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Electrical conduction system apoptosis in type II diabetes mellitus

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Abstract

Even though apoptosis is known to be associated with various cardiovascular pathologies, its presence in cardiac nodal tissue in adults was only scarcely researched. Cardiomyocyte apoptosis was associated with diabetic cardiovascular pathology. Our main objective was to test whether programmed cell death is present in nodal tissue in type II diabetes mellitus and, if present to characterize it. The study was designed as a qualitative one. We used autopsy samples of hearts from 10 patients (56 to 73-year-old, 6:4 male to female ratio), positive for type II diabetes mellitus. Samples from sinoatrial and atrioventricular nodes were stained with Hematoxylin–Eosin. For immunohistochemistry, we used primary antibodies for caspases 3 and 9, cathepsin B, and TRADD. Nodal tissue in all samples was characterized by diffuse interstitial fibrosis and chronic ischemic lesions; nuclear damage and foci of irreversible ischemic necrosis intermingled with isles of relatively morphologically normal myocytes. Sinoatrial and atrioventricular nodes were caspase-3 and -9 positive, and also cathepsin-B-positive, suggesting an overlap between apoptotic and necrotic mechanisms. Central area of the sinus node seemed to have the most severe lesions. As a conclusion, nodal apoptosis is present in nodal tissue in type II diabetes mellitus; it involves the intrinsic pathway and associated concomitant and/or post-apoptotic necrosis.

Keywords: diabetic heart, cardiac conduction system, sinus node, atrioventricular node.

Introduction

Even though apoptotic cell death was proved to be involved various cardiovascular diseases is known [1], including in electrical conduction system pathology [2], the intervention of caspases in nodal tissue apoptosis has not yet been evaluated. Only a few studies on caspase-3 expression in human myocardial infarction have been reported, which were either based on a very limited number of patients or focused their attention on the role of apoptosis in post-infarction remodeling [3].

Moreover, although much is known about the sinus node in animals, relatively little is known about the human sinus node [4, 5] and its heterogeneity is regarded as playing important roles in nodal dysfunction [4].

In mammalian cells, caspases are activated by the intrinsic or the extrinsic apoptotic pathway [6]. The intrinsic pathway is activated by various stimuli, and acts through the mitochondria [6]. The permeabilization of the mitochondrial outer membrane allows the release of cytochrome c, which induces caspase activation to orchestrate the death of the cell [7]. The release of mitochondrial cytochrome c was associated with activation of caspase-3 [8–11].

The occurrence of apoptosis, *i.e.* "suicidal", programmed cell death, in ischemic–reperfused tissue, and in the border area of myocardial infarction has been described. Apoptotic cells undergo distinct changes whereas cellular organelles and the cell membrane remain intact, which is distinctly in contrast to necrotic cell death. Numerous mediators are involved in the regulatory cascade for the apoptotic cell death. Mediators such as caspase-3 appear before execution of cell death by DNA fragmentation. The successful use of caspase inhibitors to attenuate apoptotic injury *in vitro* and *in vivo* indicates the "point of no return" to be placed downstream in the apoptotic cascade. A problem arises from the fact that the term apoptosis is used either for the entire cascade or for the last stage of DNA fragmentation [12].

Currently, the term "active" is preferred instead of "programmed" when referring to cell death processes that, once triggered, lead to a course of events processed and controlled by the dying cell [13].

Cell death is followed by cell elimination. In multi-cellular animals, cell elimination is mainly carried out by apoptosis. When removal by scavengers does not occur, the apoptotic process continues until a transition to necrosis ensues leading to cell elimination by cell disruption. This terminal disruption of apoptotic cells was designed as secondary necrosis. Secondary necrosis was viewed in the initial reports as a separate process occurring after completion of apoptosis and, thus, it has frequently been called post-apoptotic necrosis. If the necrotic outcome is considered part of the apoptotic program of cell elimination, it must be called "late apoptosis" [13].

