis* H37RA (Difco Lab., Detroit, MI, U.S.A.). The rats were immunized with 0.1 ml of emulsion by subcutaneous injection into footpads. EAM morbidity was achieved in 100% of the rats immunized by this method.^{8,10)} Rats become immobile from day 14. Six rats were died before day 28. The remaining rats were divided as follows: DSCG was administered intraperitoneally at a dose of 24 mg/kg from day 28 until the day before sacrifice to one group of rats (group DSCG; $n=17$); while controls received saline as vehicle, (group V; $n=13$). Lewis rats without any treatment were used as age-matched normal controls (group N; $n=10$). Throughout the study, animals were looked after carefully in accordance with the guidelines for animal experiments of our institute.¹⁰⁾

Histopathology After 60 d of immunization, the rats were sacrificed and their hearts were isolated. The hearts were fixed with 10% formalin in PBS, and embedded in paraffin, and several transverse sections were cut from the mid ventricles, and stained with hematoxylin-eosin (H-E) and Azan-Mallory. Infiltration of inflammatory cells was examined in the H-E stained slides viewed under a high-power light microscope. The area of myocardial fibrosis was measured quantitatively by a color image analyzer (CIA-102; Olympus Tokyo, Japan), using the differences in the color (blue fibrotic area opposed to red myocardium) of the photomicrographs of Azan-Mallory stained slides. The results are presented as the ratio of the fibrotic area to the area of myocardium.

Mast Cell Staining and Quantitation Histochemical staining with toluidine blue was performed to identify mast cells. For toluidine blue staining, slides of paraffinized sections of the mid ventricles were dewaxed, rehydrated and incubated with 0.05% w/v toluidine blue for 30 min followed by counterstaining with 0.01% w/v eosin for 1 min. Metachromatic staining of mast cell granules was handy to

* To whom correspondence should be addressed. e-mail: watanabe@niigata-pharm.ac.jp

identify these cells. Mast cell density was quantified by counting the number of toluidine blue-positive mast cells per field ($100\times$). At least 15 fields were included from each slide for counting.

Western Immunoblotting for Transforming Growth Factor (TGF) β 1 Protein lysate was prepared by homogenizing left ventricular tissue in 1 ml of lysis buffer [8 M urea, 7 mM sodium dodecyl sulfate (SDS), 0.8% tritonX-100, 3% 2-mercaptoethanol, 5 μ g/ml aprotinin, and 5 μ g/ml leupeptin]. Then, samples were incubated at 37 °C for 1 h, centrifuged at 15000 rpm for 10 min at 4 °C and supernatants were stored at -80 °C until use. Total protein concentration of samples was measured by bicinchoninic acid method. For Western blot experiments, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and identified with the primary antibody's, *i.e.* anti-transforming growth factor β 1 (anti-TGF β 1) rabbit polyclonal antibody and anti-glyceraldehyde-3-phospho dehydrogenase (anti-GAPDH) goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After primary antibody incubation, bound antibody was visualized with horseradish peroxidase (HRP) coupled secondary antibody (Santa Cruz) and chemiluminescence developing agents (ECL Plus, Amersham, Piscataway, NJ, U.S.A.).

Immunohistochemistry for Collagen-III Formalin-fixed, paraffin-embedded cardiac tissue sections were used for immunohistochemical staining. After deparaffinization and hydration, the slides were washed in Tris-buffered saline (TBS; 10 mmol/l Tris-HCl, 0.85% NaCl, pH 7.5) containing 0.1% bovine serum albumin (BSA). Endogenous peroxidase activity was quenched by incubating the slides in 0.6% H_2O_2 /methanol. To perform antigen retrieval, the sections were pretreated with trypsin for 15 min at 37 °C. Blocking was done with normal serum. After overnight incubation with goat polyclonal anti-collagen III antibody (Santa Cruz) at 4 °C, the slides were washed in TBS and HRP conjugated secondary antibody (Santa Cruz) was added and incubated at room temperature for 45 min. The immunostaining was visualized with the use of diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin.

