Electron microscopic analysis of the specific granule content of human atria

An investigation of the role of atrial pressure and atrial rhythm in the release of atrial natriuretic peptide

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Summary

Knowledge about the stimulus for the release of atrial natriuretic peptide (ANP) from human atria is incomplete. Atrial stretch is known to be a stimulus and atrial tachyarrhythmias are thought to be another. The effects of atrial size (by two-dimensional echocardiography) and atrial fibrillation on the atrial specific granule content of human atria were studied to gain insight into the secretory mechanisms of ANP. An electron microscopic analysis of the atrial granule content was used to study 12 patients — 5 with mitral stenosis and sinus rhythm, 3 with mitral stenosis and atrial fibrillation and 4 controls. Granules were counted using a free count and montage method.

This is the first report of such a morphometric analysis in humans. Granule counts were significantly raised in the patients with mitral stenosis compared with controls ($P < 0,014$). This observation probably reflects a high turnover state induced by elevated atrial pressures. Further support for this conclusion is provided by the demonstration of a positive correlation between granule counts and left atrial size ($r = 0,86$; $P < 0,01$). The tendency for higher counts in patients with atrial fibrillation may be related to the rhythm disturbance itself, but clinical and echocardiographic data suggest more severe atrial pressure overload in this group.

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A specific granule population found exclusively in atrial cardiocytes was first described by Kisch in 1956.[1] Recent immunocytochemical and biochemical studies have proved that, despite their morphological heterogeneity, all the granules store the same product, namely atrial natriuretic peptide (ANP).[2,3]

The concept that the heart is an endocrine organ[4,5] generated great interest and much has been discovered about the nature and effects of this natriuretic, diuretic and vasorelaxant peptide.[6] However, the mechanism of ANP release from the atria is poorly understood. The degree of stretch[7] of atrial cardiocytes is known to be a stimulus for the release of ANP from the atria, thereby determining their granularity.[2,8] Based on the clinical observation that diuresis often accompanies supraventricular tachyarrhythmias,[9] it is believed that ANP release is related to atrial rhythm. However, little detail is known about this relationship.

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Conditions such as mitral stenosis provide a chronic stimulus for increased release of ANP. The contribution of increased synthesis in providing the additional ANP necessary to maintain elevated serum levels when chronically stimulated has been investigated.[10,11] However, little is known about the contribution of changes in the content of intracellular stored ANP under these conditions. Morphometric analysis of the number of atrial-specific granules in the atria[12-14] can be used to quantitate the amount of ANP stored in the atria. In rats the morphological distribution of atrial-specific granules has been linked to intra-atrial pressures.[2,15,16] In these examples, high-density distribution of atrial-specific granules were associated with low atrial pressures and wall tension, implicating decreased release of stored ANP as the mechanism responsible for the high granule counts.

A study was undertaken to examine the effects of atrial size and atrial rhythm on atrial granularity. Atrial granularity was assessed by an electron microscopic morphometric study. Atrial size was measured by echocardiography. Patients with normal mitral valves and patients with mitral stenosis (in sinus rhythm or atrial fibrillation) were studied to gain insight into the mechanisms responsible for the release of ANP.

Patients and methods

Twelve patients undergoing cardiac surgery were studied (Table I). All patients were examined clinically and had ECG, chest radiography and echocardiography performed pre-operatively. The control group also underwent cardiac catheterisation. They were divided into three groups: group I — the control group consisted of 4 male patients aged 35 - 59 years with ischaemic heart disease; they were all in sinus rhythm, had normal mitral valves, were not in cardiac failure,

TABLE I. LEFT ATRIAL SIZES AND ATRIAL-SPECIFIC GRANULE COUNTS IN 12 PATIENTS

Group	Patient	LA/Ao	Granule count	
			Median	Mean ± SE
I	1	1	20	81 ± 69
	4	1,1	132	95 ± 40
	7	Not done	154	170 ± 24
	10	1,3	125	119 ± 11
II	2	1,9	242	201 ± 41
	5	1,65	197	191 ± 19
	8	1,6	146	163 ± 36
	11	1,9	225	250 ± 51
	12	1,9	230	211 ± 30
III	3	2,5	336	359 ± 54
	6	2,1	168	152 ± 23
	9	2,1	565	489 ± 114

and left ventricular end-diastolic pressures were not raised; group II — 5 female patients aged 19 - 35 years with mitral stenosis; they were all in sinus rhythm, had tight mitral stenosis and were classified as ⩾ class II (New York Heart Association); and group III — 3 female patients, aged 33 - 44 years with mitral stenosis and atrial fibrillation; they all had tight mitral stenosis and were classified as ⩾ class III (New York Heart Association).