Here, we shall use the term "apoptosis" to describe the entire sequence of events identified within the cardiac nodal tissue as being associated with an active form of cell death and "secondary necrosis" to describe the necrotic outcome of the apoptotic program.

Our main objective was to test whether programmed cell death is present in nodal tissue in type II diabetes mellitus and, if present to characterize it. Subsequently we tested caspases involvement in cardiac nodal cell death, we evaluated the apoptotic pathway that may be involved during the cardiac nodal apoptosis and tried to identify whether or not cell elimination involves a process of secondary necrosis.

Materials and Methods

Our study included autopsy samples of hearts from 10 patients 56 to 73-year-old, 6:4 male to female ratio, positive for type II diabetes mellitus, non-insulin dependent – diabetes was diagnosed in these cases seven or more years before death. The control group consisted of eight cases 44–56-year-old, sex ratio 5:3, non-diabetic violent deaths with a short survival period. Acute myocardial infarction was used as exclusion criteria in both groups. The investigation was approved by the Institutional Ethics Committee.

Anatomical identification of nodal tissue was done using the terminal crest as a landmark for the sinus node and the triangle of Koch as a landmark for the atrio-ventricular node. Tissue samples were fixed in 10% buffered formalin, paraffin-embedded, and cut at 4 μm for Hematoxylin–Eosin stains. For immunohistochemistry, additional sections were cut. We used primary antibodies: CPP 32/Caspase 3 IgG1, monoclonal, clone JHM62, Novocastra NCL-CPP32, Caspase 9 IgG1 kappa, monoclonal, clone 2C9B11, Novocastra NCL-CASP-9, Cathepsin B IgG2b, monoclonal, clone CB131, Novocastra NCL-CATH-B and TRADD IgG1 kappa, monoclonal, clone 18A11, Novocastra NCL-TRADD. Positive controls were used, according to the specifications: for caspase 3 – tonsil, for caspase 9 – large bowel, for cathepsin B – skin and for TRADD – EBV-positive Hodgkin's disease. Sections untreated with primary antibodies served as internal negative controls. Successive sections were immunostained each with a different antibody. Standard ABC technique of the Streptavidin–Biotin complexes was used and, finally, sections were counterstained with Hematoxylin.

The microscopic slides were analyzed and micrographs were taken and scaled using a Zeiss working station, which is described elsewhere [14, 15].

Results

Nodal tissue in all diabetic samples was characterized by diffuse interstitial fibrosis and chronic ischemic lesions; often found were pericellular edema, with cytoplasmic retractions of the nodal myocytes from the connective stroma, nuclear damage karyorrhexis, foci of advanced, irreversible ischemic necrosis cell lysis, with ruptured plasmalemmas, huge intra- and pericellular edema and important karyolysis, and isles of relatively morphologically normal myocytes. Immunostaining for caspase 3 (C3) revealed two main patterns of lesions of nodal myocytes NMs (Figure 1): (*i*) there were strongly C3-positive NMs which either had altered or missing nuclei, and (*ii*) NMs with "ghostly" appearances, associated in variable degree with the previous morphological aspect, all embedded within a fibrous stroma. The latter NMs presented a discrete-to-absent C3-positive reaction suggesting to be cells at the end of the cell-death cascade involving C3. There were also necrotic NMs with important calcium deposits. C3-positive NMs presented various stages of nuclear shrinkage (Figure 2), from blebbing to karyorrhexis. Swollen NMs with autophagolytic patterns were also identified being either C3-weakly positive or C3-negative.

