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Myocardial necrosis due to vitamin D_3 overdose — scanning electron microscopic observations

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Our studies were carried out on the hearts of virgin female Wistar rats treated with 100.000 i.u. of vitamin D_3 (calciol) per os *for 3 consecutive days. Multifocal cardionecrosis was established macroscopically in 70% of the vitamin D-treated rats on the 7th day of the experiment when the rats were in the acute phase of intoxication.*

Using a scanning electron microscopy (SEM), we received three-dimensional information about the structural changes to the rat myocardium damaged by high doses of vitamin D_3. The images of necrotic hearts revealed significant disruption of the structural integrity of the myocardium linked to fragmentation of the cardiac muscle bundles and a visible disruption of the extracellular matrix (ECM) components. In healthy hearts, the structural integrity of the myocardium and the dense network of the extracellular matrix were well preserved. In parallel, the effect of an increasing concentration of free Ca^{2+} on the total proteolytic activity of the heart muscle homogenate of the healthy and necrotic rats was investigated at neutral pH. These data showed that following vitamin D_3 intoxication, the proteolytic processes in the rat hearts occurred in Ca^{2+} overload or saturation. On the basis of our morphological and biochemical results we can suggest that calcium-activated neutral proteinases may have contributed to the structural alteration of the extracellular matrix components and were in this way involved in vitamin D-induced cardionecrosis.

Key words: cardiac myocytes, calciol, toxicity, extracellular matrix, calpain

INTRODUCTION

A number of recent clinical and experimental observations suggest that vitamin D_3 (calciol) plays an important role in maintaining cardiovascular function, either directly through its nuclear and membrane-associated receptors in the cardiac muscle, or indirectly through its influence on circulating levels of calcium or other regulatory factors [14]. The generation of this wide array of biological responses is connected with the unusual conformational mobility of calcitriol [5].

Hypervitaminosis D is an example of toxic myocardial damage which produces multifocal necrosis [3, 22]. The changes in the myocardium caused by toxic doses of vitamin D_3 consist primarily of degeneration and focal necrosis of the myocardial fibres [21]. Through electron microscopy fragmentation and loss of the myofibrils have been observed, associated with an accumulation of calcium within the necrotic muscle fibres between the sarcolemma and basement membrane, and also extracellularly. In addition, the authors observed fo-

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cal stimulation of both cardiomyocytes and interstitial cells with a vivid proliferation of the endoplasmic reticulum.

Heart myocytes are enmeshed in a complex array of extracellular matrix components, which are organised in 3 levels and are responsible for cardiac cell alignment and myocardial structural integrity. The sheath that surrounds the heart muscle contains collagen fibres and elastin. The groups of myocytes are surrounded by a weave-like network of bundles associated with the elastic modulus of the ventricular wall, while the connections between the cells are composed of struts of collagen (Types I and III) as well as combinations of elastic fibres, collagen fibres, and microfibrils. The rest of the ECM is filled with a polyanionic lattice of a mixture of collagen fibrils, microthreads, and granules. Therefore, the complex network of collagen throughout the heart is composed of a hierarchy of fibrils and fibres ranging from 10 nm to 2–3 μm in diameter [4, 15]. This collagen network can be broken down during some pathological processes, such as myocardial infarction, ischaemia and isoproterenol-induced myocardial necrosis [4, 13, 23, 25].

Biochemical studies of vitamin D-overdosed rats have shown that ultra-structural changes in the myocardium were associated with a dramatic increase in calcium concentration in the blood serum and heart muscle [12, 18, 22], and were intensified by the proteolytic action of calcium-activated endogenous enzymes [11, 19].

The present study was focused on a scanning electron microscopic examination of the rat heart damaged by toxic doses of vitamin D_3. Based on micrographs of necrotic hearts and biochemical results previously [19] and currently presented, we have sought a correlation of the morphological changes with the biochemical factors involved in the formation of vitamin D-induced multifocal cardionecrosis.

