Identification and localization of adrenomedullin-storing cardiac mast cells

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Received December 6, 2005; Accepted January 5, 2006

Abstract. Adrenomedullin (AM), a potent vasodilatory hypotensive peptide, is expressed in the heart, where it is known to play a protective action. Light-microscopy immunocytochemistry (ICC) demonstrated the presence of AM immunoreactivity not only in the coronary-vessel wall and ventricular myocytes of the human and rat heart, but also in sparse voluminous cells located in the perivascular space. These cells displayed the same location of toluidine blue-positive mast cells, and electron microscopy ICC showed AM-immunogold staining over the granules of rat cardiac mast cells. The incubation of rat left ventricle fragments with the mast-cell histamine releaser compound 48/80 evidenced groups of AM-positive cells undergoing degranulation and caused an increase of approximately 50% in the AM concentration in the incubation medium. Collectively, our findings provide evidence that at least a subset of cardiac mast cells are able to synthesize and store AM, and upon stimulation to release it near coronary arterioles and venules.

Introduction

Adrenomedullin (AM) is a long-lasting vasodilatory hypotensive peptide, which is abundantly expressed in human and rat coronary vessels and cardiomyocytes (reviewed in refs. 1,2). In the course of immunocytochemical studies on the effect of endothelin-1 (ET-1) on AM content and distribution in the rat heart (3,4), we observed the presence of sporadic voluminous intensely AM-positive cells prevalently located in the perivascular space. Although the bulk of evidence indicates that AM is secreted constitutively from the cells of cardiovascular system, this finding was not surprising because other tissues have been reported to possess AM-storing cells: e.g. F cells of pancreatic islets, diffuse endocrine cells of the rat stomach, apocrine cells of the mammary gland and type I glomus cells of the rat carotic body (1,5,6).

Subsequent studies showed that these AM-positive cells stain with toluidine blue, and undergo a decrease in number is response not only to ET-1, but also to the mast cell degranulator compound 48/80 (7). Based on these findings and on their prevalent location near blood-vessel walls, the hypothesis has been advanced that AM-storing cells are cardiac mast cells. However, this contention lacks definitive proof. Hence, we carried out additional light and electron microscopic immunocytochemical studies and functional experiments to address this issue.

Materials and methods

Tissue and reagents. Adult male Sprague-Dawley rats (140-160 g body weight) were purchased from Charles-River (Como, Italy). Rats were decapitated, and their heart promptly removed. Bioptic fragments of the heart interventricular septum were obtained from patients undergoing periodic control after heart transplantation. The study protocol was approved by the local Ethics Committees for Human and Animal Studies. Human and rat AM, and rabbit anti-rat AM1-50 and anti-human AM1-52 polyclonal antibodies were obtained from Phoenix Pharmaceuticals (Belmont, CA). Secondary goat anti-rabbit IgG peroxidase-conjugated antibodies were provided by Dako (Glostrup, Denmark) or Chemicon International (Temecula, CA), and secondary goat anti-rabbit IgG 20-nm immunogold-conjugated antibodies by British Biocell International (Cardiff, UK). Sigma Fast 3',3'-diaminobenzidine 0.7 mg tablets (DAB tablet set), compound 48/80, phosphate-buffered saline (PBS) and all other laboratory reagents were puchased from Sigma-Aldrich Corp. (St. Louis, MO).

Light microscopy. Left ventricle wall of the heart of 8 rats was chopped into 1-mm^3 fragments, which were put in Krebs-Ringer bicarbonate buffer (pH 7.4) and incubated for 30 min in the presence or absence of compound 48/80 (50 μg/ml). The incubation was carried out at 37˚C in an atmosphere of

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Key words: adrenomedullin, mast cells, heart, human, rat

