Histochemical study of cardiac mast cells degranulation and collagen deposition: interaction with the cathecolaminergic system in the rat

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Although their role in the cardiovascular system is still largely unknown, mast cells are present in the myocardium of both experimental animals and humans. Interestingly, catheco-laminergic nerve fibres and mast cells are often described in close morphological and functional interactions in various organs. In the present study we investigated the effects of chronic interference with β-adrenergic receptors (via either sympathectomy or β-blockade) on cardiac mast cell morphology/activation and on interstitial collagen deposition. In rats subjected to chemical sympathectomizy with the neurotoxin 6-hydroxydopamine (6-OHDA) we observed a significant increase of mast cell density, and in particular of degranulating mast cells, suggesting a close relationship between the cardiac catecholaminergic system and mast cell activation. In parallel, chronic 6-OHDA treatment was associated with increased collagen deposition. The influence of the β-adrenergic receptor component was investigated in rats subjected to chronic propranolol administration, that caused a further significant increase in mast cell activation associated with a lower extent of collagen deposition when compared to chemical sympathectomy. These data are the first demonstration of a close relationship between rat cardiac mast cell activation and the catecholaminergic system, with a complex interplay with cardiac collagen deposition. Specifically, abrogation of the cardiac sympathetic efferent drive by chemical sympathectomy causes mast cell activation and interstitial fibrosis, possibly due to the local effects of the neurotoxin 6-hydroxydopamine. In contrast, β-adrenergic blockade is associated with enhanced mast cell degranulation and a lower extent of collagen deposition in the normal myocardium. In conclusion, cardiac mast cell activation is influenced by β-adrenergic influences.

Key words: mast cells, collagen, sympathetic inactivation, propranolol, rat.

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Paper accepted on March 6, 2006

European Journal of Histochemistry
2006; vol. 50 issue 2 (Apr-Jun):133-140

Mast cells are nucleated cells normally distributed in tissues throughout the body, that have been known for a long time to play an important role in allergic reactions. Recent experimental observations suggest that they may also exert important effects in many other different disease processes, such as tissue remodelling, wound repair (Abd-El-Aleem *et al.,* 2005), and pathological fibrosis (Bradding and Holgate, 1999), in many organs and under many different physiopathological conditions.

Mast cells are also found in the normal heart tissue, and several reports have suggested a possible role of myocardial mast cells in the pathogenesis of the cardiac damage that may be observed in the setting of myocardial ischemia, heart transplantation-associated fibrosis, hypertensive heart disease and dilated cardiomyopathy (Estensen *et al.,* 1985; Petrovic *et al.,* 1999; Patella *et al.,* 1998). In particular, it has been recently reported that mast cells are activated to degranulate since the earlier stages of cardiac hypertrophy (Shiota *et al.,* 2003; Palladini *et al.,* 2003) and increased numbers of these cells have been reported in the myocardium of animal models of hypertension, myocardial infarction and myocardial remodelling (Stewart *et al.,* 2003).

The main stimuli for the myocardial hypertrophic remodelling are mechanical overload and activation of neurohormonal systems such as the renin-angiotensin-aldosterone axis and the sympatho-adrenergic system (Willenheimer, 2000). Catecholaminergic nerve fibers and mast cells are often described in close morphological and functional interactions in various organs (Bergerot *et al.,* 2000) and it has been recently described a close relation between these cells and the catecholaminergic innervation in the parietal pleura (Artico *et al.,* 1998). In the interaction between mast cells and tissue remodelling, however, it has to be considered that beyond the possible variation in

the absolute number of tissue mast cells, it is their functional status to be physiopathologically relevant, inasmuch as after being activated, mast cells undergo degranulation with the release of several mediators in the extracellular space. It is therefore crucial to assess both the absolute number and the activated/inactivated ratio in order to estimate the possible involvement of mast cells in a given disease process. Moreover, activated mast cells have been extensively demonstrated to induce fibrosis and collagen deposition via the release of several mediators, such as cytokines and TGFβ (Puxeddu & Levi-Schaffer, 2002).

