

A novel technique for isolating functional mast cells from the heart

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Abstract. *Objective and Design:* The purpose of this study was to determine the feasibility of adapting peritoneal and pleural mast cell isolation techniques to recover cardiac mast cells that retain their functional response to the secretagogue, compound 48/80.

Methods: Using a novel protocol in rats, viable epicardial mast cells were recovered by aspiration of HBSS injected into the pericardial space. Functionality of these cells was determined by ELISA quantification of histamine release in response to compound 48/80, calcium ionophore A23187 and substance P. Mast cell phenotype was determined based on the presence of chymase and tryptase demonstrated by immunofluorescence, alcian blue-safranin staining, and Western blotting.

Results: Mast cells isolated in this manner have low basal rates of histamine release and are highly responsive to these secretagogues. These epicardial mast cells were of the connective tissue type, which is consistent with previous reports characterizing cardiac mast cells isolated from the heart by enzymatic dispersion techniques.

Conclusions: This novel pericardial aspiration technique facilitates the straightforward characterization of isolated epicardial mast cell functionality in a controlled *in vitro* environment, furthering our understanding of their contribution to myocardial disease.

Keywords: Mast cell – Histamine – Compound 48/80 – Heart

Introduction

It is well established that cardiac mast cells play an important role in the pathological remodeling of the myocardium in cardiac disease [1–17], making it imperative to develop an understanding of their function. Our laboratory has dem-

onstrated that cardiac mast cells are responsive to specific secretagogues (i.e., compound 48/80 & endothelin) in the intact heart [1, 3, 4, 18]. Conventional techniques for cardiac mast cell isolation involve enzymatic digestion of the myocardium using bacterial collagenase [19–24]. However, we recently demonstrated that collagenase isolation of cardiac mast cells causes cellular degradation resulting in spontaneous histamine release, yielding mast cells that are minimally responsive to secretagogues [20]. Therefore, a technique allowing for the isolation of cardiac mast cells which remain functionally responsive to secretagogues would provide a powerful tool to fully elucidate the role of mast cells in cardiac remodeling.

The isolation of mast cells from the peritoneal or pleural cavities is accomplished using injected buffers and mechanical dispersion, thereby avoiding the problems associated with enzymatic digestion and yielding fully functional cells. Accordingly, the purpose of this study was to determine the feasibility of using an isolation protocol based upon peritoneal and pleural mast cell isolation techniques to recover cardiac mast cells from the pericardial space. Further, we hypothesized that an isolation protocol not requiring the use of enzymatic dispersion would yield greater numbers of mast cells, improve purity, and retain cell functionality; all of which are essential for a more complete characterization of cardiac mast cells.

Materials and Methods

Animal Model

All experiments were performed using 8 week old, adult male Sprague Dawley (Hsd:SD) rats housed under standard environmental conditions and maintained on commercial rat chow and tap water *ad libitum*. These studies conformed to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the University's Institutional Animal Care and Use Committee. Rats from which mast cells were isolated were deeply anesthetized with sodium pentobarbital (50mg/kg) administered by intraperitoneal injection.

