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CALCIFICATION IN AGING CANINE AORTIC VALVE

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Abstract

Aging changes of aortic valves are thought to underlie the mechanism of calcification, which leads to calcific aortic stenosis in humans. The study of calcification in the aging valvular connective tissue has been hindered by the lack of a suitable animal model. In search of the model, canine aortic valves demonstrated age changes including calcification remarkably similar to those in humans. The mechanism of calcification was studied in the aortic valves of aged Beagles by electron microscopy. Fibroblasts in the canine aortic valves showed the most prominent age changes. The cells accumulated numerous residual bodies and appeared to disintegrate. The resultant membranous cellular degradation products which sequestered in the extracellular space were the nidi of calcification. It appeared that the membrane of cell debris played an important role in calcification. Canine aortic valve is an ideal model for the study of calcification in relation to aging of the valvular connective tissue.

Key Words: Calcification, Aortic Valve, Cellular Degradation Products, Cell Aging, Canine.

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Introduction

The occurrence of calcification in the aging vascular connective tissue has long been known. The vascular calcification causes a variety of clinical syndromes including calcific aortic stenosis. Furthermore, there is a view that the vascular calcification may play an important role in the development of atherosclerosis (Kramsch et al, 1981; Blumenthal et al, 1950). With the recent control of rheumatic heart disease, age associated degeneration of the aortic valves remains to be a major threat for the development of calcific aortic stenosis.

Calcification in aging human aortic valves has received particularly early attention. Moenkeberg (1904) ascribed the degenerative changes and calcification of aortic valves in old age to continuous mechanical stress. Sell and Scully (1965) observed a decrease in the number of fibroblasts and a gradual deposition of calcium in close topographic association with lipid accumulation in human aortic valves with an increase of age. Subsequent electron microscopic studies demonstrated the valvular calcification to result from senescent changes of fibroblasts of the aortic valves (Kim, 1976). The study of calcific aortic stenosis, however, has been hindered mainly due to the lack of a suitable animal model. In a preliminary study, canine aortic valves showed degenerative changes and calcification remarkably similar to humans. In order to further evaluate aging changes as the underlying mechanism of dystrophic calcification, an electron microscopic study of aortic valves of senescent dogs was performed.

Materials and Methods

A 16.5-year-old female Beagle (Gerontology Research Center, Baltimore), five male Beagle dogs 12 years of age and a two-year-old male Beagle as a control (Buckshire Corp., Perkasies, PA.) were used for the study. The dogs were sedated with intramuscular acepromazine maleate (0.25 mg/lb) (Fort Dodge Lab. Inc., Fort Dodge, IA), anesthetized with intravenous pentobarbital sodium and sacrificed by exsanguination. A longitudinal strip from the midportions of the left coronary cusps of the aortic valves were cut into 1 x 1.5 mm pieces. The pieces were immediately fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 4 hours at 4° C, postfixed in 1% OsO_4 in 0.2% s-collidine buffer for

one hour and dehydrated in gradient concentrations of ethanol and propylene oxide. The pieces were embedded in Polybed 812 (Polyscience, Inc., Warrington, PA.) using silicone rubber embedding molds under a dissecting microscope for accurate positioning and avoidance of sectioning of the endothelial surface. Thin sections obtained from representative blocks from each valve were stained with uranyl acetate and lead citrate and examined in a JEOL 100CX coupled with a Si detector and a Tracor-Northern NS 880 multichannel analyzer. The presence of calcification was determined by x-ray microanalysis.

Results

In the senescent canine aortic valves, fibroblasts in the fibrosa, a dense layer of collagenous tissue, showed the most notable changes. By light microscopy of toluidine blue stained 1 μm thick sections, fibroblasts appeared to have decreased in number and increased in size in the valves of aged dogs. Fibroblasts in the aortic valve of a two-year-old were slender and elongated (Fig. 1). The nuclei were regular in size and shape. The nucleus frequently contained a centrally located round nucleolus. There were moderate amounts of endoplasmic reticulum and other membranous organelles in the cytoplasm. Lysosomes were scanty to absent. The extracellular space did not contain cell debris or calcific deposits.