We therefore hypothesized that in the left ventricular myocardium mast cell activation may be influenced by the cardiac sympathetic system, that we have recently shown to play an important role in cardiac fibrosis induced by chronic pressure-overload (Perlini *et al.,* 2005). In order to test this working hypothesis, rats were chronically subjected to either abrogation of the central sympathetic nervous system by chemical sympathectomy or to β-receptor blockade by propranolol administration under normal hemodynamic conditions. After 10 weeks, the effects of these interventions on total mast cells density, degranulating vs. non-degranulating mast cell ratio and interstitial collagen deposition were evaluated in left ventricular sections.

Materials and Methods

Animals

The study was conducted on 30 male Sprague-Dawley rats delivered by Charles River Italia (Calco, Italy) at the age of eight weeks. Eleven rats were subjected to chronic chemical sympathectomy (SSx), whereas 10 animals were subjected to chronic propranolol administration (SBB). The control group (SVh) included 9 eight-week old rats without any treatment.

Animal care complied with the Principles of Laboratory Animal Care, formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (NIH pubblication n° 86-23, revised 1989, authorization n° 00577, Paris, France). The animals were housed under controlled environmental conditions, with food and water ad libitum. All procedures involving animals and their care were conducted in conformity with the institutional guidelines in compliance with international policies.

Treatment

Chemical sympathectomy

Rats were subjected to chemical sympathectomy by chronic treatment with the noradrenergic fiber-specific neurotoxin *6-hydroxydopamine* (6-OHDA, 100 $mg*kg^{-1}$), (Ferrari *et al.,* 1996) which was administered twice a week for 10 weeks. Control group received vehicle injections according to the same schedule.

Chronic propranolol administration

A parallel group of rats were subjected to chronic propranolol administration at the dose of 40 $mg*kg^{-1}$ per day for 10 weeks. The drug was dissolved into the drinking water.

Echocardiographic evaluation

After 10 weeks, rats were anesthetized by intraperitoneal ketamine (75 mg/kg) plus xylazine (15 mg/kg) and subjected to a complete echocardiographic study (Hewlett-Packard Sonos 5500; 12-MHz transducer) to obtain endocardial left ventricular (LV) internal dimensions at end-diastole and end-systole as previously described (Perlini *et al.,* 2005). Finally, the animals were killed by an anesthetic overdose, the heart was quickly excised and the LV was weighted, indexed to body weight and expressed as g/100 g body weight.

Mast cell density

Formalin-fixed LV tissue samples were paraffin embedded and 6 μm sections of the entire cross section of the LV were cut and stained with Toluidine blue. For each animal the total mast cells population was counted in the entire LV cross section (Olympus BX41, 400X) in 3 random sections by two different investigators. The results were expressed as mean value ± standard error of the mean (SEM). The density, expressed as number of mast cell per mm^2, was calculated dividing the number of observed mast cells by the digitised tissue area of each histological section (12.5X; Image ProPlus, Silver Spring, MD). Mast cell degranulation was defined as the presence of metachromatic granules close to the surface of the cell or staining of about half or less of the cytoplasm with Toluidine blue, as described by Huang et colleagues (Huang *et al.,* 2002).

In order to quantitatively describe possible regional differences in mast cell density, the left ventricular wall thickness was divided in an exter-

nal (*epicardial*) and an internal (*endocardial*) section. Mast cell number was evaluated in 10 randomized fields for each section (epicardial and endocardial).

Toluidine blue staining

Deparaffinised sections were incubated for 2 minutes with Toluidine blue solution (1% Toluidine blue dissolved in ethanol 70%) in NaCl solution 10%, washed three times in distilled water, dehydrated and mounted with Entellan.