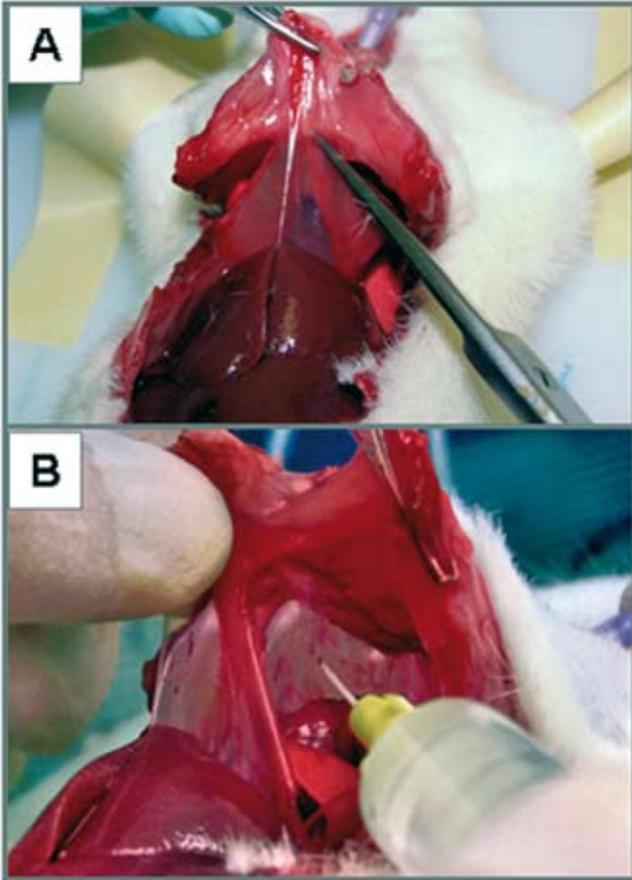


Fig. 1. A) Depicts the bilateral incisions allowing elevation of the chest wall and the dissection of the diaphragm to gain access to the pericardium. B) Depicts the chest wall reflected medially and insertion of the catheter into the pericardial sac. (Note: the sternopericardial ligament was rendered opaque to improve visualization.)

Isolation of Cardiac Mast Cells

Anesthetized rats were secured on a board inclined at an angle of approximately 30° (head raised) from the benchtop. A tracheotomy was performed and the rat ventilated for the duration of the procedure. Following this, a ventral midline incision was made in the abdomen and extended to the level of the xiphoid cartilage, taking care not to cut the diaphragm. Starting at the caudal aspect of the sternum, bilateral thoracic incisions were made beginning approximately 2-3 cm lateral to the sternum, piercing the diaphragm, and continuing cranially toward the axilla; being careful not to perforate the pericardium. In the next step it is important to avoid cutting the diaphragmatic pericardial attachment. The falciform ligament (located on the abdominal side of the diaphragm) can be used as a land mark indicating the attachment point of the pericardium on the thoracic side of the diaphragm. After completing the bilateral thoracic incisions, the remaining diaphragm was cut laterally to medially along the ventral aspect of the thoracic wall leaving the falciform ligament intact (Figure 1A). The left ventral thoracic wall was then retracted medially to expose the heart still encapsulated by the pericardium. At this point, it can be observed that the pericardium wraps around both sides of the heart and converges to form a double layer of tissue connecting to the sternum, referred to as the sternopericardial ligament. The teflon tip (with needle removed) of a 24-gauge Baxter intravascular over the needle quick-cath® catheter (1.6 cm) attached to a 10 cc syringe was then inserted into the pericardium at a point in the middle

of the sternopericardial ligament (preferably more cranial than caudal, Figure 1B). Note that it is not unusual for the catheter to unintentionally pass through both layers of pericardium and the catheter tip be outside the pericardial space (analogous to passing through the back wall of the vessel in venipuncture). When this happened, application and/or subtraction of slight tension to the pericardium by gripping the falciform ligament while the catheter was slowly withdrawn induces separation of the two pericardial layers, thereby allowing the tip of catheter to be re-introduced into the pericardial space. Room temperature Hanks Buffer [HBSS composed of 1) Hanks calcium and magnesium free salt solution, 2) HEPES (13 mM), 3) 607 units/mL of deoxyribonuclease I (Sigma Chemical Co., St. Louis, MO) and 4) an antibiotic-antimycotic mixture of penicillin G sodium, streptomycin sulfate, and amphotericin B (Gibco-BRL, Life Technologies, Grand Island, NY); pH 7.4] was then gradually introduced into the pericardial sac, filling it to maximal capacity as determined by distention of the pericardial tissue to the point just prior to buffer leaking from the puncture site (approximately 3-3.5 mL). At this stage in the isolation, the beating of the heart provides gentle mechanical dispersion. After the HBSS was injected, the catheter was once again removed and the anterior end of the rat was manually inclined to achieve as upright of a position as possible, facilitating collection of the HBSS at the apex of the heart. Another quick-cath® catheter tip was then inserted into the pericardial sac either through the original entry hole or by a second puncture and the HBSS gently suctioned off into an empty syringe, taking care not to tear the pericardial sac. This process of buffer injection and aspiration was repeated two to three times. The extracted buffer was placed into tubes on ice pending centrifugation for 10 min at 200 x g (4°C). After centrifugation the supernatant was collected and frozen at -80°C for subsequent testing of spontaneous histamine release, while the cell pellet was reconstituted in 1 mL of Hyclone buffer [HBSS containing magnesium sulphate (1.1 mM), calcium chloride (1.3 mM), and phenol red (Hyclone, Logan, UT)]. To determine the number of mast cells obtained from the isolation process, an aliquot from each sample was stained with toluidine blue for 20 min before determining mast cell counts using a hemocytometer. Upon completion of the mast cell isolation process, the heart was removed and a transverse section of the mid-left ventricular myocardium was fixed in formalin for subsequent histological evaluation.