MATERIAL AND METHODS

The experiments were performed on n = 28 virgin female Wistar rats, weighing about 200 g. The animals were kept under standard laboratory conditions of a 12-hour light/dark cycle with food and water available *ad libitum*. The care and treatment of the animals were in accordance with the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes".

To develop cardiac necrosis, the rats (n = 18) were given 100.000 i.u. of vitamin D_3 *per os* for 3 consecutive days. Ten rats were used as controls. On the 7th day after the first calciol dose (in the acute phase of intoxication), the rats were sacrificed. A macroscopic assessment of the extent of the cardiac muscle lesion was based on the scale, described earlier [20], ranging from 0^0 to 5^0 (0^0 — unchanged myocardium, 5^0 — profound cardionecrosis). 70% of vitamin D-treated rats exhibited signs of visible cardionecrosis (4^0 to 5^0). Their hearts were the subject of SEM and biochemical investigations.

Scanning electron microscopy (SEM)

The hearts of vitamin D_3-treated (n = 8) and untreated (n = 4) rats were prefixed by two methods:

— after the decapitation of n = 6 rats, the hearts were rapidly removed from the thorax, and small blocks excised from the anterior wall of the left ventricle above the apex were immediately placed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), at 0^0C for one day. The remaining parts of the hearts were used for the biochemical assays.

— n = 6 hearts were fixed by transcardial perfusion through the left ventricle. The animals were deeply anaesthetised with intraperitoneally administered Nembutal (30 mg/kg body weight). Initially, the vascular system was rinsed with heparinised 0.9% NaCl at 37^0C, followed by a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) with 0.03% $CaCl_2$. The hearts were rapidly removed from the thorax and small blocks taken from the anterior wall of the left ventricle above the apex were transferred directly into 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 0^0C for one day.

Next, the specimens were postfixed in 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) and dehydrated through graded ethanols. After being washed in acetone, they were critical point dried through carbon dioxide in a Balzers Critical Point Dryer. The exposed surfaces of the tissue were gold-coated in a sputtering device (Edwards) and, without any additional staining, examined in a JEM-1200 EX II electron microscope equipped with an EM-ASID11 Scanning Image Observation Device.

To confirm the development of focal cardionecrosis in the vitamin D-overdosed rats, slices taken from these areas were processed for histological examination. They were fixed in 10% formalin for 2 days and embedded in paraffin. Microtome sections (20 mm) were stained with haematoxylin and eosin and examined under the light microscope.

Assay of proteolytic activity

The proteolytic activity of necrotic (n = 8) and healthy (n = 8) hearts was determined by a modification of Kar and Pearson's method [9]. Heart muscle homogenate pooled from 2 animals killed by decapitation, was incubated in a medium containing 20 mM Tris/HCl buffer (pH 7.5), 0.15 M KCl, 0.1 mM EDTA, 1 to 10 mM $CaCl_2$, and 2 mg/ml casein yellow (nitrate casein) as a substrate. The protein concentration in the incubated probe was about 2 mg/ml. The reaction was terminated with 10% $HClO_4$. The products of casein-yellow hydrolysis were measured spectrophotometrically at 428 nm. A unit of activity was defined as that amount of enzyme which caused a change in the absorbance of 0.001 per 18 hours at 37^0C. Protein concentration was measured by the biuret method with bovine serum albumin as a standard [7].

RESULTS

Morphology

The administration of high doses of vitamin D_3 resulted in the development of necrotic lesions of various extents in rat hearts. On the 7^{th} day of the experiment (in the acute phase of intoxication), 70% of vitamin D-treated rats exhibited macroscopically visible heart necrosis (4^0 to 5^0) and these became the subject of investigation. These necrotic hearts contained numerous irregular pale spots (1 mm to 2 mm in diameter) and revealed a marked decrease in elasticity.

The light microscopic observations in the vitamin D-treated rats showed multiple scattered foci of necrosis in the entire myocardium but predominantly subendocardially and subepicardially. Some of the necrotic myocytes were partially disintegrated and fragmented (Fig. 1). In the control rats there were no morphological changes in the myocardium.