On the slides immunostained for caspase 9 (C9), of the samples of sinus node, the following histopathological pattern was identified: a the nodal core, centered by the sinus node artery, had a higher degree of perivascular and nodal fibrosis with foci of calcification, with a moderate C9-positive immunoreaction; b the nodal mantle kept better the myocytary architecture and it was strongly C9-immunostained (Figure 3, A and B). Such topographically different pattern of C9 immunostaining was not noticed in the atrioventricular node where almost all AV node cells appeared ghostly and intensely C9-positive (Figure 3, C and D). Specifically, the C9-positive NMs appeared with a highly immunostained peripheral halo, mostly but not exclusively eccentric (Figure 3). All nodal cells, either nucleated or ghostly were all positively immunostained with cathepsin B (Figure 4). Sinus node artery often appeared to be affected with intimal and medial lesions (Figure 4). TRADD immunolabeling was negative on all samples.

Control samples presented a decreased level of nodal fibrosis and negative caspase -3, -9, and cathepsin B immune reaction.

Discussion

Apoptosis has fundamental roles in the development of the electrical conduction system. The sinus node, atrio-ventricular node, and the bundle of His all suffer major anatomical transformations soon after birth in which apoptosis plays major roles. Sinoatrial node remains practically unchanged from the middle of the first trimester until first two weeks after birth [16], when its structure shifts from a mass of P-cells distributed around a large sinoatrial artery into a network of transitional cells connecting small groups of P-cells [17], process which will end normally in the first few years of life. A process

of symmetrical fibrosis occurs simultaneously – the collagen fibers within the sinoatrial node become denser and thicker, but their appearance remains symmetrical, suggesting a controlled process in which apoptosis seems to play an important role. AV node and His bundle suffer a similar process – their adult characteristics are formed throughout a slow, controlled process in which apoptosis plays important role [18, 19].
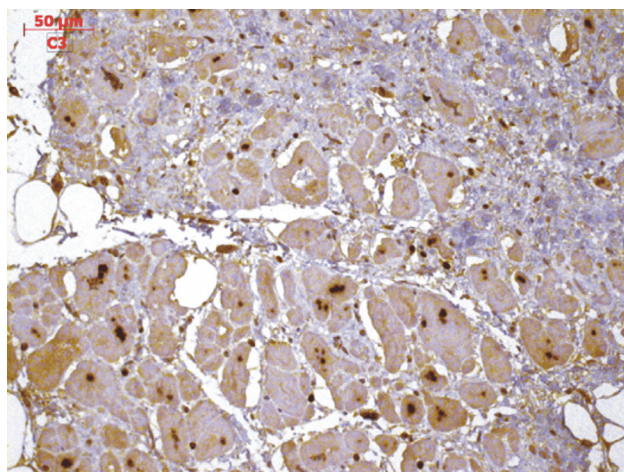

Figure 1 – *Sinus node, human adult diabetic heart. Nodal myocytes immunopositive for caspase 3 and nodal "ghost cells" devoided of nuclei.*
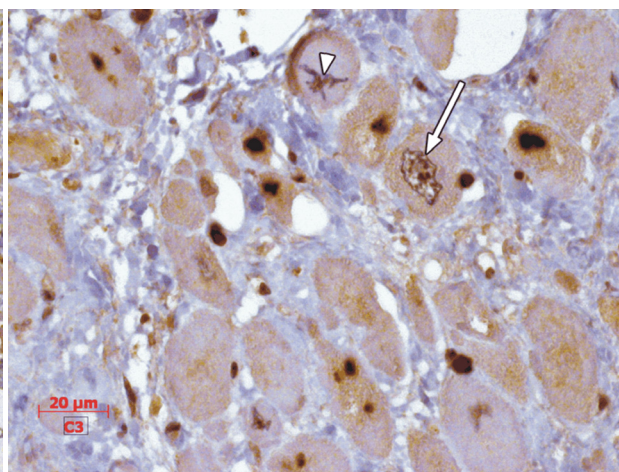