Echocardiography was performed pre-operatively on all patients (excluding patient 7, Table I) (Hewlett-Packard system: HP77020AC — combined two-dimensional and M-mode investigation). Patients were examined in the left lateral decubitus position. Standard parasternal, apical and subcostal windows were used and the left atrial dimensions were determined on the parasternal long and short axis views.[17]

Clinical and echocardiographic assessment confirmed normal mitral valves in the control group and tight mitral stenoses in all patients in groups II and III. To compensate for variations in patient size, the left atrium to aortic root diameter ratio (LA/Ao)[17,18] was used as a parameter of left atrial pressure overload. The presence of sinus rhythm or atrial fibrillation was confirmed by ECG.

Left auricles were obtained during mitral valve and coronary artery bypass surgery and were immediately sent to the electron microscopy laboratory. Each auricle was carefully opened, fixed in 2,5% buffered glutaraldehyde for 24 hours and stored in phosphate buffer at 4°C. Using a dissection microscope, longitudinal fibres were isolated, and ten blocks of tissue from different areas in each patient were trimmed. The specimens were post-fixed with osmium tetroxide in veronal buffer, dehydrated and embedded in Spurr's resin. Semi-thin sections (1 μm) were cut and stained with toluidine blue for light microscopic examination. Sections from three blocks, containing only cells cut parallel to their long axis, were then selected for the final preparation of the thin sections (60 - 90 nm). These sections were stained with uranyl acetate and lead citrate and mounted on H-2 London 200 finder grids (Maxtaform grids by Graticules Ltd, UK).

The best quality single gridspace, containing the largest area of longitudinally orientated cardiocytes with nuclei, was selected from each of the final three grids (blocks). This was done at a magnification of × 1500 to eliminate bias in respect of the degree of granulation. Marked grids are essential for locating gridspaces accurately. A Hitachi H-600 electron microscope was used for viewing.

Two methods were used to analyse individual gridspaces, both covering the same surface area (11 000 μm^2).

1. **Montage method.** Sixteen serial electron microphotographs were taken, covering the entire gridspace except a small edge; 20 × 25 cm prints at a final magnification of 9 000 were made and assembled as a montage (Fig. 1). While photographing, it was essential to have the magnified gridspace square on the screen. All well-defined granules were counted (Fig. 2). Cell organelles, especially lipofuscin, were easily differentiated. A 4 × magnifier and transparent overlay with horizontal lines facilitated counting.

2. **Free counting method.** A free count of the specific granules was made directly from the electron microscope at a magnification of 10 000. Counted granules were plotted on a sketch of the individual myofibres to avoid multiple counting of individual granules.

Three gridspaces were analysed in this way for every patient, thereby covering a total surface area of 33 000 μm^2. To ensure representative sampling, the three gridspaces were taken from different blocks of tissue.

Statistical methods. Non-parametric tests were used because the samples were small.[19] Paired measurements were analysed using the non-parametric Wilcoxon signed-rank test and the non-parametric Mann-Whitney *U*-test was used to compare independent samples. Correlation was determined by Spearman's rank-order coefficient of correlation. Linear regression was obtained by the least-squares method.