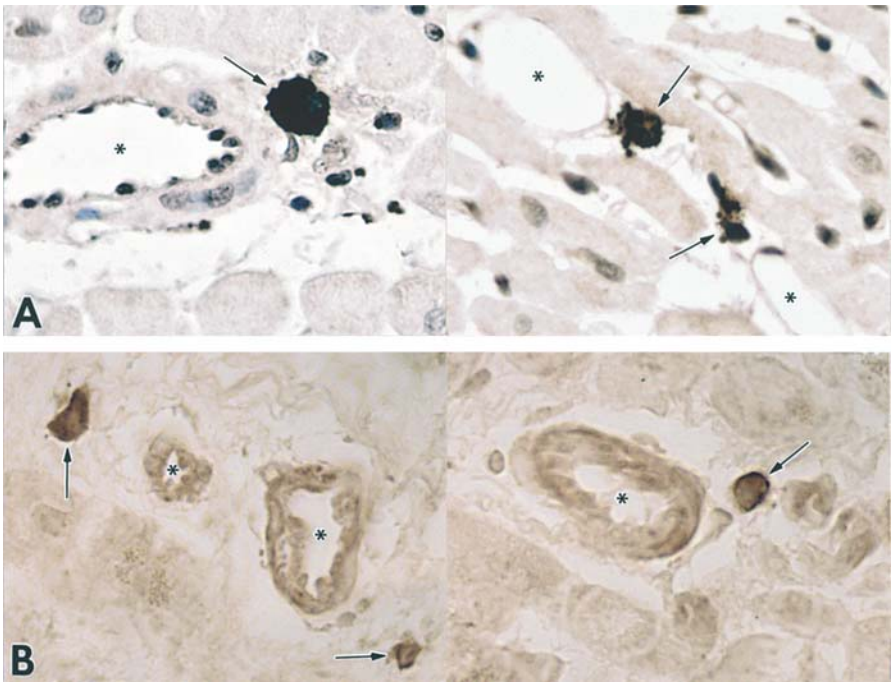

Figure 1. Six-μm thick paraffin sections of the rat (A) and human heart (B), showing AM-storing (DAB-positive) cells (arrowheads) located near coronary venules and arterioles (*), respectively. Moderate DAB positivity is also displayed by cardiomyocytes and arteriole walls (B). Magnification x500.