Figure 2 – *Sinus node, human adult diabetic heart, immunohistochemistry for caspase 3. Extensive interstitial fibrosis and immunopositive nodal myocytes, with nuclear blebbing (arrow), karyorrhexis (arrowhead) and nuclear dissolutions leading to anuclear necrotic nodal myocytes "ghost cells".*
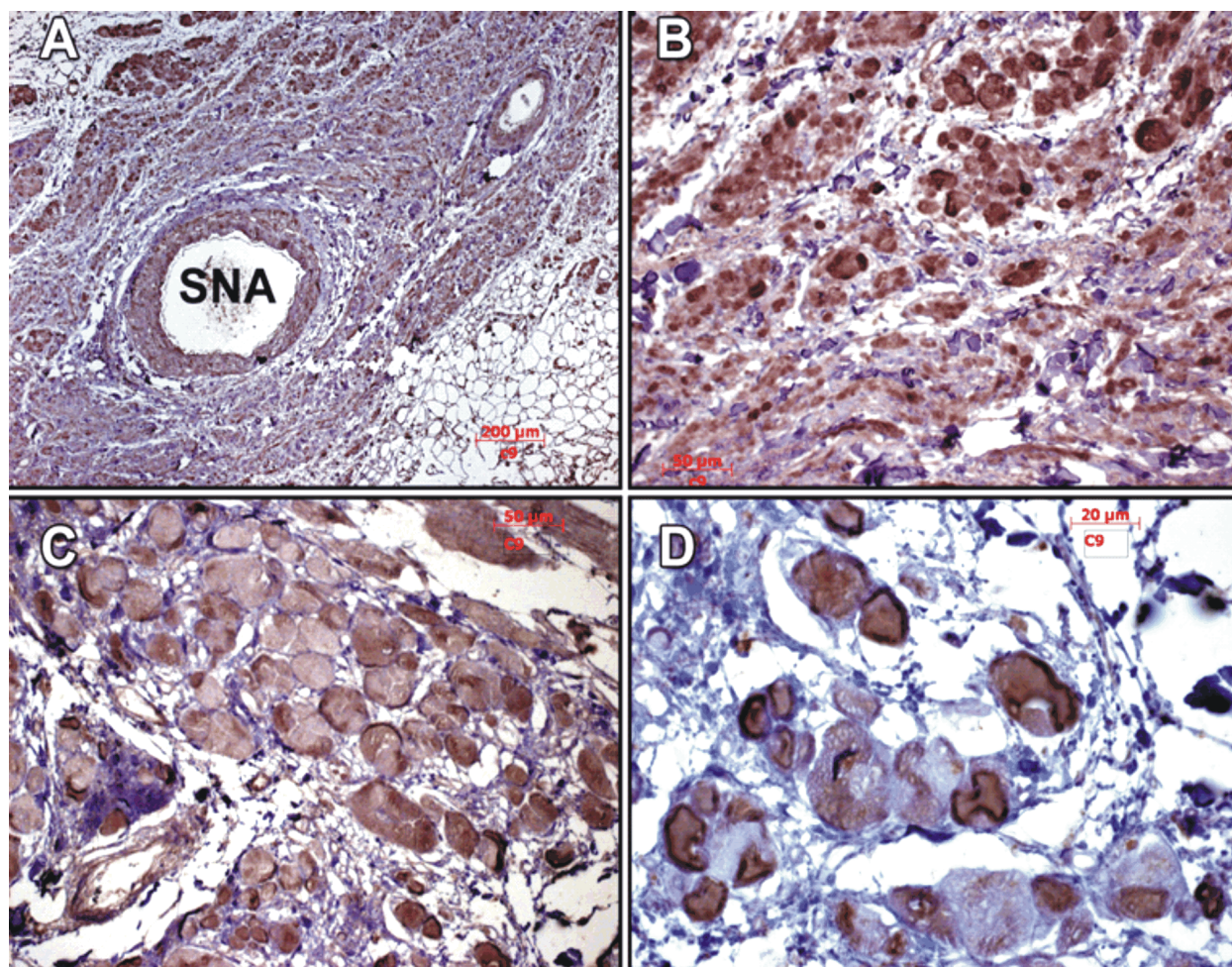