The cells from the older valves were larger and thicker (Fig. 2). They frequently gave rise to multiple and short cell processes. Nuclei were very irregular in size and shape with deep indentations of their membranes. Chromatin granules were irregularly clumped with a tendency of condensation along the nuclear membrane. Nucleoli were reticulated and peripherally located. In the enlarged cytoplasm, cell organelles appeared irregularly distributed. Large areas of the cytoplasm were frequently occupied by cytofibrils which appeared to have pushed other cell organelles to the other areas of the cytoplasm. Particularly notable was an accumulation of a large number of residual bodies in the 'senescent' fibroblasts. Certain cells contained only residual bodies but no other organelles (Fig. 3). In the vicinity of these senescent cells, there were accumulations of numerous membranous cellular degradation products (CDP) (Figs. 2-6). Certain CDP were morphologically similar to the intracellular residual bodies (Fig. 3). There were also clusters of CDP which retained the size and shape of a cell in the same valvular tissues (Fig. 4). Occasionally, degenerate cells which had partly emptied their contents, namely CDP, to the surrounding space were seen. The morphology of CDP was very complex. They ranged from 50 nm to several microns in diameter.

Calcific deposits were seen exclusively in association with CDP (Figs. 2-6). The crystals were either in the lumen of the CDP vesicles or in apposition to the inner or outer surfaces of the CDP membrane. Of particular interest is the frequent occurrence of large sized vesicles of several microns in diameter (Fig. 6). The wall of the large vesicles was thickened to approximately 300 nm. The thickening appeared to be due to a combination of folding of the membrane and deposition to the membrane of organic substance which has yet to be identified. Needle shaped crystals containing calcium and phosphorus were frequently imbedded radially in the thickened wall of the large vesicles (Fig. 6). X-ray microanalysis of the thick wall of the vesicles with the needles yielded peaks of calcium and phosphorus in addition to the elements which were also present in the background (Fig. 7).

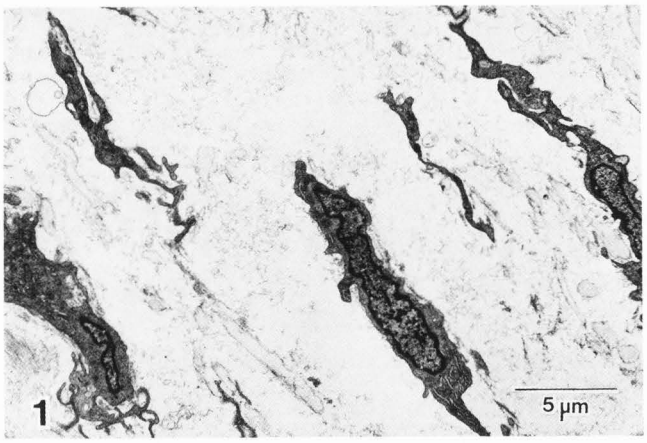
Discussion

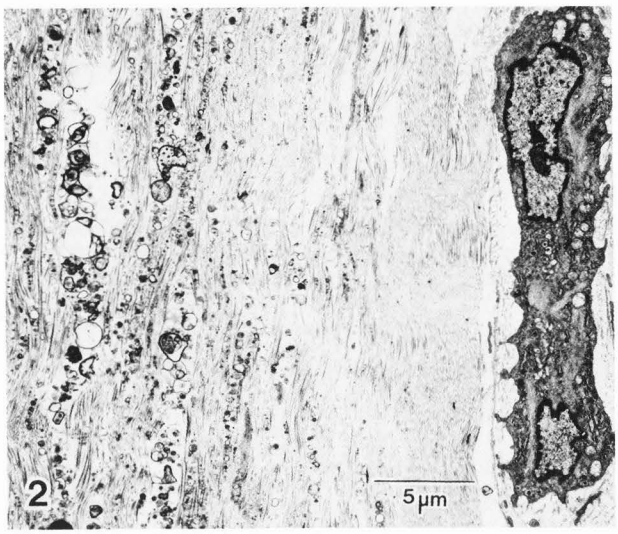
The mode of calcification in canine aortic valves is remarkably similar to that of human aortic valves. Membranous CDP derived from senescent and degenerated fibroblasts in the fibrosa were the exclusive nidi of calcification. On the basis of morphological evidence, exocytosis, budding of the plasma membrane and the disruption of the entire degenerated cells, all appeared to result in the accumulation of CDP. A gradual decline in the number of cells with age has been shown to be associated with an accumulation of lipid and calcium in the connective tissue of human aortic valve (Sell and Scully, 1965). Although morphometric analysis was not performed in this study, a similar decrease in the number of cells was noted by light microscopy. It, therefore, can be said that senescent cells which are no longer able to divide undergo degeneration and sequester in the extracellular space as CDP. Similar calcification of membranous CDP was observed in a variety of pathological calcifications in human and experimental animals in vivo and in vitro (Kim, 1983a, 1985).