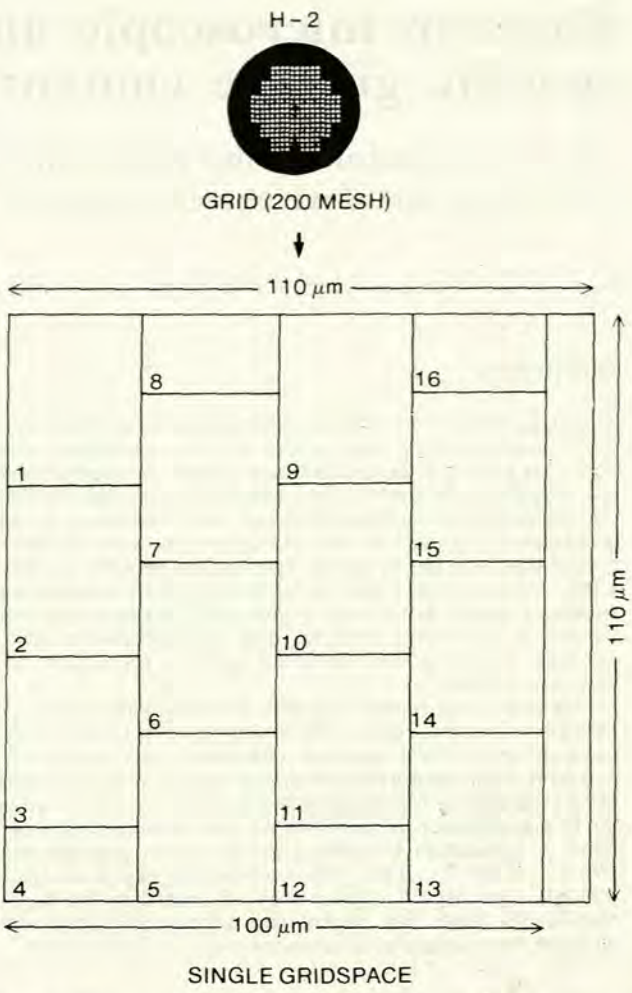

Fig. 1. Schematic diagram illustrating a grid and montage assembly of a single gridspace (area covered = 110 μm × 100 μm) using 16 prints, each at a final magnification of × 9 000.

Results

Comparing the two methods used for granule counting (free count — montage) in 8 patients (Table II) no statistical differences were found, indicating that both methods were reliable and had comparable results ($P > 0,1$; nonparametric Wilcoxon signed-rank test).

Three different granule counts were obtained for each of the 12 patients in this study. For the first 3 patients, only montages were used. Thereafter, free counts were done on each gridspace analysed. One of these in each patient (patients 5 - 12) was complemented by a montage (Table II).

The median values of the patients in each group (Table I) were compared using exact non-parametric Mann-Whitney *U*-tests. Granule counts in patients with mitral valve lesions (groups II and III) were significantly elevated compared with control (group I) ($P < 0,014$). The lowest values were found in group I with much higher values in group III (Fig. 3). This tendency was also demonstrated by the mean values (± SE) for the three groups: I = 116 ± 21; II = 203 ± 16; III = 333 ± 61. However, the intergroup differences were not statistically

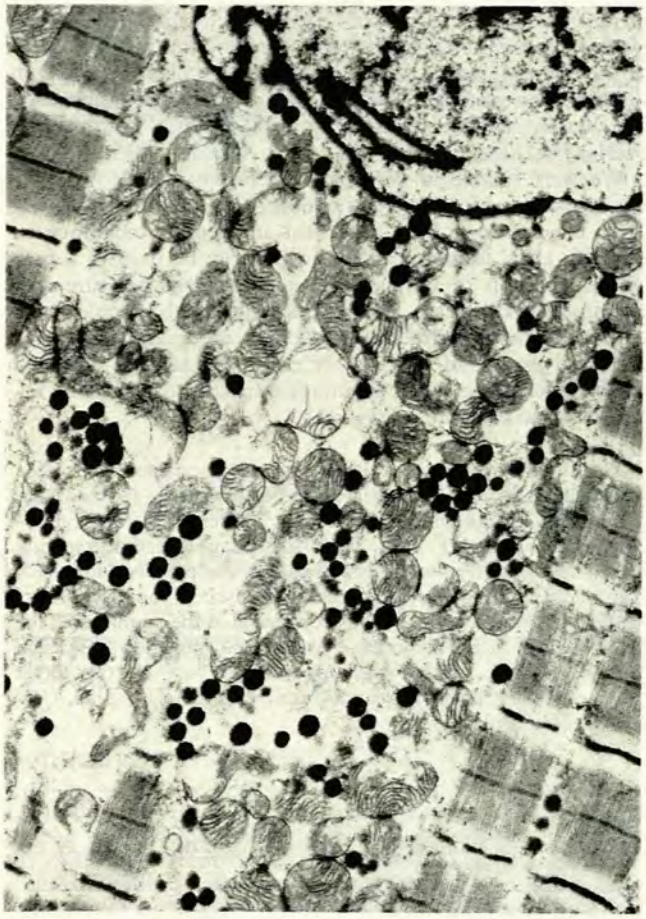

Fig. 2. Montage electron microphotograph demonstrating atrial-specific granules. The granules are membrane-bound with electron-dense cores and vary in diameter (100-450 nm). They are found between the myofibrils and are concentrated in greatest numbers in the paranuclear zones (× 9 000).