Figure 3 – *Immunohistochemistry for caspase 9. (A and B): Immunopositive sinus node myocytes, in the mantle of the node the architecture of the nodal myocytes is better preserved; the nodal core presents an important interstitial fibrosis, with small foci of dystrophic calcification. (C and D): Immunopositive atrioventricular node myocytes, with a high degree of fibrosis. SNA: Sinus node artery.*
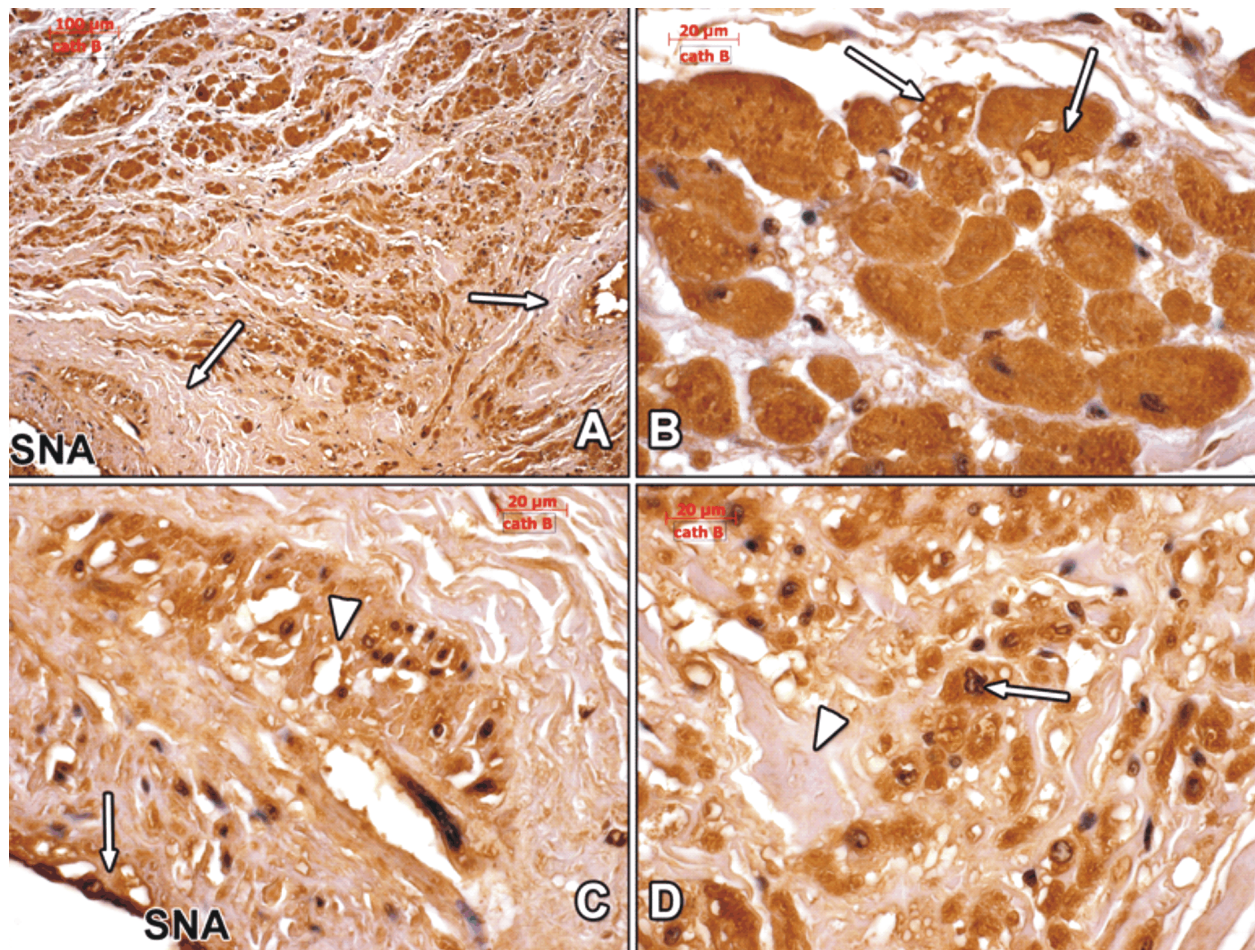
Figure 4 – *Sinus node, human adult diabetic heart, immunohistochemistry for cathepsin B. Immunopositive nodal myocytes of a sinus node presenting important perivascular fibrosis (A, arrows) at the level of the sinus node artery SNA. The architecture of the nodal myocytes is better preserved in its mantle but it does not exclude the presence of autophagolytic processes with the activation of cathepsin B (B, arrows). Intimal C, arrow and medial (C, arrowhead) lesions, with vacuolizations and positive immunoreactions are evidenced in the wall of the SNA. Nodal sclerosis is more pregnant in the nodal core (D, arrowhead) where nodal myocytes with shrunken nuclei arrow, immunopositive for cathepsin B, are identified.*
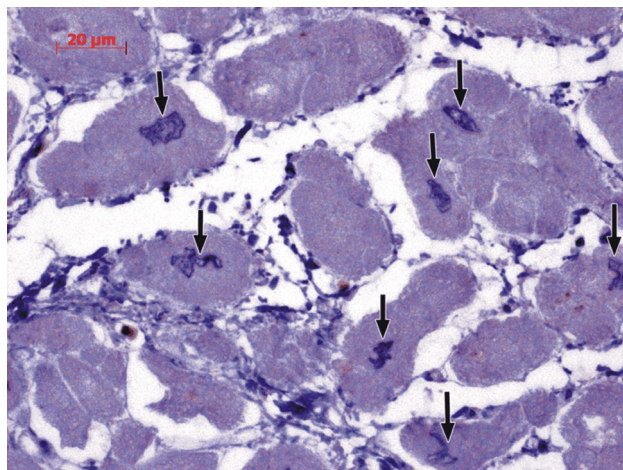