Mast Cell Functionality: Compound 48/80 Treatment

To determine mast cell functionality, the isolated cells were analyzed for histamine concentration and response to the secretagogues, compound 48/80, the calcium ionophore A23187 and substance P. Epicardial mast cell isolates were aliquoted so that approximately 4000 mast cells were contained in each tube. This number of cells was chosen in order to achieve histamine values within the standard curve of the assay. Each tube was then brought to a total volume of 500 µL with additional Hyclone buffer. These tubes were then centrifuged at 200 x g for 10 min and 200 µL of the supernatant removed in order to measure spontaneous histamine release prior to treatment. The pellets were resuspended in the remaining 300 µL of buffer by gentle pipetting. The samples were situated in a shaking water bath and 200 µL of Hyclone buffer containing compound 48/80 (Sigma Chemical Co., St. Louis, MO) were added to each sample to achieve concentrations of 0, 0.3, 1, 3, 10, 30 and 300 µg/mL and incubated at 37 °C for 20 min at 60 rotations per min. Additional samples were incubated with the calcium ionophore A23187 (10 µg/mL) and substance P (10 µM) using previously reported concentrations of these compounds [19, 22]. The samples were then placed on ice for 10 min to stop the reaction and after cooling were centrifuged (4 °C, 5 min, 660 x g) and the post-treatment supernatant removed and stored at -80 °C for measurement of histamine release in response to compound 48/80. The remaining pellets were resuspended in 500 µL of Hyclone buffer and sonicated twice at 10 seconds per sonication to release all of the cellular contents in order to determine the amount of unreleased histamine remaining within the mast cells. All measures of histamine concentration were performed using a Neogen® Veratox Histamine ELISA (Lexington, KY), in accordance with Neogen's protocol manual and analysis on a microplate reader using a double wavelength of 620 nm and 450 nm.

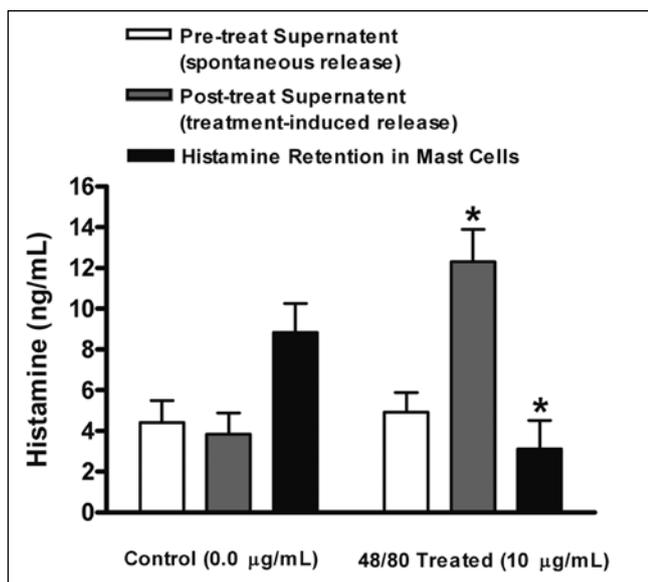


Fig. 2. Depiction of basal and compound 48/80 (10 µg/ml) induced histamine release from epicardial mast cells. * $p \leq 0.02$ versus corresponding control.