TABLE II. COMPARISON OF ATRIAL-SPECIFIC GRANULE COUNTS FROM IDENTICAL GRIDSPACES IN 8 PATIENTS USING THE MONTAGE AND FREE-COUNT METHODS

Patient	Montage	Free count
5	216	221
6	170	168
7	220	216
8	140	146
9	504	565
10	128	135
11	358	348
12	210	230

significant. Owing to the logistical problems involved in a study of this nature, the numbers of patients were limited and this must be considered when interpreting the data statistically.

The relationship between the granule counts and left atrial size was also investigated. The LA/Ao obtained pre-operatively by echocardiography was used as an index of left atrial pressure overload (Table I). There was a clear positive correlation between atrial size and granule counts (Fig. 4) ($r = 0,86$; $P < 0,01$).

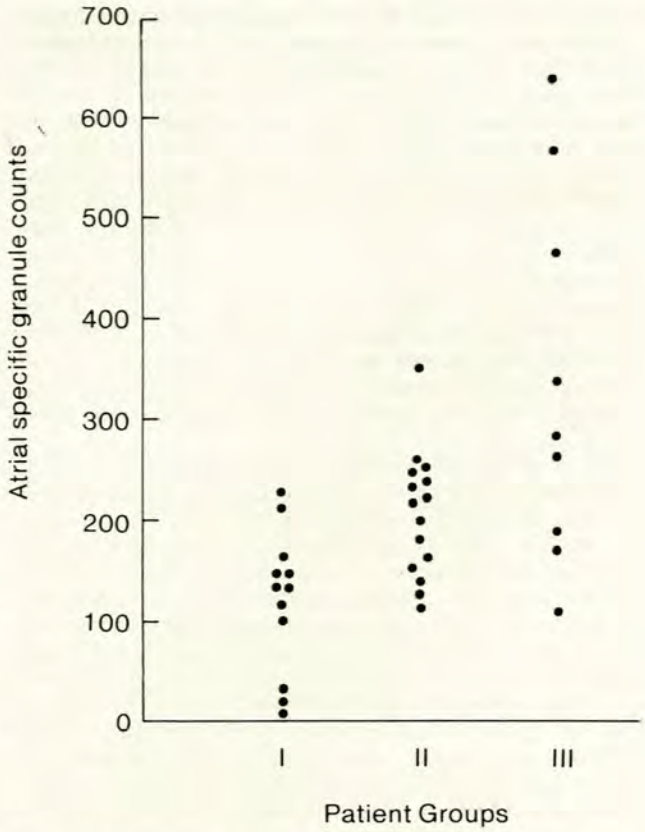

Fig. 3. The distribution of atrial-specific granules in the three patient groups (I = controls; II = mitral stenosis/sinus rhythm; III = mitral stenosis/atrial fibrillation).

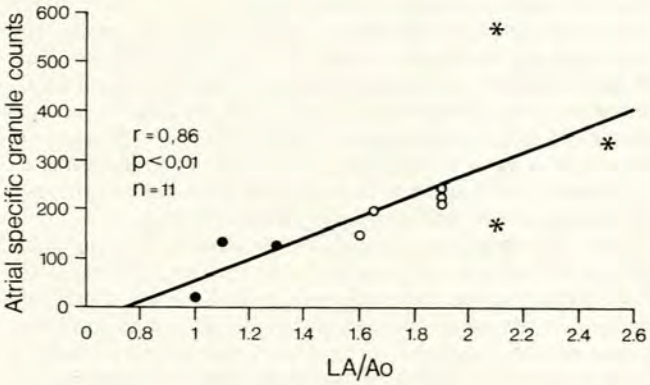

Fig. 4. The relationship between left atrial size and atrial-specific granule counts. Atrial size is expressed in terms of the echocardiographically determined left atrial diameter/aorta root diameter ratio (LA/Ao) • = controls; o = mitral stenosis/sinus rhythm; * = mitral stenosis/atrial fibrillation).