Collagen quantification

Light microscopic examination (400X) was performed on 8 μm thick formalin-fixed LV sections stained with *Sirius Red* to measure the percent of interstitial collagen. Sirius Red stained sections were digitised and analysed (Image ProPlus, Silver Spring, MD) to determine the percent area of collagen deposits from 10 randomly chosen fields (40X) within the LV section. For each animal 5 sections were examined. Areas that enclosed signs of scar fibrosis or blood vessels were excluded from analysis. In parallel, *Masson's trichrome staining* was performed to obtain a more detailed cytomorphological pattern.

Sirius red staining

Deparaffinised sections were incubated for 5 minutes with 1% aqueous phosphomolybdic acid solution, washed rapidly 3 times in distilled water and then stained for 90 minutes with 2% *Sirius Red F3BA* in water. Then sections were washed 3 times in absolute alcohol, clarified with xylol, dehydrated and mounted with Entellan balsam.

Masson's trichrome staining

Deparaffinised sections were incubated with Bouin solution (75 mL picric acid, 25 mL formaline 37%, 25 mL acetic acid) for 1 hour at 60°C. Sections were cooled down for 10 minutes at R.T. and subsequently washed with tap water. After 10 minutes in fresh Weigert's Hematoxilin staining, sections were washed for 15 minutes in distilled water and then incubated 7 minutes with acid fucsin (1% acid fucsin in a solution 1:50 of acetic acid). A wash in distilled water preceded the incubation in a 2.5% aqueous phosphotungstenic phosphomolybdic acid solution for 15 minutes. Then, collagen fibers were counterstained in anilin blue solution (2.5% anilin blue in 2% water solution of acetic acid) for 10 minutes. Sections were finally washed in distilled water, clarified by 1% acetic acid solution for 5 minutes, dehydrated and mounted with Entellan.

All chemical products were purchased from Sigma-Aldrich Corporation.

Immunoistochemistry

Deparaffinised sections (5 μm) were incubated with 3% H_2O_2 for 5 min. for blocking endogenous peroxidase activity. Sections were incubated with primary antibody anti-tryptase (Serotec) for 30 min. at room temperature. Sections were washed with PBS and then incubated with peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulines for 30 min. at room temperature. The sections were finally treated with 3-amino-9-ethylcarbazole (AEC) for 3 min. and counterstained with aqueous hematoxylin. Prior to IHC staining all sections were submitted to protein digestion pre-treatment with trypsin.

Data analysis

Results are expressed as mean values ± standard error of the mean (SEM). Differences among the 3 experimental groups in mast cell density and collagen data were analysed by ANOVA followed by a Tuckey-Bonferroni post-hoc test (StatView 4.5, Abacus Concepts Inc., Berkeley, CA, USA). A $p<0.05$ was taken as statistically significant.

Results

Mast cells

Morphological analysis

Mast cells were identified by metachromatic granules with Toluidine Blue staining in all left ventricular sections from the 3 experimental groups. Mast cells were mostly round shaped in the proximity of blood vessels (Figure 1a), whereas they displayed an elongated shape in the interstitial regions (Figure 1c). In addition, mast cell often showed reduced numbers of granules and disorganised granule content, suggesting an ongoing degranulation process. Thus, the analysis at higher magnifications allowed the identification of two different morphological cell types: degranulating mast cells, characterized by numerous extracellular metachromatic granules (Figure 1b) and/or by a poor intracytoplasmic granule content, and non-degranulating mast cells without any granule in the close

Table 1. Mast cell density (cell/mm²) and interstitial collagen in control (SVh), sympathectomized (SSx) and propranolol treated (SBB) rats. n = no. of rats.

Group	n	Collagen deposition (%)	Total mast cell cell/mm²±SD	Degranulating mast cell cell/mm²±SD	Non-degranulating mast cell cell/mm²±SD
SVh	9	1.20±0.14	2.56±0.26	1.35±0.14	1.21±0.21
SSx	11	2.22±0.26*#	3.00±0.18	1.93±0.14	1.07±0.08#
SBB	10	0.51±0.07*	2.89±0.25	2.36±0.23*	0.53±0.05*

p<0.05 vs control; # p<0.05 vs SBB

Table 2. Left ventricular weigth index (g/100 g), end-diastolic and end-systolic dimensions (mm) in control (SVh), sympathectomized (SSx) and propranolol treated (SBB) rats. n = no. of rats.

