Molecular Pathology of Cardiac Diseases Liable to Cause Sudden Death

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A sudden death (SD) always has a tragic impact both on families and on the general community. Although cardiac diseases liable to result in SD are mostly well identified, the etiopathogenetic understanding of most of these disorders is poor. To improve presymptomatic diagnosis, prevention and treatment, we need to define these disorders at the molecular and cellular levels.

This chapter will address the general principles of molecular pathology in cardiovascular medicine. The most common pathological substrates of SD and the current knowledge of the molecular basis of cardiovascular diseases liable to result in cardiac arrest will be reviewed as well.

Molecular Biology in Cardiovascular Medicine

The field of molecular biology was considered to have started in the 1950s, with the discoveries of the structure of DNA, the structure and mechanism of tRNA and the breaking of the genetic code [1-3]. The field has now entered its golden era with the development of recombinant technologies which are now utilized in virtually every subspeciality of diagnostic medicine and pathology [4]. Wide application of these techniques results from their sensitivity, specificity, speed, and relatively inexpensive cost. Although the ethics and economics of some molecular tests will spark intensive discussion, recombinant DNA technologies are likely to play an ever-increasing role in disease diagnosis and pathogenesis.

Unfortunately these new technologies were not immediately adopted in the field of cardiovascular disease. The reasons for this reluctance were mainly due to some organic features of the heart: (1) adult myocytes are differentiated cells, no longer capable of proliferating and thus not of primary interest in molecular biology; (2) most genomic mutations associated with hereditary cardiopathy usually cause lethal diseases, giving little access for future molecular approaches; (3) tumors, which are uncontrolled forms of proliferation and quite useful for pro-

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A. Raviele (ed.), *Cardiac Arrhythmias 1999 - Vol.1*

viding information regarding development, are very rare in the heart; (4) it has only recently become possible to obtain intact DNA and RNA using fresh cardiac tissue for in vivo studies through endomyocardial biopsy. Since the 1980s molecular biology techniques have been used more and more accurately, offering unprecedented opportunities for improved diagnosis, detection, prevention and treatment of various forms of cardiovascular disease [5].

The most important subfields in which these techniques are successfully used are summarized in Table 1.

Table 1. Recombinant DNA technologies: major applications in cardiovascular fields

In vivo morpho-functional analysis of protein
Production of proteins present in low quantity and generation of new specific drugs
Sensitive and specific detection of different pathological processes using molecular hybridization assays and new molecular techniques
Identification and isolation of disease-causing genes (molecular genetic)
Better understanding of molecular cardiac development

Structural-Functional Analysis of Protein and Generation of New Specific Drugs

Before the widespread use of recombinant DNA technologies the protein function was tested only in an indirect way: protein isolation from tissue, purification and in vitro evaluation of its biochemical kinetics (studying its affinity for the substrate and production of each new protein).

Up to today the function of a specific protein could not be tested precisely. Nowadays using recombinant DNA technologies (such as molecular cloning and gene sequencing) it is possible to modify the gene which codes the specific protein to produce a non-functioning protein or on the contrary, to induce greater expression using a promoter. Cultured cells have typically been used to study in vivo mutant forms of proteins. However, for some proteins, adequate cell culture expression systems do not exist, and their expression in transgenic animals has now become an attractive alternative.

Other studies, conducted to perform structural-functional analysis of proteins in vivo, addressed the ionic sodium, calcium and potassium channels. The specific mRNA for the proteins of a given ionic channel is isolated, cloned and injected in the oocyte and then the ionic flow monitored using the "path clumping" technique.

Recombinant DNA technologies can quickly develop specific drugs with minimal or no collateral effects by transforming and specifically defining the biological properties of a protein or part of one in vivo. Tissue plasminogen activator (t-PA), used for thrombolysis in myocardial infarction, represents just one of the most important and well-known examples. It is the result of the genetic fusion of

three genes: one codes a portion of the antibody (Fab) against fibrin, a second codes the Fab of antibodies against the tissue type plasminogen inhibitor (t-PA), also known as PA1, and a third codes the catalytic unit of recombinant tissue type plasminogen activator (rt-PA) [6]. More than 40 different forms of t-PA have been produced in attempts to reduce its side effects and inprove its therapeutic efficiency.