Statistical Analysis Data are presented as mean $\pm$ standard error of mean (S.E.M.). Statistical comparison between groups was performed by one-way analysis of variance (ANOVA), followed by Tukey's or Bonferroni's method.

RESULTS

Myocardial Macroscopical Changes and Myocardial Fibrosis in DCM Dilated cardiomyopathic hearts were discolored and dilated in comparison to normal hearts (Fig. 1a). The area of fibrosis in dilated cardiomyopathic hearts (group V) was higher than that of normal hearts (group N) (Fig. 1b; Figs. 1B, A, 1d).

Cardiac Mast Cell Density and Degranulation in DCM The mast cell density in the myocardium of group V was higher than that of group N (Figs. 1E, D, 1c). Partial and complete degranulation of mast cells in dilated cardiomyopathic hearts was viewed in the midventricle sections stained with toluidine blue as shown in Figs. 1G, H, and I respectively, which was absent in normal hearts.

Myocardial Levels of Molecular Markers of Fibrosis in

DCM Western immunoblotting showed an increase in TGF β 1 levels of dilated cardiomyopathic hearts in comparison to normal hearts (lanes V and N of Fig. 2A respectively, and Fig. 2B). Midventricle sections of group V showed stronger immunoreactivity for collagen-III than those of group N (Figs. 1N, M).

Association between Cardiac Mast Cell Density and Myocardial Fibrosis in DCM We observed co-localization or identical locus for toluidine-blue positive mast cells and fibrosis, with the serial sections obtained from dilated cardiomyopathic hearts of same sample. The serial sections from hearts obtained from DCM rats were stained with H-E for inflammatory infiltration (Fig. 1J), for mast cells with toluidine blue (Fig. 1K) and for fibrosis with Azan-Mallory staining (Fig. 1L). Altogether it revealed that mast cells were infiltrated in the area of myocardium of DCM where fibrosis took place.

A positive correlation was obtained between % area of myocardial fibrosis and cardiac mast cell density. The R^2 value was 0.946 (Fig. 2C).

Effect of DSCG on Cardiac Mast Cell Density and Degranulation Administration of DSCG reduced cardiac mast cell density in the myocardium of group DSCG in comparison to hearts from group V (Figs. 1F, 1c). Both partial and complete degranulation of mast cells was absent in the midventricles of group DSCG.

Effect of DSCG on Myocardial Fibrosis and Molecular Markers of Fibrosis in DCM Administration of DSCG reduced % area of myocardial fibrosis significantly in comparison to group V (Figs. 1C, 1d).

Immunohistochemical analysis of group DSCG samples revealed a decrease in myocardial levels of collagen-III (Fig. 1O). Immunoblotting for TGF β 1 showed a significant reduction in its level with DSCG administration (lane D of Fig. 2A and Fig. 2B).

DISCUSSION

In this report, we showed increased infiltration and degranulation of mast cells in rats with DCM in addition to an increase in myocardial fibrosis. Furthermore, administration of DSCG, a mast cell stabilizer, reduced myocardial fibrosis by reducing infiltration and degranulation of mast cells in the myocardium of postmyocarditis rats.

In the present study, the development of DCM in the late phase of autoimmune myocarditis after myosin immunization is apparent from the dilated and discolored myocardium. Myocardial fibrosis, the hallmark of DCM, is observed in dilated cardiomyopathic hearts as indicated by Azan-Mallory staining and increased myocardial TGF β 1 and collagen-III levels. Apart from the increased cardiac mast cell density, we observed increased mast cell degranulation in the midventricle sections of cardiomyopathic hearts of rats. Previously, a significant correlation has been demonstrated between arrhythmias with mast cell density and degranulation in cardiomyopathic hearts of *Trichinella spiralis* challenged rats.¹¹ The mast cell degranulation products are either proinflammatory, hypertrophic or fibrogenic in nature. Histamine, the principle mediator of mast cells, has been found to stimulate the proliferation of fibroblasts in dose dependent manner.¹² TGF β 1, a marker for fibrosis is stored in mast cell granules,

Fig. 1.