Group	n	LV weight index (g/100 g body weight)	End-diastolic LV internal dimension (mm)	End-systolic LV internal dimension (mm)
SVh	9	1.92±0.05	5.70±0.13	2.00±0.14
SSx	11	2.03±0.07	6.65±0.22*#	2.95±0.29*
SBB	10	2.13±0.06	5.93±0.13	2.31±0.13

p<0.05 vs control; # p<0.05 vs SBB.

extracellular space.

Some regional differences were noticed in the localization of these cells within the LV sections. In particular, in sympathectomized rats mast cells were most often localized in perivascular and interstitial areas, with a co-localization with collagen deposition as highlighted by Sirius Red staining (Figure 1i, l). Interestingly, we observed a trend to an higher mast cell density in the external (epicardial) section of the left ventricular wall when compared to the internal (endocardial) section in sympathectomized rats (SSx) and even more so in propranolol-treated rats (Figure 3), although the difference between the 3 experimental groups was not statistically significant.

Mast cell density

In the LV of control rats (SVh) a lower number of mast cells (2.56±0.79 cell/mm²) was found in close proximity of blood vessels and in the pericardium, equally distributed in degranulating (1.35±0.14 cell/mm², 52.7%) and non-degranulating (1.21±0.21 cell/mm²) cells. In chronically sympathectomized rats there was a clear trend to an increase in total mast cell density (3.00±0.15 cell/mm²), although it fell short of statistical significance. Sympathectomy was associated with an higher density of degranulating mast cells (1.93±0.14 cell/mm²), accounting for 64% of the total mast cell count. A further significant increase in the number of activated mast cells was observed in chronically propranolol-treated animals, with a degranulating mast cell density reaching 2.36±0.23 cell/mm² (81.3% of the total mast cell count) (p<0.05 vs. vehicle-treated control rats). All data are summarized in Table 1.

Immunohistochemistry

All histological sections showed tryptase-positive mast cells, although their number was higher in the experimental groups subjected to chronic β-blockade or sympathectomy, indicating a larger extent of mast cell degranulation (Figure 1 m, n, o, p).

Collagen deposition

The Masson Trichrome staining showed differet patterns of interstitial collagen deposition in our samples as illustrated in Figure 1g,h. The semiquantitative analysis performed on LV Sirius Red stained sections (Figure 1 e,f) showed a significant increase in interstitial collagen deposition in sympathectomized animals (SSx) when compared to the control group (SVh): 2.22±0.26% versus 1.20±0.14% (p=0.04). In contrast, propranolol treatment was associated with a significant decrease in collagen deposition (0.51±0.07%) when compared to both the untreated (p= 0.002) and sympathectomized groups (p=0.02). All data are summarized in Table 1.

LV weight and internal dimensions

As shown in Table 2, LV weight index was similar in the 3 experimental groups, indicating that neither sympathectomy nor β-blockade were able to influence cardiac weight when compared to vehicle treatment. However, sympathectomy induced significant LV chamber dilation.

Discussion

The main finding of the present study is that in the normal myocardium β-blockade is associated with a large increase in the proportion of degranulating mast cells. A clear cut increase in degranulating mast cells is not necessarily the result of an increase in total mast cell density. Indeed, we did not find a substantial change in total mast cell number. This demonstrates that in the normal