Sensitive and Specific Detection of Different Pathological Processes Using New Molecular Assays

The principle of all molecular hybridization assays is the complementary base pairing between two nucleic acid strands. In situ hybridization (ISH) provides the direct detection of nucleic acid in cellular material in which simultaneous morphological analysis can be performed.

Since its introduction in 1985, the polymerase chain reaction (PCR) has led to a veritable revolution in molecular biology [7]. It has been referred to as the "molecular biologist's photocopying machine" as it allows millions of copies of any specific DNA sequence to be generated within a few hours. The reaction consists of an in vitro enzymatic amplification of a defined DNA sequence by repeated rounds of heat denaturation, primer annealing and DNA polymerase-mediated primer extension. The amplified DNA can then be seen as a distinct band after standard agarose gel electrophoresis, and the specificity of detection can be increased by subsequent hybridization or DNA sequencing. According to the nested PCR technique, a second pair of primers "internal" to the original primer pair is used in a subsequent series of amplification cycles. Using this strategy, the sensitivity is enhanced from 100 to 1000 times, to a such an extent that even a single copy target can be detected in a complex background of 300 000 cells or more. Recently a number of strategies have also been reported for carrying out nested PCR in a single tube [8, 9].

One of the most important and frequent applications of these novel techniques is for the identification of microbial pathogens. Given the extreme sensitivity of these techniques, particularly of PCR, a single copy of a gene can be readily detected from extremely small amounts of tissue, such as small fragments of endomyocardial biopsies. The significance of detection of viral genomes in heart tissue is reduced by latency, common to some viruses such as herpes viruses. Reverse transcriptase PCR of specific viral mRNA is usually performed to detect active viral replication, in other words the infective state of the virus. The decision to develop and apply PCR for routine diagnosis of myocarditis must be considered in relation to the low cost, speed, sensitivity and reliability of more conventional culture and/or serological methods [10]. PCR holds its greatest promise for extending diagnosis beyond simple infective agent detection. Important genetic characteristics such as virulence and responsiveness to chemotherapy are amenable to direct analysis by PCR. This area of endeavor is proceeding rapidly, and it is likely that many types of microrganisms will be categorized pathologically according to subtle genetic changes.

PCR may detect virtually all the common genetically inherited diseases in which the defective gene has been identified, such as Duchenne muscular dystrophy and hypertrophic cardiomyopathy [11, 12].

PCR can also trace the inheritance of diseases in which the defective locus has only been defined in terms of linkage to other cellular genes. Using PCR, allelic forms of many cellular genes can be identified by sizing selected introns between the coding exons [13]. This approach should provide much more detailed linkage studies than are presently possible using restriction fragment length polymorphism (RFLP) analysis.

Sequencing of PCR-amplified genes has led to the discovery of disease-associated single-nucleotide differences in the genes like those encoding histocompatibility antigens [14, 15] and various hormone and growth-factor receptor molecules. PCR-based analysis will undoubtedly lead to the identification of a vast array of genetically based diseases as well as provide insights into disease pathogenesis.

A variety of types of samples may be used in PCR. Extracted nucleic acid may be amplified with ease, even in a partially purified sample. Extracted DNA or RNA from formalin-fixed, paraffin-embedded samples obtained either at autopsy or at surgery are successfully used as templates for PCR. This could permit ever more frequent application, including in retrospective studies.

Apoptosis is a mechanism by which cells respond to damage by triggering a program of cell death [16-18]. Apoptosis has only recently been recognized as a component of many common cardiac pathologies such as chronic heart failure, viral myocarditis and ischemia [19-21]. Since apoptosis involves the fragmentation of chromatin, several researchers have used DNA polymerases or terminal transferases to end-label DNA strand breaks by the incorporation of biotinylated nucleotide in situ end labeling (ISEL) [22, 23]. Labeled nuclei are then identified by the addition of a streptavidin-peroxidase conjugate and an appropriate peroxidase substrate. These methods have several potential advantages: greater sensitivity (more nuclei are detected as being apoptotic), greater specificity (there is less equivocation since apoptotic nuclei are clearly marked); and easy quantitation (the labeled nuclei in tissue sections may be counted by automated image cytometry).