Histology: Mast Cell Staining

Left ventricular tissues were fixed in 10% buffered formalin and subsequently processed for routine histology, with paraffin embedded 5 µm cross-sections from each heart stained with the mast cell specific stain, pinacynol-erythrosynate [25]. The number of mast cells present on the epicardial surface of the ventricular cross-section were determined in hearts that had undergone the isolation procedure, as well as in control hearts that had not, in order to determine if the isolated population of mast cells originated from the epicardial surface of these hearts.

Histology: Mast Cell Phenotype Characterization

Untreated aliquots from the original mast cell isolation were used to confirm whether epicardial mast cells were of the mucosal- or connective tissue-type. Slides were prepared using 100 µL of the isolation sample, diluted with 900 µL of 4% paraformaldehyde in PBS and spun onto a glass slide for 10 min at 600 rpm using a cyto-spin centrifuge (Cytotech, Sakura). To characterize the phenotype, the isolated cells were stained with alcian blue and counterstained with safranin (Sigma Chemical Co., St. Louis, MO) and compared to comparably stained cardiac mast cells obtained by enzymatic digestion as previously described [20]. Additional slides were blocked in 1.5% BSA at room temperature for 30 min and incubated at 4°C overnight with primary antibodies directed against chymase (Mast Cell Protease 1, goat anti-rat polyclonal antibody) or tryptase (Mast Cell Tryptase rabbit anti-rat polyclonal antibody), both at a 1:100 dilution (Santa Cruz BioTech., CA). Omission of primary antibodies and staining with an irrelevant IgG of the same isotype served as the negative controls. After the primary antibody incubation, slides were washed with PBS and incubated in a 1:1,000 dilution of donkey anti-goat or goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 or 594, respectively (Invitrogen) for one hour before being imaged using a confocal laser scanning microscope at a magnification of 400X.

Additionally, the cellular content of tryptase and chymase was demonstrated by Western blotting. Cytosolic protein samples, together with the MagicMark XP molecular ladder (Invitrogen), were run on a 10% Tris-HCL gel and then transferred, at 4°C, to a nitrocellulose blotting membrane overnight. Blots were then probed with mast cell tryptase and chymase antibodies (Santa Cruz BioTech., CA) and imaged on radiographic film.

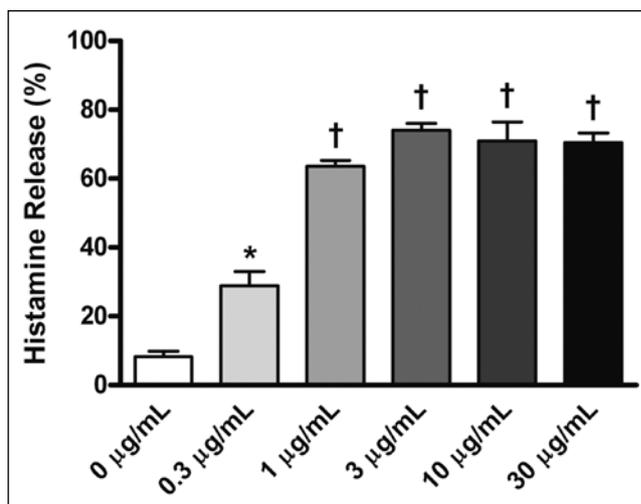


Fig. 3. Percent of total histamine release induced by incubation of epicardial mast cells with increasing concentrations of compound 48/80. * $p \leq 0.05$ versus control. † $p \leq 0.001$ versus control.