A scanning electron microscopic (SEM) examination of the control hearts showed structural integrity of the myocardium (Fig. 2). Cardiac myocytes were organised into tight muscle bundles in a regular arrangement. The dense network of the extracellular matrix (ECM) components surrounded and well interconnected the myocytes. The rats receiving high doses of vitamin D_3 exhibited disorganisation of the structural arrangement of the cardiac myocytes (Fig. 3). We observed a narrowing of the muscle bundles along with their disintegration and fragmentation. The disorders of the ECM components were visible. Muscle bundles were separated by the dilated extracellular space with a loosened network of collagen bundles. Partial loss of the collagen fibres that surround the myocytes was revealed (Fig. 4).

The type of damage to rat hearts was the same, irrespective of the method by which they were fixed.

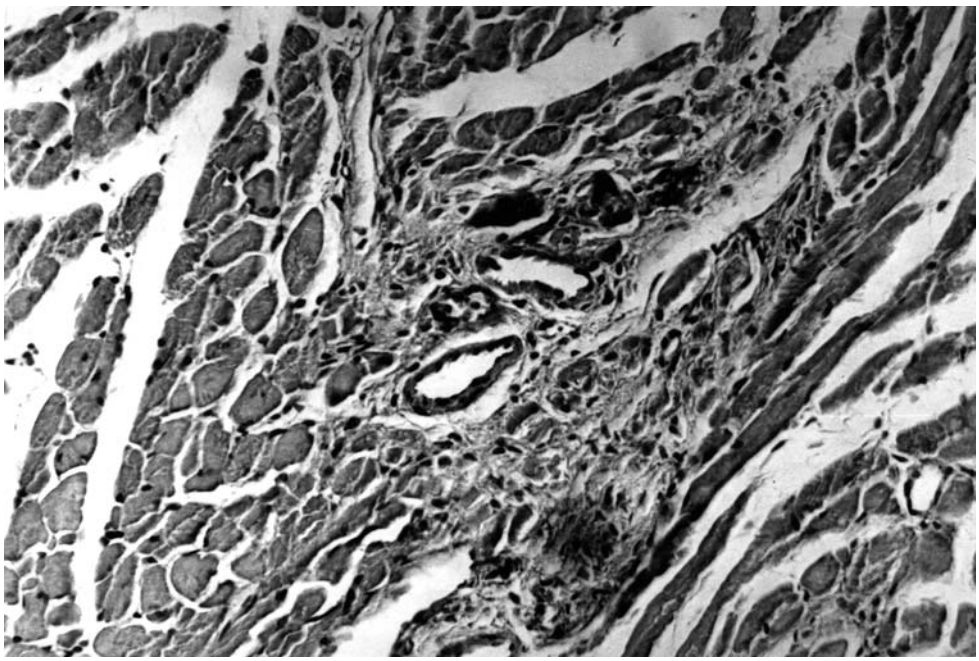

Figure 1. Micrograph of the myocardium of a vitamin D_3-treated rat. In the centre is the area of the focal necrosis with fragmentation of the myocytes. Haematoxylin and eosin staining. × 250.

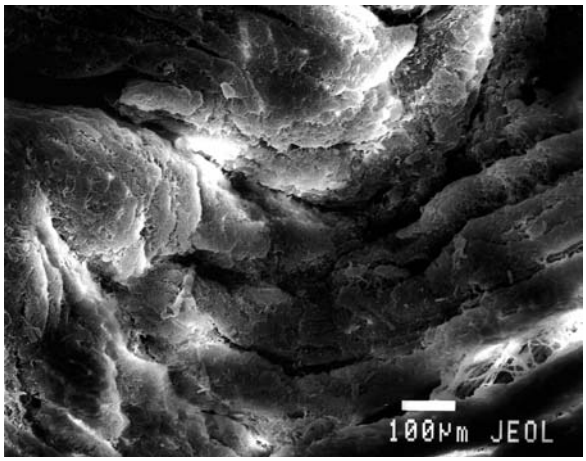

Figure 2. Scanning electron micrograph of control rat myocardium showing the structural integrity of the myocytes. Cardiomyocytes are well organised into tight muscle bundles with a regular arrangement. A dense network of the extracellular matrix (ECM) components surrounds and interconnects the cells.