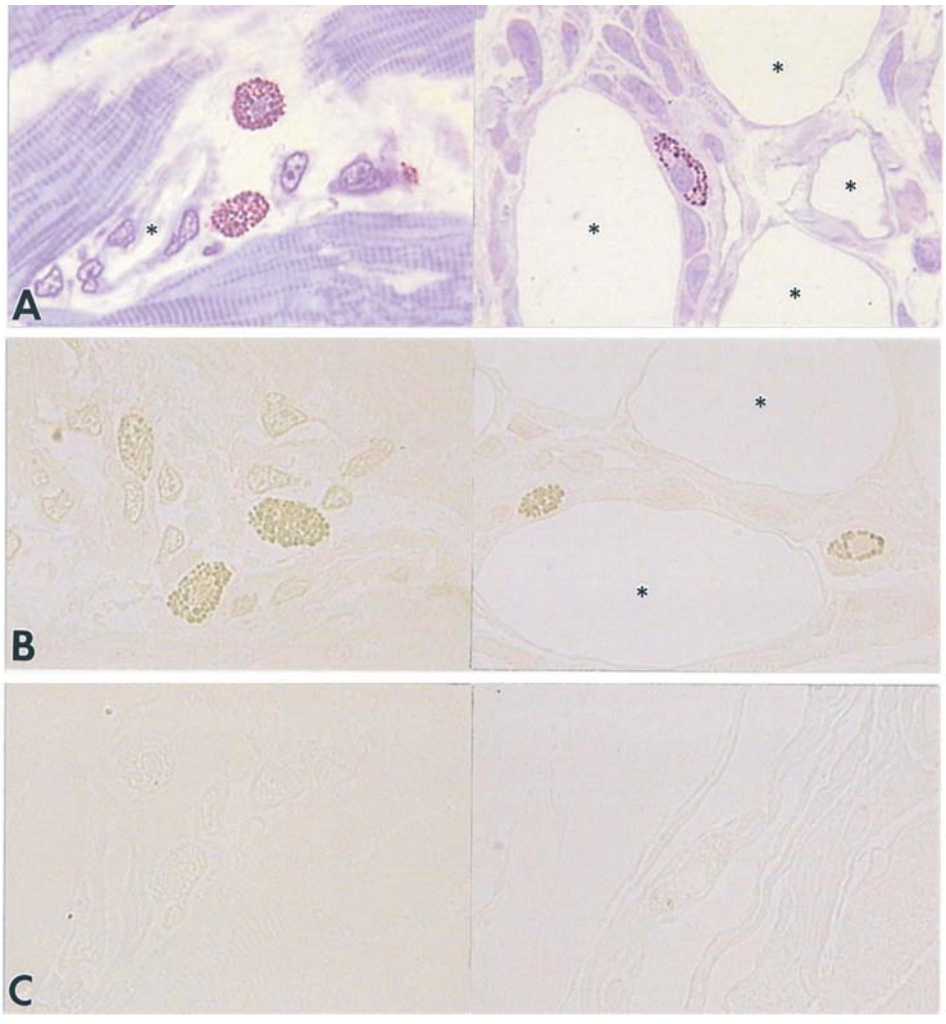

Figure 2. Two-μm thick plastic sections of the rat heart, showing that toluidine blue-stained (A) and AM-immunostained cells (B) have the same location in the perivascular space near the coronary vessels (*). Negative control carried out by omission of the primary antibody (C). Magnification x1,000.

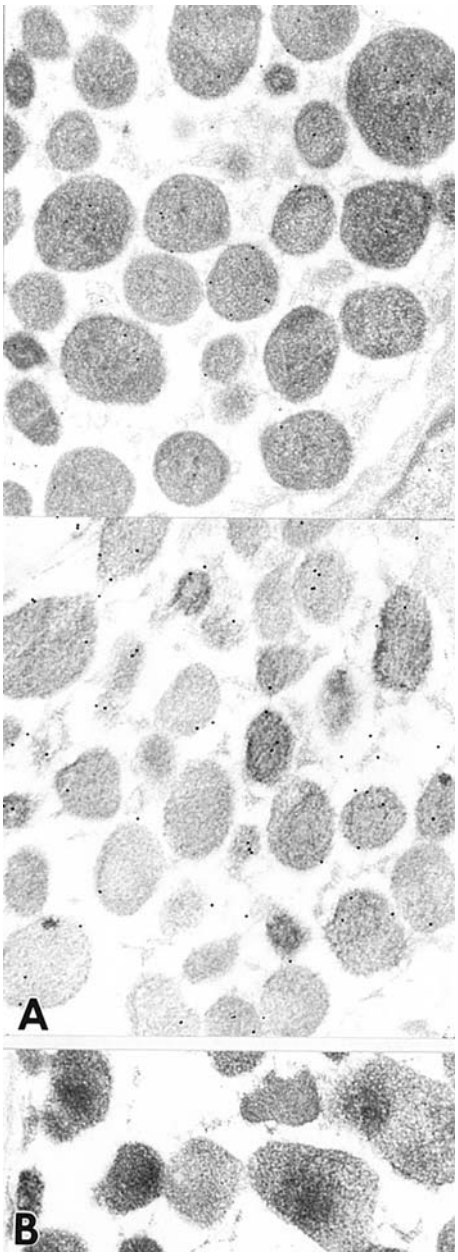

Figure 3. Electron micrograph of a mast cell of the rat heart, showing the almost exclusive granular location of AM-positive material, as evidenced by the 20-nm immunogold particles (A). Negative control carried out using primary antibody preabsorbed with AM excess (B). Magnification x25,500.

95% air-5% CO_2, then fragments were weighed and incubation medium was collected and stored at -80˚C until AM RIA. Rat heart and human heart biopsy fragments were fixed in 4% paraformaldehyde in PBS for 5 h, and then embedded in paraffin. Six-μm thick sections were cut, and stored for immunocytochemistry (ICC).