Figure 5 – *Shrunken nuclei (arrows) of sinus node myocytes immunonegative for TRADD, human adult diabetic heart.*
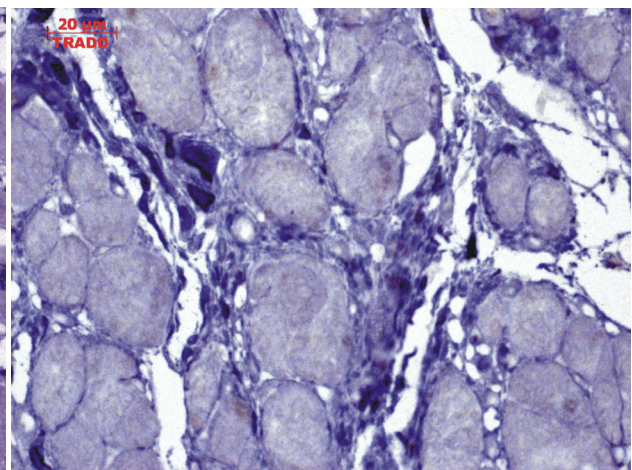
Figure 6 – *Atrioventricular nodal myocytes immuno-negative for TRADD, human adult diabetic heart.*

In adulthood, increased apoptotic activity in the electrical conduction system was usually studied in relation with either AV-blocks or long QR syndromes. In a syndrome characterized by gradually progressive development of heart block ending with fatal arrhythmias [20], James TN *et al.* found a total absence of the AV node, virtually absent myocytes in the internodal and interatrial pathways and extensive destruction of the sinus node, normal His bundle and ventricular myocardium; each affected structure contained abundant apoptosis [21].