Statistics

All data are expressed as mean \pm SEM. Grouped data comparisons were made by one-way analysis of variance (ANOVA) using SPSS 11.5 software (SPSS Inc., Chicago, IL). When a significant F test ($p \leq 0.05$) was obtained, intergroup comparisons were analyzed using Bonferonni post-hoc testing, with differences considered to be significant at $p \leq 0.05$.

Results

Mast Cell Counts: Isolated and Left Ventricular Tissue

Mast cell counts were carried out on toluidine blue-stained aliquots of the mast cell preparations immediately following the isolation process. On average, each isolation yields $113,080 \pm 5,872$ mast cells. In order to determine if the population of mast cells thus isolated originated from the epicardial surface of these hearts, the number of mast cells on the epicardial surface was determined. Histologic evaluation demonstrated a 40% decrease in the number of mast cells on the epicardial surface (i.e., left ventricular free wall) of hearts that underwent the isolation process relative to normal control hearts (18 ± 2 mast cells, $n = 14$ and 30 ± 4 mast cells, $n = 9$, respectively; $p < 0.01$).

Mast Cell Functionality

Histamine release in response to the secretagogue, compound 48/80, was analyzed in order to assess the functionality of epicardial mast cells following the isolation process. There was a relatively small basal release of histamine present in the pre- and post-treatment supernatants (Figure 2). However, incubation with compound 48/80 induced a dose-dependent release of histamine (Figures 2 and 3), reaching a maximal 73% at concentrations as low as 3 µg/mL of compound 48/80, substantially greater than the basal release seen in the untreated

Table 1. Comparison Of Histamine Release Induced By Different Secretagogues.

Secretagogue	Epicardial Isolation (% histamine release)	Enzymatic Isolation (% histamine release)
Compound 48/80 (10 µg/mL)	71.0 ± 5.5	1.7 ± 0.4 [22] ~2.0 [31] 1.4 ± 0.2 [19]
A23187 (10 µg/mL)	59.2 ± 3.3	30.1 ± 2.7 [22] 14.0 ± 2.0 [19]
Substance P (10 µM)	15.8 ± 2.3	0 [22]

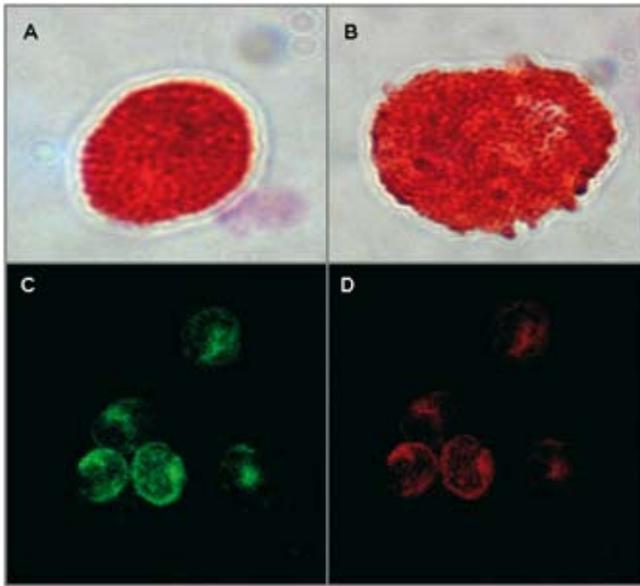


Fig. 4. Connective tissue-type morphology of rat cardiac mast cells stained with alcian blue and safranin isolated by A) enzymatic dispersion or B) pericardial aspiration methodologies. Magnification, 1000X. Representative immunostaining of epicardial mast cells isolated by pericardial aspiration with antibody directed against C) chymase and D) tryptase. Magnification, 400X.

controls. The histamine release induced in response to treatment with compound 48/80 at the 300 µg/mL concentration was comparable to that achieved at the 3 µg/mL dosage (data not shown). The calcium ionophore A23187 also induced a substantial release of histamine ($p < 0.001$ vs. untreated control). Although not as substantial, the 15.8% release of histamine induced by substance P was significantly greater than control ($p < 0.05$ vs. untreated control). Table 1 compares histamine release induced by secretagogues using our new method of isolation and the traditional enzymatic approach.