Electron microscopy. Left ventricle fragments of other rats were fixed overnight in 4% paraformaldehyde-0.2% glutaraldehyde in 0.1 M phosphate buffer, dehydrated, and then embedded in the acrylic resin L.R. White (London Resin Company, London, UK). Sections (2-μm) were cut with a Reichert-Jung SuperNova ultramicrotome, stained with toluidine blue and observed with a light microscope to recognize mast cells (8). Adjacent 60-nm ultrathin sections were then cut and collected on 400-mesh nickel grids. Sections were stored until ICC.

Immunocytochemistry. Paraffin (6-μm) and plastic (2-μm) sections were exposed to 3% hydrogen peroxide for 5 min at room temperature to inhibit endogenous peroxidase, washed in PBS and then transferred to 0.1 M sodium citrate buffer at 90˚C for 5 min to unmask antigens. After cooling, section were washed in 0.1 M PBS, preincubated in 0.1 normal goat serum at room temperature for 20 min, and then incubated with the anti-AM primary antibodies (1:1000 dilution, paraffin sections; 1:100 dilution 2-μm sections) at 4˚C overnight. After repeated PBS washing, sections were incubated for 30 min at room temperature with the secondary antibody (1:40 dilution). Sections were rinsed, and the reaction developed for 5 min with Sigma DAB tablets, and stopped with water. Negative controls were carried out by similarly treating adjacent sections and either omitting the primary antibody or by using primary antibodies pre-absorbed with antigen excess (9). Specimens were observed and photographed with a camera-connected Leitz Laborlux microscope. Ultrathin sections were floated for 15 min at 90˚C on a drop of PBS to unmask antigens, then they were processed as detailed above, using anti-rat AM primary and immunogold-conjugated secondary antibodies (dilution 1:100). After rinsing and washing in PBS and bidistilled water, grids were counter-stained with 2% uranyl acetate for 2 min at 50˚C and then with Sato's lead for 30 sec at room temperature. Specimens were observed in a Hitachi H-300 transmission electron microscope.

Adrenomedullin assay. AM concentration was measured using the AM1-50 RIA kit purchased from Phoenix Pharmaceuticals. AM was extracted from incubation media, and purified on Sep-Pak C18 cartridge, following the manufacturer's protocol: sensitivity, about 5 fmol/ml; cross reactivity, rat AM, 100%, and human AM and other peptides, 0%; intra- and interassay CVs, 6% and 8%, respectively. Data were normalized per mg of incubated tissue fragments, and expressed as means ± SD (n=8). The statistical comparison was done by ANOVA followed by the Dunnet test.

Results

ICC carried out on paraffin sections showed the presence of sparse AM-positive cells near coronary vessels of both rat and human heart. AM immunostaining was also detected in the wall of coronary arterioles and in cardiomyocytes (Fig. 1). Using 2-μm sections, AM-storing cells had the same juxta-vascular location of toluidine blue-stained mast cells (Fig. 2). However, not all mast cells were AM positive. Electron microscopy demonstrated that anti-AM immunogold was located over the granules of rat cardiac mast cells (Fig. 3).

The incubation with the compound 48/80 decreased the number of both toluidine blue- and AM-positive cells in the rat left ventricle (data not shown), in keeping with our expectations (7). Sometimes, groups of AM-storing cells undergoing degranulation were observed: granules were less compactly arranged in the cytoplasm and AM positivity was