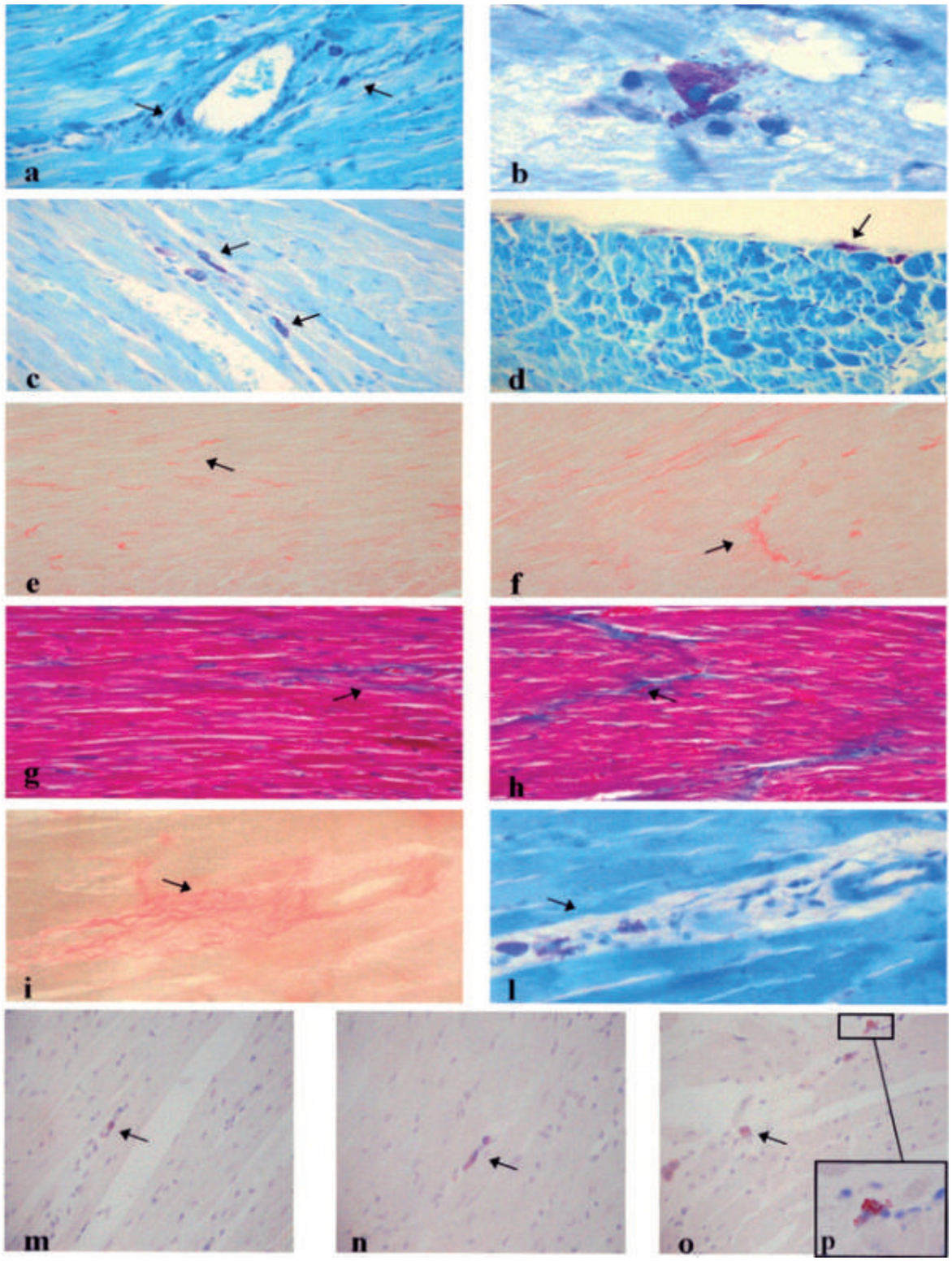
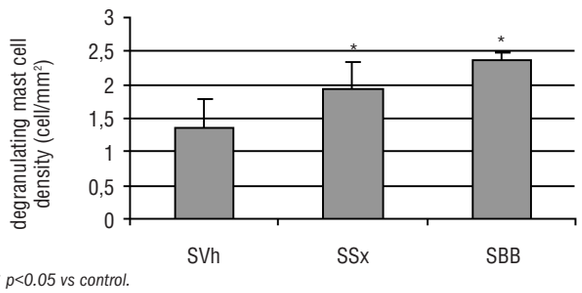
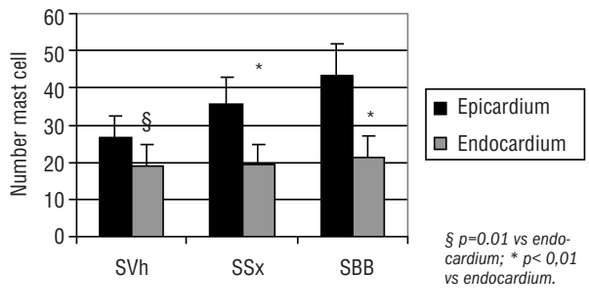