The residual histamine content remaining within the mast cells following compound 48/80 stimulation was measured after sonication of the cell pellet. As can be seen in Figure 2, compound 48/80 mediated degranulation significantly reduced the amount of residual histamine remaining in the mast cells as compared to untreated control cells. The significant reduction in residual histamine remaining in the mast cells after treatment with compound 48/80 at the 1, 3, 30 and 300 µg/mL concentrations was comparable to the maximal response that was achieved with the 10 µg/mL dose (data not shown).

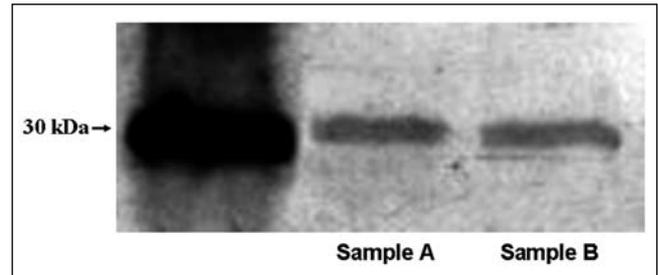


Fig. 5. Representative Western blot demonstrating chymase in the cytosolic fraction of epicardial mast cells isolated by pericardial aspiration.

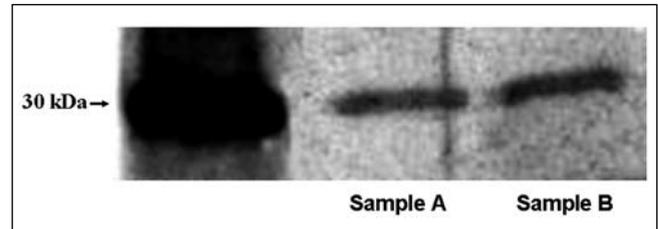


Fig. 6. Representative Western blot demonstrating tryptase in the cytosolic fraction of epicardial mast cells isolated by pericardial aspiration.

Mast Cell Characterization

The isolated epicardial mast cells were confirmed to be of the connective tissue-type classification based upon their characteristic red-orange coloration after alcian blue-safranin staining and positive immunofluorescent labeling for both tryptase and chymase (Figure 4). Western blotting for mast cell tryptase identified a band corresponding to approximately 30 kDa as depicted in Figure 5. Separate Western blots probed for mast cell protease I (chymase) identified a band corresponding to approximately 30 kDa shown in Figure 6. These are consistent with previously identified molecular weights for isoforms of mast cell tryptase and chymase [26, 27].

Discussion

Previously described techniques for isolation of cardiac mast cells have required enzymatic degradation of myocar-

dial tissue [19–24]. Based upon results obtained from those studies, the supposition has been put forward that cardiac mast cells are not responsive to a number of secretagogues. However, we knew from previous studies in the intact heart that cardiac mast cells respond robustly to specific secretagogues (i.e., compound 48/80 & endothelin) [1, 3, 4, 18, 20]. Thus, the original concept for this new isolation technique stemmed from our frustration in working with enzymatically obtained mast cells and not being able to reproduce our *ex vivo* findings [20]. This difference appears to be attributable to cellular damage incurred during the collagenase incubation, with enzymatic digestion resulting in spontaneous release of large amounts of histamine during the isolation process.

In contrast to enzymatic methods for cardiac mast cell isolation, the well established methodology for obtaining pleural or peritoneal mast cells is straightforward, and because enzymatic dispersion is not required, these mast cells are not damaged and remain functionally responsive to secretagogues. This new approach for isolating epicardial mast cells described herein avoids the use of enzymes by introducing the HBSS culture media directly into the pericardial sac. A large number of mast cells present in the myocardium (i.e., 25–30%) are localized on the epicardial surface of the heart (unpublished data). Therefore, by using the pericardial sac as a structure analogous to the peritoneal cavity, we have developed a protocol that consistently produces a high yield of functional epicardial mast cells. Importantly, as can be seen in Figure 4, the mast cells thus obtained are phenotypically consistent with the tryptase and chymase positive MC_{TC} subset of connective tissue mast cells previously described in myocardial tissue [20, 22, 24, 28, 29].