Identification and Isolation of Disease-Causing Genes

Through the application of recombinant DNA technology, the study of human genetic disorders has undergone a substantial surge in activity. Many cardiovascular diseases recognize genetic factors that can contribute to their pathogenesis. According to a survey of the human conditions caused largely by mutations in a single gene (currently more than 16,000 have been identified) [24], a large number (7-8%) affect the cardiovascular system. This suggests that potentially 5,000 or more genes are involved in the embryology, differentiation and maturation of cardiovascular structures and in all the processes that regulate cardiovascular function.

A genetic marker is a trait whose inheritance can be followed within a family. This trait becomes a marker for genes located within the neighboring chromosomal region. If an individual has inherited the marker from a given chromosomal region, by inference, the individual should have inherited all of the genes located within that chromosomal region.

DNA sequence variations between individuals are routinely ascertained using restriction enzymes. Each restriction enzyme recognizes and cleaves double-stranded DNA at a specific sequence of nucleotides. Variation in the presence of restriction enzyme sites results in the generation of different-size fragments of DNA (restriction fragment length polymorphism, RFLP). These fragments of DNA are detected by a procedure using gel electrophoresis, transfer of the separated DNA to a filter membrane, and hybridization to a radiolabeled probe (southern blotting) [25-27].

Genetic linkage may be defined as the nonrandom assortment of two DNA markers (defined as multiallelic polymorphic loci) within a family because of their physical proximity on the same chromosome. The closer two markers are within the same segment of chromosomal DNA, the less likely it is that a recombination event will occur between them and the more tightly linked they are. In any genetic linkage study, informative meioses are those that occur in an individual who is heterozygous at the marker loci. With their high level of polymorphism and their ability to detect both alleles in heterozygotes, RFLPs are particularly well suited for genetic studies. Linkage relationships (or the lack of them) are expressed mathematically as the logarithm of the odds (LOD score) in favor of linkage at given distance between the marker and the phenotype loci. LOD scores of 3.0 or greater are considered statistically significant, while LOD scores of ≤ 2.0 statistically exclude two markers as being linked. LOD scores between 2.0 and 3.0 are considered inconclusive. Several single-gene disorders have been linked to DNA markers over the last few years, such as those identified as causing dilated cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy and long QT syndrome.

Better Understanding of Molecular Cardiac Development

Cardiogenesis is one of the most critical steps in embryonic development.

A large body of genetically based studies have elucidated the principles and pathways that guide cardiovascular development in both invertebrate and vertebrate species. Different transcriptional regulators (GATA factors, MEF-2 family, etc.) have been found in specific steps of cardiac morphogenesis [28, 29] from the establishment of left-right asymmetry in the primitive heart tube, looping morphogenesis, right and left ventricular chamber specification, trabeculation, outflow tract septation, conotruncal development, functional maturation of the ventricular chamber, expansion of the compact zone and endocardial cushion formation. In this regard, a variety of genetic systems, including fruit flies (*Drosophila*), zebrafish, frogs, chicks and mice are used to identify and better understand the complex stages of cardiovascular growth and development [30].

Molecular Basis of Cardiovascular Substrates at Risk of Sudden Death

Whereas SD in the adult is mostly due to atherosclerotic coronary artery disease often associated with previous myocardial infarction [31, 32], a large spectrum of cardiac substrates may underlie SD in the young [33-37]. We recently calculated an overall prevalence of SD of 0.8/100,000 per year in the young [38]. Based upon the Veneto region study project on juvenile SD, cardiovascular SD accounted for more than 80% of the collected cases, and about one third of SDs were due to a congenital heart defect present since birth [37]. As to the pathophysiologic mechanism, cardiac arrest may be mechanical or arrhythmic in nature. Table 2 reports the main causes of cardiovascular SD in our series of cases collected since 1979 [38].