James, working on sinoatrial nodes supplied by Bokeriia [22] from patients with long QT-syndrome [23] found the following: narrowed small sinoatrial arteries, degenerated neural elements both nerves and ganglia in and near the SN without associated inflammation, typical apoptosis affecting nodal cells, pleomorphic micromitochondriosis [24]. Again James, working on the heart of a two-month old child who died of intractable right ventricular failure Uhl anomaly [25] associated with complete heart block found a normal AV node, separated from an intensely apoptotic His bundle by a dense band of collagen fibers; the right ventricular myocardium was completely destroyed throughout apoptosis [26]. Frustaci A *et al.* found an association between increased apoptotic activity measured by the TUNEL method in Brugada patients with SCN5A mutation carriers [27]. A direct correlation with the electrical conduction system has not yet been made but this correlation seems plausible. Ottaviani G and Matturri L, researching the histology of the electrical conduction system in sudden intrauterine unexplained death SUIUD, found increased apoptotic activity in the AV node, bundle of His and initial tract of the bundle branches, but the difference between SUIUD and normal controls was not significant [28].

In acquired cardiovascular pathologies, the apoptosis in the electrical conduction system did not get as much attention as in the above-mentioned situations, as it was usually correlated with myocardial remodeling after acute or chronic coronary or cardiac events. Gottlieb RA *et al.* [29] described cardiomyocytes apoptosis in ischemia/reperfusion injuries in rabbit hearts; later studies found apoptosis to be a specific characteristic of reperfusion injuries as compared with necrosis [30], which usually characterizes acute, anoxic myocardial injuries, subsequently appearing more frequently in ischemia/reperfusion injuries compared to ischemic only injuries and in border zones of acute myocardial infarction AMI, where the cardio-myocytes are less affected by the acute central anoxia which leads to myocardial necrosis [31]. Cardiomyocytes apoptosis occurs months after an acute myocardial infarction [32], with a higher rate in the peri-infarcted area [33, 34] and lower in remote myocardial areas higher though than in normal controls, which slowly decreases over time and leads to ventricular remodeling with progressive chamber dilatation, wall thinning, systolic and diastole dysfunction, increased electrical heterogeneity, and increased frequency of ventricular tachyarrhythmias. These events are caused mostly by the replacement of death cardiomyocytes with fibrous/adipose tissue [35–37], although other mechanisms like caspase leakage from apoptotic cells with a consecutive decrease in contractile activity of normal cardiomyocytes are involved as well [38]. Recently, apoptosis associated with myocardial infarction was found in other cardiac cells as well in neutrophils, possible playing an important role in their disappearance after AMI [39, 40], in mononuclear cells, vascular endothelial cells and myofibroblasts from the granulation tissue in subacute myocardial infarction, etc. [3, 41].

In heart failure, apoptosis is increased throughout many mechanisms – ischemia/reperfusion injuries, cellular calcium overload, increased free oxygen radicals, p53,

Fas-ligand, angiotensin and AT1 receptors, catecholamine levels, TNF-α, ANF/BNP, volume and pressure overload of cardiomyocytes, decreased coronary reserve [42], etc., most of them being caused by either the subsequent cause/s of CHF AMI, arterial hypertension, mitral regurgitation, diabetic cardiomyopathy, CMM, DCM, etc. or compensatory mechanisms left ventricular hypertrophy, left ventricular dilation, increased RAAS activity, chronically increased intracellular calcium, increased sympathetic response. Increased apoptotic activity in the insufficient heart is associated with increased remodeling; therefore, any drugs, which can interfere with either apoptosis or its causes, can decrease cardiac remodeling and subsequently decrease the severity of CHF. Hyperglycemia is known to increase cardiac concentration of reactive oxygen species, which leads to abnormal signal transduction, abnormal gene expression and apoptosis *via* p53 and the activation of the cytochrome-c-activated-caspase-3 pathway [43, 44]. It also decrease VEGF expression in the myocardium, leading to a decreased capillary density in the myocardium [45], increased endothelial cell apoptosis, increased myocardial ischemia and ischemia-reperfusion associated apoptosis.