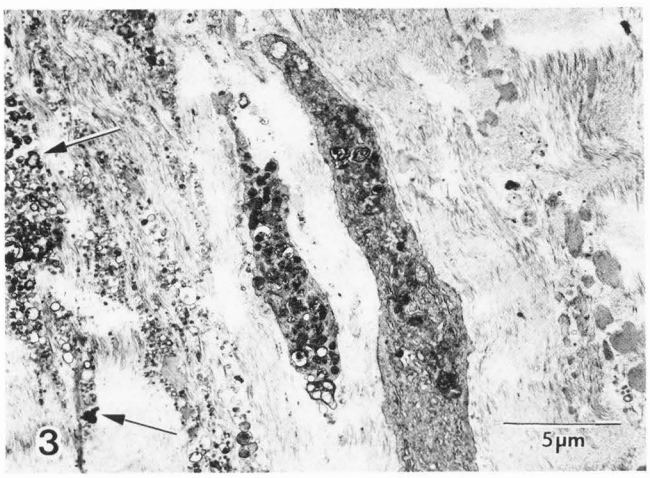
Membranous CDP in the canine aortic valves were morphologically similar to matrix vesicles which have been shown to be the nidi of calcification in both physiological and pathological calcifications (Anderson, 1985; Bonucci, 1981). Matrix vesicles are believed to accumulate calcium and phosphate by a cell-mediated active process which utilizes cell derived energy (Anderson, 1985; Wuthier, 1982). However, morphological evidence in this study, i.e., calcification as a result of the cell degeneration, suggests that calcification may occur without the cell derived energy. In fact, calcification occurs commonly in a variety of necrotic tissues. Similarly, devitalized rat aorta by repeated freeze thawing and renal tissue injured by anoxia calcified in experimental conditions (Kim, 1983b, 1984). The so-called crystal ghosts described in cartilages (Bonucci, 1981) were not seen in this study.

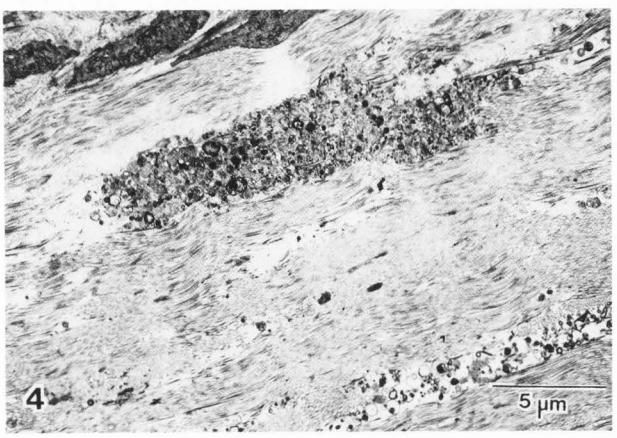
The mode of calcification of CDP appeared complex. The calcific deposits were seen in the lumens as well as in apposition to the surface of membranous CDP. Similar deposits at the surface of membranous vesicles were seen in a variety of dystrophic calcifications in humans including atheromatous plaques (Kim, 1985). In view of wide spread evidence that the surface of membranous CDP serves as the nidi of calcification, it is theorized that devitalized membranes in the form of CDP may serve as a substrate for heterogeneous nucleation of calcium apatite. Exposure of the high intracellular phosphate to the extracellular calcium upon cell injury (including cell aging) is also believed to have a role in calcification (Kim, 1985).