Macroscopy of dilated myocardium: Representative photographs of dilated cardiomyopathic heart (left panel) and normal heart (right panel) were shown (a). Myocardial fibrosis of gross sections: Representative photographs of Azan-Mallory stained gross sections from dilated cardiomyopathic heart (left panel) and normal heart (right panel) were shown (b). Blue area in the sections indicates area of myocardial fibrosis. Scale bar is 1 cm.

Quantification: Mast cell density of group N, group V and group DSCG were plotted (c). The % area of myocardial fibrosis of group N, group V and group DSCG was plotted (d). The values are mean $\pm$ S.E.M. ** $p < 0.01$ vs. Group N; ## $p < 0.01$ vs. Group V, (Tukey's). □ Group N, ■ Group V, ▤ Group DSCG.

Myocardial fibrosis: Photomicrographs of Azan-Mallory staining from group N (normal rats) (A), group V (myosin-injected rats administered with vehicle) (B) and group DSCG (myosin-injected rats treated with DSCG) (C) were shown ($\times 100$). They are representative photographs of respective group. Blue area in the sections depicts area of myocardial fibrosis. Cardiac mast cell density: Increase in mast cell density with group V (E) relative to that of group N (D) and group DSCG (F) was observed. Myocardial sections were stained with 0.05% w/v toluidine blue and counterstained with 0.01% w/v eosin in order to view mast cells (shown by arrows) ($\times 100$). They are representative photographs of respective group. Mast cell degranulation: Degranulation of mast cells was only observed in the cardiac sections of group V (myosin-injected rats administered with vehicle). Photomicrographs for partial degranulation and complete degranulation of group V were shown (G, H and I respectively). The degranulating mast cells are violet in color. ($\times 1000$). Accumulation of mast cells in the area of fibrosis: Serial sections from same sample of group V (myosin-injected rats administered with vehicle) were stained with three different stains. Infiltration of inflammatory cells was viewed in the sections stained with hematoxylin-eosin (shown by arrows) (J). Increase in mast cell density was shown in the sections stained with 0.05% w/v toluidine blue and counterstained with 0.01% w/v eosin (shown by arrows) (K). Area of myocardial fibrosis was shown with Azan-Mallory staining (blue color indicates fibrosis) (L) ($\times 100$). Myocardial levels of collagen-III: Cardiac sections from group N (M), group V (N) and group DSCG (O) show brown colored positive immunostaining with anti-collagen-III antibody (shown by arrows) ($\times 400$). They are representative photographs of respective group.

upon stimulation is released in active form¹³) and known to modulate the phenotypic characteristics of fibroblasts and proliferate them, it also elevates collagen synthesis and deposition.^{14,15} Tumor necrosis factor α , a preformed cytokine stored in mast cell granules, is released from variety of mast cell preparations^{16,17} and is reported to induce fibroblasts¹⁸ and participate in adverse cardiac remodeling of DCM.¹⁹ Role of mast cell proteases in fibrosis has been documented in several other studies.^{20–22}

A significant correlation is obtained between myocardial fibrosis and cardiac mast cell density in the present study.

Administration of DSCG also led to the reduction in myocardial fibrosis as evident from decreased area of myocardial fibrosis with Azan-Mallory staining and reduced myocardial collagen-III and TGF $\beta 1$ levels. Involvement of mast cells in the progression of myocardial fibrosis of DCM in rats can be further confirmed by the identical locus of fibrosis and infiltration of mast cells by the use of serial sections of midventricles of same samples with different staining.

The dose of DSCG was selected from a previous report in which the same dose prevented cardiac remodeling in rats with aortocaval fistula.²³ Since myosin immunized rats with

Fig. 2.