Avoiding the enzymatic dispersion of tissue minimizes spontaneous histamine release attributable to cellular degradation and does not alter the functional responsiveness of epicardial mast cells, as evidenced by the significant histamine release triggered by treatment with concentrations of compound 48/80 as small as 0.3 µg/ml (Figure 3). These findings are in stark contrast to our results obtained using cardiac mast cells isolated by enzymatic methods, in which 10 µg/ml of compound 48/80 elicited the release of less than 2% of the histamine from cells [20]. As can be seen in Table 1, other studies using enzymatically isolated cardiac mast cells have also reported negligible response to compound 48/80 (i.e., <2% histamine release), even after incubating the cells overnight to allow for recovery [19, 22]. This is also true of the response to the calcium ionophore and substance P. However, in contrast to the existing literature indicating that cardiac mast cells do not respond to substance P [22, 30], the current findings indicate the neuropeptide substance P can trigger a significant response in epicardial mast cells. Accordingly, the heterogeneity of cardiac mast cells from mast cells of other tissues alluded to in the literature may to some extent be an artifact of the enzymatic methodology that was used to obtain the cells. Nevertheless, the supposition of heterogeneity of connective tissue mast cells is reinforced by our recent study using this new technique which established that cardiac mast cells do not degranulate in response to atrial natriuretic peptide [31], unlike peritoneal mast cells which we and others have shown

to be activated by atrial natriuretic peptide [32, 33]. Thus, the utility of epicardial mast cell isolates is readily apparent, and the response of cardiac mast cells to secretagogues must be re-examined given these new observations.

In addition to retaining cellular function, the pericardial isolation technique consistently produces a two fold greater recovery of mast cells compared to previous observations using enzymatic mast cell isolation [20]. Along with the observed overall increase in mast cell yield, the cell preparation from the pericardial sac was found to be relatively pure in that no myocytes, fiber or epithelial cells were present. This allowed for the elimination of the density gradient centrifugation and multiple washes previously required to filter out remnants of digested cardiac tissue, and thus minimized the stress to which the isolated mast cells were subjected. Differential staining of the resulting isolate demonstrated a population of lymphocytes (~60%) and macrophages (~15%) in addition to mast cells (~25%). This already has substantially greater purity than the average of 12% attained after density gradient centrifugation reported by Patella et al. [22], and allows for examination of mast cell response to secretagogues under controlled conditions. However, we are currently working on methods to further purify these samples to allow measurement of mast cell derived secretory products other than histamine.

Based on problems identified during our initial attempts to isolate mast cells from the epicardial region, a number of improvements to refine the isolation technique have been incorporated. To make the protocol viable, a practical method was required to provide access to the pericardium while simultaneously keeping it intact. The teflon tip of the Baxter Quick-cath[®] proved to be strong enough to puncture the pericardium, but soft and flexible enough not to tear the pericardial sac or damage the heart. Increasing the angle of elevation by raising the rat's head increased the amount of injected buffer that could be aspirated from the pericardial sac (as the buffer drained more efficiently to the caudal aspect of the pericardial sac producing a pooling effect). This adjustment led to a marked increase in the number of mast cells isolated from each heart, as well as increasing the consistency of mast cell numbers obtained from each isolation.

In summary, we have described the development of a novel technique for isolating viable epicardial mast cells from the pericardial sac which respond to stimulation with compound 48/80 in a comparable manner to that reported in the intact heart [1, 3, 4, 18]. The need to more extensively characterize the functional role of mast cells in the heart is essential to furthering our understanding of their contribution to myocardial disease. Accordingly, this novel technique for the isolation of functional epicardial mast cells presents an outstanding opportunity by which to characterize and study cardiac mast cells under controlled *in vitro* conditions. This technique is especially timely given recent implication of mast cells in the chronic inflammatory processes [34].

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