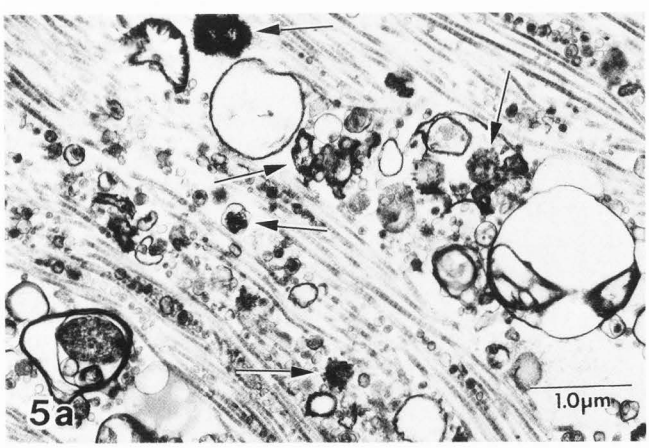
Of particular interest is the frequent occurrence of calcification in the thickened wall of the large sized CDP. On the basis of their size, they are











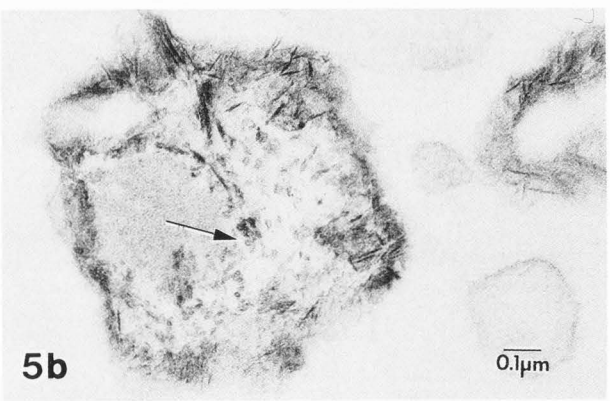


Fig. 1. Aortic valve of a 2-year-old dog. Fibroblasts are bipolar and elongated. The extracellular space is free of cell debris.

Fig. 2. Aortic valve from a 12-year-old dog. A fibroblast shown is larger and thicker. Numerous membranous cellular degradation products (CDP) are seen in the extracellular space.

Fig. 3. Aortic valve of a 12-year-old dog. Senescent fibroblasts contain large numbers of residual bodies which are similar to CDP in the extracellular space. Calcific deposits are seen at CDP (arrows).

Fig. 4. Disintegration of an entire cell in the same valve.

Fig. 5a. An area in Fig. 2. Calcific deposits are seen at CDP (arrows). **b.** A closer view of calcified CDP. In addition to needle shaped crystals, spheroidal particles with empty centers are also present (arrow).

presumed to have originated from the plasma membrane. Red cell ghosts incubated in rat peritoneum in millipore chambers produced similar thick walled vesicles with calcification (Kim, 1983c). The thickening is evidently due to folding of the membrane and the deposition of electron lucent substance. The mechanism of calcification in the thickened wall apparently differs from that of other CDP calcification. The needle shaped crystal appeared to develop in the thickened wall as opposed to the deposition of the crystals in the lumen or on the surface of the trilamellar membrane of CDP. Large vesicles which calcified were observed in a variety of human dystrophic calcifications as well (Unpublished data). They were particularly prevalent in the atheromatous plaques. Interestingly enough, similar thick-walled structures with calcification were also seen in decalcified urinary stones (Kim, 1983c).

Summary

In aging canine aortic valves, membranous CDP derived from senescent and degenerated cells were the nidi of calcification. The mode of CDP calcification was complex. In view of the frequent occurrence of the calcific deposits in apposition to the membranous surface of CDP, the membranes of CDP play a primary role in calcification by serving as a substrate for heterogeneous nucleation of apatite. Calcification resulting from cell degeneration is unlikely to involve an active cell-mediated process. Frequent calcification of large sized and thick walled vesicles also indicate that certain alterations of the membrane predispose to calcification. Canine aortic valve is an ideal model with which to study the mechanism of age related calcification which progresses to calcific aortic stenosis in human presumably due to their longer life span and the superimposition of atherosclerosis.

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Discussion with Reviewers

H.C. Anderson: The microprobe tracing suggests that about equal amounts of Ca and P are present, but such would not be the case for hydroxyapatite which has a Ca:P molar ratio of approximately 1.6. Can the Ca:P be estimated from this data?

KPR Pritzker: By microanalysis, calcium and phosphorus were demonstrated in the calcifications. However, the crystal nature of the calcifications was not demonstrated.