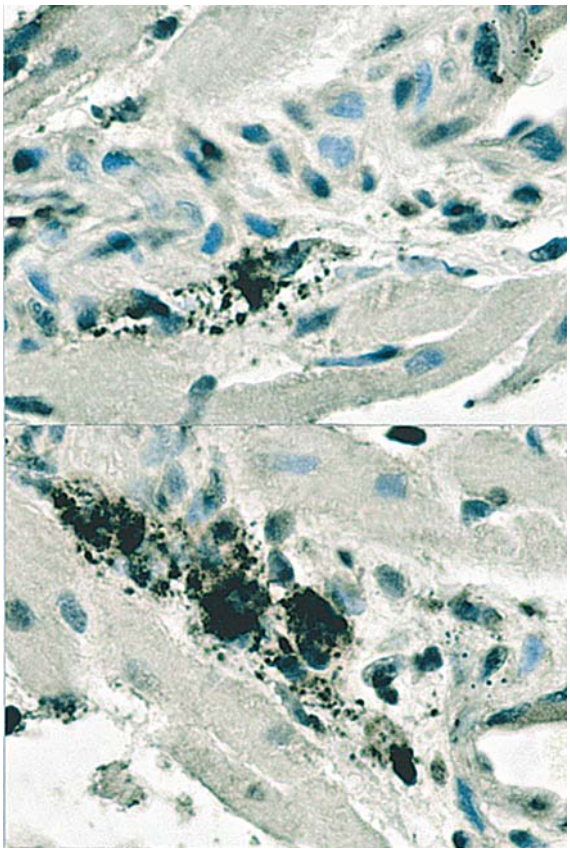

Figure 4. Paraffin sections (6-μm) of rat left ventricle fragments incubated for 30 min in Krebs-Ringer bicarbonate buffer not containing (upper panel) or containing 50 μg/ml compound 48/80 (lower panel), showing AM-storing (DAB-positive) cells located near coronary vessels (*). Compared with the controls (Fig. 1), AM-storing cells of compoud 48/80-treated fragments occasionally show a less compact arrangement of granules, some of which appear to be released in the perivascular space. Magnification x650.

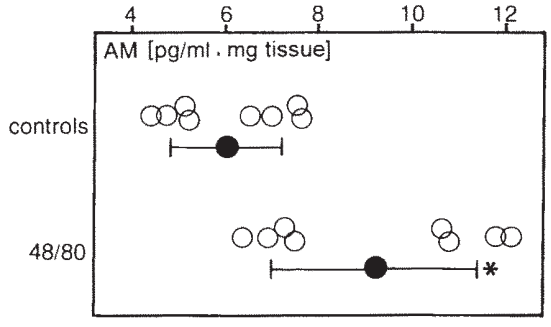

Figure 5. Effect of compound 48/80 on the AM concentration in the incubation media of rat left ventricle fragments. Solid circles indicate mean values ± SD. *P<0.01 from control group.

present in the perivascular extracellular space (Fig. 4). RIA showed that incubation with compound 48/80 raised by about 50% AM concentration in the incubation medium of rat left ventricle fragments (Fig. 5).

Discussion

Our present study confirms the presence of AM-storing cells in the rat heart (3,7), and extends this finding also to the human cardiac tissue. Moreover, it provides proof that these cells belong to a subpopulation of cardiac mast cells. In fact: i) AM-positive cells stain like mast cells with toluidine blue (8); ii) AM-positive material is contained in cardiac mast cell granules; and iii) compound 48/80, a specific histamine releaser and mast cell degranulator (10), induces degranulation of AM-storing cells and raises AM concentration in the incubation medium of rat heart fragments.

These findings are not totally unexpected, because the functional heterogeneity of mast cells is well recognized. Mast cells, in addition to storing and releasing histamine and heparin, are also able to synthesize growth factors (11) and other regulatory peptides, including ET-1 (12), CRH (13) and renin (14). Our results indicate that AM may be added to the list of peptides stored in mast cells, thereby confirming the earlier finding that rat peritoneal mast cells express the AM gene (15).

The functional relevance of our observations remains to be elucidated. However, we recall that AM exerts a major cardiac protective action, its expression being up-regulated in various heart diseases, including left ventricular hypertrophy, myocardial infarction and congestive heart failure (2,16). The beneficial effects of AM ensue from its NO-mediated coronary vasodilatory action (17,18), its ability to counteract cardiac fibrosis (19-21), and its relevant proangiogenic activity (22,23). Compelling evidence is available that mast cells are involved in the regulation of coronary vascular tone (24), myocardial remodeling (25,26), and angiogenesis (27-29). The perivascular location of these AM-storing mast cells and their previously shown capability to respond to vasoactive stimuli as ET-1 (7) are consistent with their important paracrine role in the regulation of coronary circulation under both physiological and pathophysiological conditions.

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