Figure 3. Scanning electron micrograph of vitamin D_3-treated rat myocardium. Degenerated muscle bundles which have undergone disintegration and fragmentation. Profound dilatation of the intercellular space with looseness of the extracellular matrix network.

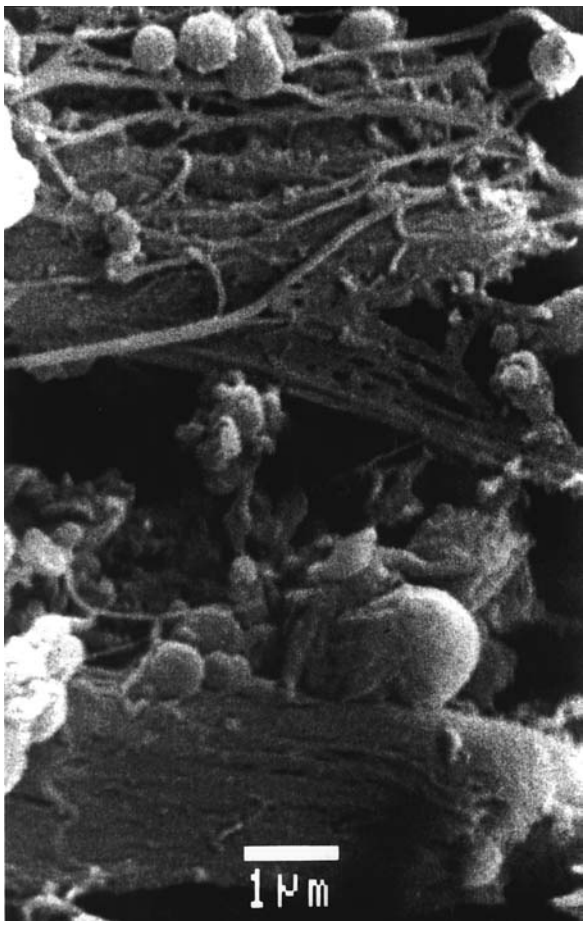

Figure 4. Scanning electron micrograph of vitamin D_3-treated rat myocardium. At the top collagen fibrils are quite normal in appearance. Below, muscle fibre is devoid of collagen matrix.

Proteolytic activity

The effect of an increasing concentration of free Ca^{2+} on the proteolytic activity of the heart muscle homogenate of the healthy and necrotic rats is shown in Figure 5. The results obtained indicate that the caseinolytic activity of the heart muscle homogenate of the healthy rat was significantly stimulated by free Ca^{2+} of between 1 and 10 mM. Maximum activation occurred at 5 mM Ca^{2+} concentration and was approximately 60% higher than proteolytic activity in the absence of Ca^{2+}. There were no effects

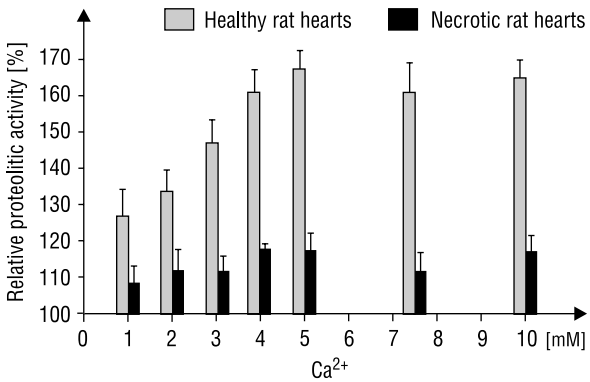

Figure 5. Effect of Ca^{2+} concentration on proteolytic activity in the healthy and necrotic rat hearts. Homogenates of rat hearts were prepared and incubations were carried out as described under Material and Methods. Values are means ±SD for 4 separate experiments; 100% proteolytic activity of the rat hearts homogenate in the absence of Ca^{2+}.

of the increasing concentration of free Ca^{2+} on the proteolytic activity of the heart muscle homogenate of the vitamin D-treated rats.