HR Schumacher: How do the authors explain the calcium to phosphorus ratio that appears to be close to 1:1 on the single elemental analysis shown? What was the range of ratios found in early and denser calcifications?

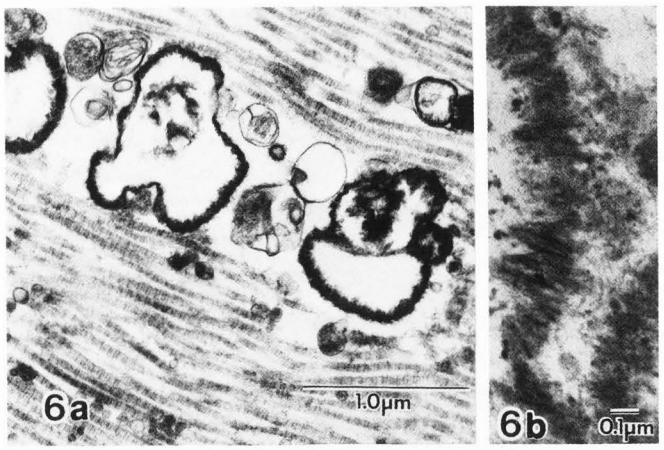
Authors: An Ideal Ca:P ratio of apatite may occasionally be obtained by x-ray analysis of pure aggregates of needle shaped crystals especially in experimental calcifications. However, the determination of Ca:P ratios of minute calcific deposits is not a simple issue for the following reasons. In addition to the limitations in instrumentation which call for the ZAF corrections, the Ca:P ratio may be affected by crystal imperfection; possible coexistence of different types of crystals and non-crystalline deposits such as octacalcium phosphate, whitlockite, brushite and amorphous calcium phosphate; considerable dissolution of calcium and phosphate in conventional EM preparations using aqueous fixatives; and the presence of calcium and phosphorus in the background organic matrix. Therefore, the determination of Ca:P ratio requires a comprehensive study including statistical analysis. Such an extensive analysis was not the purpose of this study. The analysis was meant not to confirm the type of crystalline deposits but the presence of calcification.

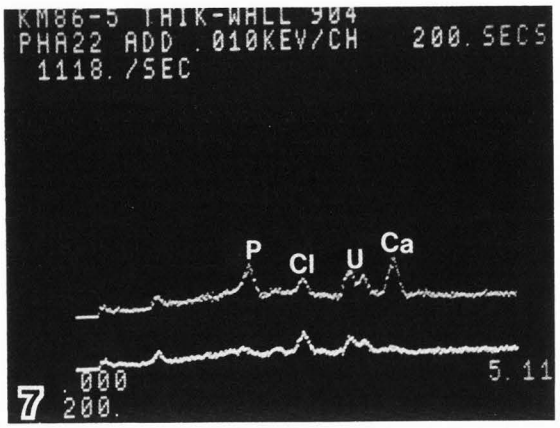
Occasionally, x-ray analysis of calcified vesicles in the canine or in the human vascular connective tissue yielded the phosphorus peak taller than the calcium peak. It is suspected that the presence of phosphorus in the background organic matrix is the major cause of the demonstrated low Ca:P ratio.

K.P.R. Pritzker: Was any calcification associated specifically with collagen or matrix vesicles?

HR Schumacher: Did the authors see definite vesicles with just a few apatite crystals. Was there ever any calcification of collagen or elastin?

Authors: Yes, membrane bound vesicles containing scanty needle shaped crystals were frequently seen. However, there were also vesicles containing round or granular electron dense deposits. X-ray analysis of these deposits is pending. Although a possible involvement of collagen in calcification cannot be excluded by a morphological survey, isolated





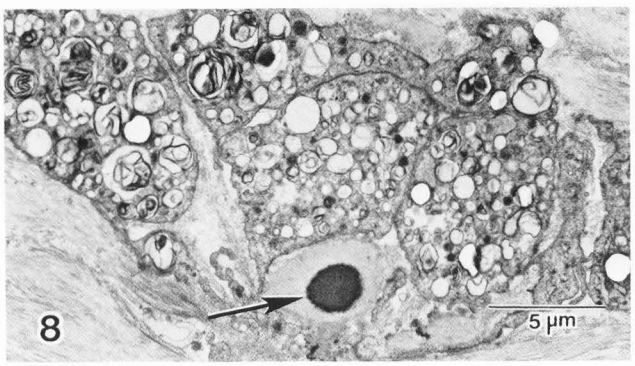


Fig. 6a. Calcified large vesicles with thickened wall. **b.** Portions of large vesicles showing radially arranged needle shaped crystals embedded in the thickened wall.