Discussion

The stimuli for ANP release accepted at present include atrial stretch[7] and atrial tachyarrhythmias.[20] Patients with mitral stenosis with or without atrial fibrillation are a good study model to examine the effects of increased atrial pressure and disturbance of atrial rhythm on the secretion of ANP. Assessing the atrial-specific granule content in this patient group provides insight into the mechanisms which ANP levels are controlled.

Certain aspects of our method for the morphometric analysis of atrial-specific granules in humans need to be emphasised. Previous morphometric studies have all been done in rats. Rat studies have the advantage of relatively large atrial-specific granules compared with humans (average diameters 0,42 μm and 0,25 μm respectively).[21] This observation and the fact that atrial-specific granules stain specifically with lead-haematoxylin-tartrazine makes light microscopic studies in rats feasible.[22] With light microscopy, the large sampling size compensates for cell-to-cell variations in granularity. However, this method is unsatisfactory in humans because of the smaller average size[21] and lower affinity of these granules for the stain.[23] Although far smaller areas are sampled using electron microscopy, this method is accurate and precise and several successful quantitative electron microscopic studies have been reported in which sampling difficulties have been overcome.[2,14,24]

The procedure was standardised throughout. One person was responsible for all the electron microscopy and granule counting, minimising observer error. Our sampling is random and unbiased and thus representative, compensating for cell-to-cell and area variations. Continuous areas of tissue were examined and not just selected regions. The total surface area per patient (33 000 μm^2) was far larger than in previous studies. Free counting of specific granules is much cheaper and quicker than the montage method and just as reliable, and can confidently be used as a suitable alternative. The problem of variable quality of fixation obtained with immersion fixation is inherent to a clinical study of this nature but this did not prove to be a major obstacle in our study.

Clinical observations supporting the role of atrial stretch and atrial tachyarrhythmias in stimulating ANP release include increased serum ANP levels in chronic renal failure,[25] congestive heart failure[26] and atrial tachyarrhythmias.[25,27] These observations have been substantiated by experimental studies.[7,28] However, little is known about the mechanism whereby atrial tachyarrhythmias and atrial stretch elevate ANP serum levels. The question that arises is how the atria provide the additional ANP needed to sustain the high serum levels found in these situations. Increased synthesis[10,11] has been implicated. However, little is known about the role of changes in the amount of ANP stored in atrial-specific granules to provide an additional supply of the peptide. This study is the first to contribute in this regard by assessing the atrial-specific granule content in patients with chronically distended atria.

Since the degree of granularity reflects ANP synthesis, storage and release, the increased granule content in patients with mitral stenosis may reflect decreased release, increased synthesis (without an increase in release) or a high turnover state (increased synthesis and release). Concurrent measurement of serum ANP levels would be valuable in distinguishing between these possibilities. This was not done in our study, since the necessary techniques for ANP measurement were not yet available when the study was initiated. The finding that patients with mitral stenosis have higher granule counts than the control group, is probably the result of a high turnover state, since high serum ANP levels have been documented in patients with mitral stenosis.[29] This rules out decreased release or increased synthesis without concomitant increased release as explanations of the increased granule content in this group of patients. Serum ANP levels in patients with atrial fibrillation are also elevated.[20] Therefore the tendency for higher granule counts in the subgroup with mitral stenosis and atrial fibrillation (group III) than in the patients with mitral stenosis and sinus rhythm (group II) (Fig. 3), probably also reflects a further increase in the turnover of atrial-specific granules.

The correlation between atrial-specific granule counts and atrial size (Fig. 4) implies that the greater the pressure overload of the left atrium, the larger the amount of ANP stored in the atrium. Patients with mitral stenosis and atrial fibrillation tended to have the largest left atria (Table I and Fig. 4) as well as the highest granule counts (Fig. 3). It is therefore difficult to determine whether the high granule counts are related to the rhythm disturbance itself or are the result of the pressure overload of the left atria in this group.

We were unable to demonstrate exocytosis and the exact mechanism of granule content release remains unknown.

To conclude, we have described an accurate technique to quantify atrial granule content, which can be used in future studies to help to unravel the role of atrial pressure and rhythm in ANP release. Our results show a high granule count in patients with mitral stenosis. This observation supports increased storage of ANP in secretory vesicles as a compensatory mechanism whereby increased demands, such as increased atrial pressure, can be met.

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