DISCUSSION

The scanning electron micrographs presented in this paper provide a deeper insight into the morphology of multifocal necrosis in the rat heart muscle caused by overdose of vitamin D_3.

On the basis of our SEM micrographs of the necrotic rat hearts, it can be concluded that the type of damage is the same, irrespective of the method by which the tissue was fixed. This result confirms the hypothesis [19, 21] that the changes in the rat heart structure caused by the action of toxic doses of vitamin D_3 were formed *in vivo*.

SEM observations have significantly increased the available information on the three-dimensional ultra-structure of the damaged cardiac muscle, the spatial arrangement of neighbouring cells and the relationship between them. Our TEM study [19] on isolated cardiac myofibrils indicated that the earliest ultra-structural changes to rat hearts damaged by large doses of vitamin D_3 concerned the cardiac contractile system and were localised in the I-band and Z-line. In consequence of this, the processes of destruction, fragmentation and loss of the cardiac myofibrils occurred. The present SEM observations revealed the reduced size of the heart muscle bundles. This was linked with a decrease in the size of the degenerated individual muscle fibres, preceded by a reduction in the number of myofibrils. The process of degeneration of the muscle bundles was also accompanied by morphological changes to the ECM components. According to some reports [16], collagen bundles that interconnect the myocytes are anchored just lateral to the Z-line outside the cell. This indicates the regions of structural continuity and the transmission of force across the sarcolemma, which runs from the endomysial collagen struts to the Z-line.

Our SEM observations revealed dilatation of the extracellular space of the necrotic myocardium. This could only be accomplished by partial breakdown of the collagen matrix. These observations correspond to changes that occur during some other pathological processes such as myocardial infarction, ischaemia and isoproterenol-induced myocardial necrosis [4, 13, 23], in which structural abnormalities include a pronounced loss of the collagen framework.

The substances that break down the extracellular matrix are specialised proteinases, which in the healthy heart appear to be normally balanced with their inhibitors in maintaining the integrity of the myocardium. Our previous studies [19] indicated that the ultra-structural alterations of the rat hearts damaged by high doses of vitamin D_3 were accompanied by approximately twice as much activity of the proteolytic enzymes in the necrotic heart homogenate as in the control hearts.

Studies on steroid hormone $1\alpha,25(OH)_2D_3$ provide a convincing structural correlation between binding to the membrane receptor and the ability to initiate calcium transport at both the cellular and subcellular levels [14]. Vitamin D intoxication in the rat has been shown to increase calcium levels in the serum, kidney, duodenum, aorta and cardiac muscle (but to be without effect in the skeletal muscle, liver and brain). Hypervitaminosis D produces an approximately 100-fold increase in whole-heart tissue Ca^{2+} content [18], preceding the morphological changes in the myocardium [12]. It is significant that vitamin D-induced microscopic lesions of the intracellular and extracellular spaces have been characterised by more extensive rat heart degeneration than mineralisation [21]. The biochemical data presented in this paper shows that at neutral pH the proteolytic processes in the rat heart damaged by large doses of vitamin D occurred in conditions of calcium overload or saturation. Calpain (non-lysosomal calcium-activated neutral cystein proteinase) is considered to be a highly likely candidate, since it plays an important role in the pathology of several cardiac disorders, such as hypertrophy [2], ischaemia [24], hypoxia [8] and myocardium infarction [10]. Its activity in the rat kidney and heart is several times greater than in the liver and brain. Although generally believed to exist and function only in the cytoplasm, this enzyme has been detected in the extracellular space of various tissues and is more particularly associated with extracellular matrix components. M-calpain is distributed on extracellular collagen fibrils and the peripheries of elastic fibres [1]. This enzyme cleaves to the fibrillar network of fibronectin [6] and participates in the degradation of proteoglycans [17]. It is therefore possible that, following vitamin D intoxication, extracellular calcium-activated proteinases in part contribute to ECM alterations, and are involved in the development of cardiac necrosis.

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