Fig. 7. X-ray microanalysis of calcified thick wall showing calcium and phosphorus peaks in addition to other elements in the background.

Fig. 8. Electron dense deposits in the core of an elastic fiber in a 16.5 year old female Beagle (arrow).

calcification of collagen was not observed in this study. Interestingly, in a recently examined 16.5 year old female Beagle, electron dense deposits in elastic fibers were observed (Fig. 8). Preliminary x-ray analysis of the deposits failed to yield calcium or phosphorus. In view of their similarity to calcified elastic fibers, a comprehensive x-ray analysis of elastic fibers in the valves is planned.

SR Khan: Was there an increase in the amount of collagen in aging aortic valves? There appears to be more collagen in the valves of old dogs than of a young.
Authors: Yes, it is known that collagen and other connective tissue matrices increase with age.

SR Khan: What are the similarities between the matrix vesicles and membranous cellular degradation products found in aging canine aortic valves?
Authors: Both are cell derived, membrane bound vesicles and serve as nidi of calcification.

SR Khan: How can one identify the vesicles originating from the plasma membrane on the basis of their size?
Authors: As demonstrated in Fig. 4, disintegrated cells are frequently devoid of the plasma membrane. The presence of the large vesicles in the vicinity of such degenerated cells is highly consistent with the origination of large vesicles from the plasma membrane. As stated in the text, the formation of similar thick walled vesicles with calcification from red cell ghosts incubated in rat peritoneum demonstrates that the thick walled vesicles may develop from the plasma membrane.

SR Khan: The authors describe the phenomenon of crystal deposition within the "thickened wall of the vesicles." Is this thickening for real or does the membrane appear thickened because of oblique sectioning? How does the wall thicken and how does this aid in the crystal nucleation?
Authors: The thickening of the large vesicle wall involves the entire circumference of the vesicles. It is, therefore, inconceivable that the thick wall is a product of tangential cuts. Furthermore, tangentially cut membranes have a distinctive appearance. As to the origin and the mechanism of calcification of the thickened wall, they are largely obscure.

K.P.R. Pritzker: What is the evidence for senescence of fibroblasts?
Authors: The cells from senescent dogs are very different from those of a young. In a preliminary study of cultured cells by the colony size distribution (Smith JR, Pereira-Smith OM, Schneider EL. (1978). Colony size distribution as a measure of in vivo and in vitro aging. Proc. Nat'l. Acad. Sci. 75:1353-1356), the colony sizes formed by the 'senescent' cells were consistently smaller than by the cells from the young donor.

K.P.R. Pritzker: Is the presence of cell degradation products in the matrix a result of failure to clear the products deposited in the collagenous matrix?
Authors: In view of the lack of similar accumulations of CDP in other aging connective tissues, it is believed that the lack of capillary networks and in turn the lack of scavenging is responsible for the CDP accumulation in the aortic valves.

K.P.R. Pritzker: Was intracellular calcification observed? If so, was this associated with cell death?
Authors: Calcification was not observed in the cells which were still bounded by the plasma membrane.

SR Khan: Osmium has been used during the processing of the tissue and osmium should interfere with phosphorus in x-ray microanalysis. Was non-osmicated tissue used for microanalysis?
Authors: Osmicated tissue was used for microanalysis. Osmium in x-ray analysis of calcific deposits is frequently inconspicuous.

SR Khan: In the discussion the authors state that "exocytosis, budding of the plasma membrane and disruption of entire degenerated cells, all appeared to result in the accumulation of CDP". No proof of budding or exocytosis is provided in the paper.
Authors: The occurrence of numerous vesicles in the vicinity of cell processes indicates that budding is likely to give rise to the vesicles. Congestive engorgement of residual bodies is a known phenomenon in chronic cell injuries including cell aging. "Defecation" of residual bodies is generally believed to be a defense mechanism of such cells. Partial extrusion of residual bodies is frequently seen in aged aortic valves.