Figure 1. Photomicrographs of transversely sectioned left ventricle formalin fixed, paraffin embedded from chemically sympathectomized (a, b, e, g, i, l, n) and propranolol (c, d, f, h, o, p) treated rat. a, b, c, d: toluidine blue staining. Figure (b) shows an example of degranulating mast cell with numerous widespread metachromatic granules. Figure (d) represents the pericardium portion with numerous mast cells (arrows). Sirius Red (e, f) and Masson's trichrome (g, h) stained sections show an increase of interstitial collagen (arrows) in propranolol treated (f, h) than sympathectomized (e, g) animals. Sirius red (i) and toluidine blue (l) sequential stained sections show a colocalization between collagen/mast cells. Sections (m,n,o,p) immunostained with an anti-tryptase mAb show the mast cell degranulations. (a, c, d, e, f, i, l , m, n, o ; 400x; b, p:1000x).



** p<0.05 vs control.*

Figure 2. Group comparison of degranulating mast cell density (cell/mm²) in left ventricle of control (SVh), sympathectomized (SSx) and propranolol treated (SBB) rats.



*§ p=0.01 vs endocardium; * p< 0,01 vs endocardium.*

Figure 3. Comparison of number of mast cell in pericardium versus endocardium in left ventricle of control (SVh), sympathectomized (SSx) and propranolol treated (SBB) rats.

myocardium mast cell activation is modulated by the sympathetic nervous system, in particular via β-adrenergic receptor mediated mechanisms.

Marked changes in mast cell density and morphology have been observed in various chronic diseases in which autonomic nerve alterations are suspected given a large body of evidence in favour of a neuroimmune interaction involving mast cells and nervous system (Bergerot *et al.,* 2000).

Our study was focussed on the effects of sympathetic inhibition on mast cell degranulation and on myocardial fibrosis, since it is known that mast cell-derived mediators have been reported to stimulate fibroblast growth and collagen synthesis both in vitro and in vivo (Panizo *et al.,* 1995; Hara *et al.,* 1999). In particular, among the vast panel of mast cell products, histamine, tryptase and IL-4 were described to be mitogens and co-mitogens for human fibroblasts and to promote collagen synthesis in guinea pigs (Hatamochi A, *et al.,* 1985; Abe M *et al.,* 1998). Concerning the heart tissue, mast cells are the main source of histamine. Interestingly, it has been shown that both the total number of mast cells and the histamine content of the myocardium of patients with dilated cardiomyopathy are increased (Marone *et al.,* 1995, Patella *et al.,* 1998).

In the present work we reported that chemical sympathectomy increased degranulating cardiac mast cell density without significantly influencing the total number of mast cells. The enhanced mast cell activation in sympathectomized rats can be responsible of the significant interstitial collagen deposition, thereby demonstrating an important role of the cardiac mast cell-fibroblast interactions in rat myocardial tissue remodelling (Brower, 2002). However, we observed that propranolol caused a further increase in cardiac mast cell activation without any effect on interstitial fibrosis, that was indeed significantly inhibited.