Myocardial levels of TGF-β1: Western immuno-blotting images of TGF-β1 and GAPDH from group N (normal rats) (lane N), group V (myosin-injected rats administered with vehicle) (lane V) and group DSCG (myosin-injected rats treated with DSCG) (lane D) (A) were shown. The bands are representative bands of respective groups. Densitometric analysis of immuno-reactive bands for TGF-β1 and GAPDH was performed using Scion Image software and TGF-β1 data was normalized with GAPDH. Data are represented as the mean signal intensity ± S.E.M. from five experiments (B). ** $p < 0.01$ vs. Group N; # $p < 0.05$ vs. Group V (Bonferroni's). □ Group N, ■ Group V, ▒ Group DSCG. Correlation analysis: Correlation analysis was performed between myocardial fibrosis and cardiac mast cell density. The values are mean ± S.E.M. (C). ■ Group N, ▲ Group V, ● Group DSCG.

EAM develop DCM from day 28 of immunization, we have decided to start the DSCG administration on the same day. In the current study, we have not planned to explore the mechanism of stabilization of mast cells by DSCG, the reduction of degranulated mast cells by DSCG is consistent with other studies.^{24,25} DSCG is known to stabilize the membrane of mast cells, thus prevent the degranulation of mast cells. Once mast cell degranulation is prevented, the release of various mediators is also inhibited, ultimately leading to reduction in chemotaxis, inflammation, fibrosis and other remodeling process in the myocardium. With long-term DSCG treatment the inflammatory milieu is getting reduced hence the reduction in mast cell numbers was observed on day 60. Generally it is believed that c-Kit ligand released from macrophages and mast cells recruits and activates the mast cells to cause fibrosis. These explanations may fit into the current situation because the mast cells are localized in the area of fibrosis and inflammatory infiltration.

Thus, it can be suggested that inhibition of mast cells may be useful strategy to prevent the pathological remodeling such as myocardial fibrosis in postmyocarditis DCM. On the contrary, another report alerts the occurrence of phenotypic shift in mast cells of DCM hearts with long-term mechanical support,²⁶ by which the fibrogenic content of mast cells was reportedly reduced. It may explain the healing property of mast cells in the chronic stage. On the other hand, similar to

our study, a report from Hara *et al.* demonstrated reduction of myocardial fibrosis with tranilast, another mast cell stabilizer, in a pressure overload model of heart failure.²⁰ Involvement of mast cells in viral myocarditis induced heart failure has been described previously.²² However, this study may be the first report to elucidate and confirm such notion with the use of mast cell stabilizer and co-localization of mast cells in the fibrotic region in a model of postmyocarditis DCM. Myocardial fibrosis has been widely implicated for the diastolic dysfunction in DCM. Thus, finding out new therapeutic approach to prevent the progression of myocardial fibrosis of DCM based on etiopathology and pathophysiology seems to be sensible. From this study, it can be concluded that mast cells have a significant role in the pathogenesis of postmyocarditis DCM especially contributing to the progression of myocardial fibrosis, thus the treatment strategies based on mast cells appear to be promising in these conditions.

Acknowledgements This research was supported by grants from Yujin Memorial Grant; the Ministry of Education, Culture, Sports, Science and Technology of Japan; and Promotion and Mutual Aid Corporation for Private Schools of Japan. We thank Mir I. I. Wahed, Gurusamy Narasimman, Juan Wen, Prakash Paras, Punniyakoti V. T., Mayako Soga, Ken Shirai, Yusuke Nagai and Fadia A. Kamal for their assistance in this research work.