Our study found a positive reaction for caspase-3 and -9 in nodal cells in diabetic patients, which, associated with a TRADD-negative phenotype, suggests activation of the intrinsic mitochondrial apoptotic pathway. Also, at least partially apoptosis and necrosis are active in the same time, as suggested by the activation of cathepsin B; cathepsin B-positive "ghost cells" may be either viewed as the result of a coagulative necrosis or of a secondary necrosis associated with irreversible apoptotic processes. It was shown that like loss of plasma membrane integrity, lysosomal membrane disintegration also coexisted with caspase-3 activation in the same cell at early hours of ischemia. Cathepsin-B spilled into cytoplasm may contribute to development of necrotic features by digesting structural and functional proteins, whereas it reinforces apoptotic mechanisms such as Bid cleavage and caspase activation that are also activated by several other factors in ischemic cells [46].

It was shown in mice that after cerebral ischemia early and concurrent increase in caspase 3 and cathepsin B activities were followed by the appearance of caspase-cleavage products, DNA fragmentation, membrane disintegration and it was suggested that subroutines of necrotic and apoptotic cell death are concomitantly activated in ischemic neurons and the dominant cell death phenotype is determined by the relative speed of each process [46]. As in diabetic hearts, angiopathy and ischemia are constant features, and taking into account the possible neural crest origin of the nodal tissue [47] our results reasonably support a similar concomitant mechanism within the nodal tissue. However, debate continues as to whether specialized cardiac tissues have a neuroectodermal-derivation neurogenic cells or differentiate from populations present in the cardiogenic mesoderm cardiomyogenic cells [48].

Sinus node is heterogeneous in terms of its electrical activity. Evidences in rabbits suggest that from the periphery crista terminalis to the sinus node center there are changes in the shape of the action potential,

pacemaking activity, ionic current densities (*e.g.*, Na^+ channel density decreases from periphery to center), connexin expression, Ca^{2+} handling, myofilament density minimum within the electrical nodal center [4] and cell size [49]. Electrical and histological nodal center/periphery should not be confused, the latter being termed as core and mantle. The only correspondence is that the electrical center of the node located within the center of the nodal core, where the highest density of P cells is found. The mantle layer of the sinus node was on our preparates better preserved architecturally and structurally, while the nodal core showed more advanced irreversible damage with a centripetal advance of the irreversible lesions. As time, cells are still apoptotic interventions with anti-apoptotic agents may be of benefit.

These mantle-to-core differences seem justified as the blood supply of the sinus node is better ensured by the peripheral nodal networks [50]; as so, the anatomically better peripheral vascular supply of the sinus node may be compared in some degree with the reperfused border zone of human infarcts, where apoptosis was also proved to be present [51]. The core necrosis relies on an ischemic scaffold while mantle apoptosis correlates with a better vascular supply.

We do not intent to relate exclusively the cell death events of the nodal tissue with the presence of diabetes because these may be, at least partly, unspecific or age-related. It was shown for example that sinoatrial node firing is reduced with age and fibrous tissue is increased in aged atria [52]. But, no matter the age-related changes, nodal apoptosis is, if not hyperglycemia-induced, at least hyperglycemia-augmented. Hyperglycemia seems to be central to the pathogenesis of diabetic cardiomyopathy [53]; the processes associated with this cardiomyopathy are not mutually exclusive and likely act synergistically and also the nodal cell death must be considered among these.

As ischemia/reperfusion injury due to cardioplegic arrest inflicts significant damage on subendocardial myocardial conduction cells, but not on working myocardium and the structural and ultrastructural findings are consistent with coagulation necrosis, rather than apoptosis [54], it may be also presumed that apoptosis of the nodal tissue in diabetic hearts is consistently determined by hyperglycemia.

⊟ Conclusions

Both apoptotic and necrotic mechanisms are involved in the cell death of the cardiac conduction system. Apoptosis is executed on the intrinsic pathway and involves the caspases 3 and 9. Considering these changes, it appears important nodal cell death to be strongly taken into account in diabetic and aged patients.

Acknowledgments

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