Propranolol and other *first-generation* compounds, such as timolol, are non selective agents with equal affinities for blocking β1 and β2 receptors (Bristow, 2000). Two recent studies by Long & Brown (Long and Brown, 2002) and Johnson (Johnson, 2002) have reported that β2-adrenergic receptors are present on inflammatory cells, such as mast cells and monocytes, and β2-agonists inhibit the release of histamine (Barnes, 2002). Moreover, it has been recently shown that chronic treatment with formeterol or salmeterol induces β2-receptor down desensitization (Scola, 2004). On the other hand it is well known that β-adrenoreceptor antagonist propranolol promotes mast cell degranulation in the lung (Chong *et al.,* 1998). These data underscore the complex interplay between mast cells and β-adrenoceptor mediated pathways.

In our study, chronic propranolol administration caused a significant increase in the fraction of degranulating cardiac mast cells: 81.3% of the total mast cell population versus 52.7% in the control group and 64% in symphatectomized animals. Thus our findings support the hypothesis that chronic propranolol administration increases the fraction of activated cardiac mast cells, concomitant with a decrease in the concentration of interstitial collagen that we observed in treated (0.51%) rats compared to the control group (1.20%). It is therefore very likely that the increased fibrosis observed in sympathectomized rats was indeed caused by the local effects of the cardiac neurotoxin 6-hydroxydopamine.

To our knowledge, these data demonstrate for the

first time that cardiac mast cell degranulation is strongly influenced by β-adrenergic system and that mast cell-derived vasoactive peptides, such as histamine and cytokines, may play an important role in extracellular matrix remodelling. This is in line with the evidence that several different neurotransmitters can affect mast cell degranulation, although the effects of these agents on the activation state of mast cells has not yet been described (Nechushtan and Razin, 1996). In addition, it has been reported that cardiac mast cell degranulation is mediated by endothelin-1 and potentially involved to myocardial remodelling (Murray *et al.,* 2004).

Moreover, these findings support the evidence that the wide portfolio of cytokines, proteases and lipidic mediators that are released by the mast cells can actively contribute to several biological events, in health as well as in disease conditions.

In our experiments, sympathetic nerve abrogation and β -adrenoceptor blockade had completely different effects: the former was associated with both interstitial fibrosis and a non-significant trend toward an increase in the degranulating vs. non-degranulating mast cell ratio, whereas the latter was associated with significant changes in mast cell degranulation. It has to be considered, however, that chemical sympathectomy (SSx) causes a substantial decrease in norepinephine plasma concentration, while sparing adrenal epinephrine production (Ferrari *et al.,* 1996), that may stimulate both α and β-adrenoreceptors. In contrast, β-adrenergic blockade (SBB) does not change catecholamine production without interfering at all with the alpha receptor. Our data on propranolol-treated rats suggest that β -adrenergic stimulation may inhibit (or at least modulate) mast cell degranulation. Indeed it has been demonstreted that isoproterenol treatment inhibits mast cell degranulation in tracheal tissue preparations (Brown *et al.,* 1982). We may therefore hypothesize that either such an inhibitory effect is also mediated by non-neural (i.e. adrenal) derived epinephrine (since in SSx animals degranulating mast cells are comparable to vehicle treated animals) or that other (non-β) receptors positively stimulate mast cell degranulation, as evident after blockade of the inhibitory β receptor. Alternatively, it may be hypothesized that the effects of sympathetic nervous system stimulation are at least in part mediated by non-alpha/non-beta adrenoceptor mechanisms (i.e. neuropeptides released by sympathetic nerve terminals that may activate cardiac mast cells).

In conclusion, the present study demonstrated a correlation between sympathetic nerve traffic, activation of cardiac mast cells and extracellular matrix turnover. The issue is particularly intriguing in the absence of known stimuli to cardiac fibrosis, such as pressure-overload and/or activation of the renin-angiotensin-aldosterone axis, and might clarify some potentially new aspect of the pathophysiology of the balance between collagen deposition and degradation. Further studies are needed in order to clarify the precise mechanism(s) of these interrelationships, in both normal and in pathological conditions.

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