REFERENCES

- Broide D. H., Smith C. M., Wasserman S. I., *J. Immunol.*, **145**, 1838—1844 (1990).
- Ryan J. J., Klein K. A., Neuberger T. J., Leftwich J. A., Westin E. H., Kauma S., Fletcher J. A., DeVries G. H., Huff T. F., *J. Neurosci. Res.*, **37**, 415—432 (1994).
- Hawkins R. A., Claman H. N., Clark R. A., Steigerwald J. C., *Ann. Intern. Med.*, **102**, 182—186 (1985).
- Panizo A., Mindan F. J., Galindo M. F., Cenarruzabeitia E., Hernandez M., Diez J., *J. Hypertens.*, **13**, 1201—1208 (1995).
- Frangogiannis N. G., Perrard J. L., Mendoza L. H., Burns A. R., Lindsey M. L., Ballantyne C. M., Michael L. H., Smith C. W., Entman M. L., *Circulation*, **98**, 687—698 (1998).
- Li Q. Y., Raza-Ahmad A., MacAulay M. A., Lalonde L. D., Rowden G., Trethewey E., Dean S., *Transplantation*, **53**, 1047—1051 (1992).
- Kodama M., Matsumoto Y., Fujiwara M., Masani F., Izumi T., Shibata A., *Clin. Immunol. Immunopathol.*, **57**, 250—262 (1990).
- Kodama M., Hanawa H., Saeki M., Hosono H., Inomata T., Suzuki K., Shibata A., *Circ. Res.*, **75**, 278—284 (1994).
- Cooper L. T., Jr., "Myocarditis: From Bench to Bedside," ed. by Cooper L. T., Jr., Humana Press, New Jersey, 2003.
- Watanabe K., Ohta Y., Nakazawa M., Higuchi H., Hasegawa G., Naito M., Fuse K., Ito M., Hirono S., Tanabe N., Hanawa H., Kito K., Kodama M., Aizawa Y., *Br. J. Pharmacol.*, **130**, 1489—1495 (2000).
- Paolocci N., Sironi M., Bettini M., Bartoli G., Michalak S., Bandi C., Magni F., Bruschi F., *Virchows Arch.*, **432**, 261—266 (1998).
- Jordana M., Befus A. D., Newhouse M. T., Bienenstock J., Gauldie J., *Thorax*, **43**, 552—558 (1988).
- Lindstedt K. A., Wang Y., Shiota N., Saarinen J., Hyttiainen M., Kokkonen J. O., Keski-Oja J., Kovanen P. T., *FASEB J.*, **15**, 1377—1388 (2001).
- Petrov V. V., Fagard R. H., Lijnen P. J., *Hypertension*, **39**, 258—263 (2002).
- Eghbali M., Tomek R., Sukhatme V. P., Woods C., Bhambi B., *Circ. Res.*, **69**, 483—490 (1991).
- Gordon J. R., Galli S. J., *Nature (London)*, **346**, 274—276 (1990).
- Young J. D., Liu C. C., Butler G., Cohn Z. A., Galli S. J., *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 9175—9179 (1987).

- 18) Vilcek J., Palombella V. J., Henriksen-DeStefano D., Swenson C., Feinman R., Hirai M., Tsujimoto M., *J. Exp. Med.*, **163**, 632—643 (1986).
- 19) Higuchi Y., McTiernan C. F., Frye C. B., McGowan B. S., Chan T. O., Feldman A. M., *Circulation*, **109**, 1892—1897 (2004).
- 20) Hara M., Ono K., Hwang M. W., Iwasaki A., Okada M., Nakatani K., Sasayama S., Matsumori A., *J. Exp. Med.*, **195**, 375—381 (2002).
- 21) Patella V., Marino I., Arbustini E., Lamparter-Schummert B., Verga L., Adt M., Marone G., *Circulation*, **97**, 971—978 (1998).
- 22) Kitaura-Inenaga K., Hara M., Higuchi K., Yamamoto K., Yamaki A., Ono K., Nakano A., Kinoshita M., Sasayama S., Matsumori A., *Circ. J.*, **67**, 881—884 (2003).
- 23) Brower G. L., Chancey A. L., Thanigaraj S., Matsubara B. B., Janicki J. S., *Am. J. Physiol. Heart Circ. Physiol.*, **283**, H518—525 (2002).
- 24) Debbasch C., Pisella P. J., Rat P., Warnet J. M., Baudouin C., *J. Fr. Ophthalmol.*, **24**, 121—128 (2001).
- 25) Yamawaki I., Tamaoki J., Takeda Y., Nagai A., *Res. Commun. Mol. Pathol. Pharmacol.*, **98**, 265—272 (1997).
- 26) Akgul A., Skrabal C. A., Thompson L. O., Loebe M., Lefuente J. A., Noon G. P., Youker K. A., *J. Heart Lung Transplant.*, **23**, 709—715 (2004).