Table 2. Causes of sudden death in people aged ≤ 35 years in the Veneto region of Italy, 1979 to 1996. (From [38], modified)

Cause	Total	
Arrhythmogenic right ventricular cardiomyopathy	29	(10.8%)
Atherosclerotic coronary artery disease	45	(16.7%)
Anomalous origin of coronary artery	7	(2.6%)
Disease of conduction system	24	(8.9%)
Mitral valve prolapse	26	(9.7%)
Hypertrophic cardiomyopathy	17	(6.3%)
Myocarditis	22	(8.2%)
Myocardial bridge	7	(2.6%)
Pulmonary thromboembolism	4	(1.5%)
Dissecting aortic aneurysm	12	(4.5%)
Dilated cardiomyopathy	10	(3.7%)
Other	66	(24.5%)
Total	269	

Arrhythmic Sudden Death with Structural Heart Disease

Heart Muscle Disease

Hypertrophic cardiomyopathy. The natural history of hypertrophic cardiomyopathy is often marked by SD [39, 40]. Heart dysfunction appears more in the form of electrical instability than of impaired contractility. The reported incidence of SD is 2-4% a year in adults and 4-6% a year in children and adolescents. Risk factors are considered to be young age, previous syncopal episodes, a malignant family history, myocardial ischemia, sustained ventricular tachycardia on electrophysiological test and ventricular tachycardia on Holter monitoring [39]. A complex interaction occurs between left ventricular hypertrophy, left ventricular outflow pressure gradient, diastolic dysfunction and myocardial ischemia, which accounts for the great variability of clinical findings. Myocardial disarray, with myocytes spatially arranged in a chaotic manner, and interstitial fibrosis, represent an ideal substrate of inhomogeneous intraventricular conduction with potential reentry phe-

nomena [41]. Moreover, detailed pathologic studies on subjects dying suddenly demonstrated the superimposition of ischemic damage to the dysplastic myocardium, in the shape of myocyte necrosis and large fibrous scars mimicking healed infarction. The ischemic damage may occur in the absence of significant epicardial coronary artery disease, although small vessel disease as well as intramural course of the left anterior descending coronary artery have been noted [42]. The combination of myocardial disarray and replacement fibrosis has to be considered a highly malignant arrhythmogenic substrate in hypertrophic cardiomyopathy.

Recently, molecular genetic studies demonstrated that hypertrophic cardiomyopathy is a heterogeneous disease, with several missense mutations in genes encoding for proteins of the cardiac sarcomere [43]. Mutations in 7 sarcomeric protein genes have been identified in affected families (Table 3): β-myosin

Table 3. Genetic basis of cardiac diseases liable to cause sudden death

Disease	Locus	Gene	Reference
Hypertrophic cardiomyopathy	1q3	CTnT	Thierfelder et al. [48] Watkins et al. [55]
	3p	MELC	Poetter et al. [45]
	7q3*	?	MacRae et al. [50]
	11p11.2	MyPBC	Bonne et al. [53] Watkins et al. [54]
	12q23-q24.3	MRLC	Poetter et al. [45]
	14q11-q12	β-MHC	Jarcho et al. [44]
	15q2	α-TM	Thierfelder et al. [48] Watkins et al. [55]
	19p13.2-q13.2	CTn1	Kimura et al. [47]
Arrhythmogenic right ventricular cardiomyopathy	14q23-q24	?	Rampazzo et al. [64]
	1q42-q43	?	Rampazzo et al. [65]
	14q12-q22	?	Severini et al. [66]
	2q32.1-q32.2	?	Rampazzo et al. [67]
	3p23	?	Ahmad et al. [68]
Naxos disease	17q21	?	Coonar et al. [69]
Familial idiopathic VF	3p21-q23	SCN5A	Chen et al. [143]
X-linked dilated cardiomyopathy	Xp.21.2	Dystrophin	Muntoni et al. [81] Muntoni et al. [82] Milasin et al. [91] Ortiz Lopez et al. [92]
Barth syndrome	Xq28	G.4.5	Bione et al. [93]
Autosomal dominant dilated cardiomyopathy	1q32	?	Durand et al. [84]
	2p31	?	Siu [85]
	9q13-q21	?	Krajinovic et al. [86]
	10q21-q23	?	Bowles et al. [87]
	3p22-p25	?	Olson and Keating [89]
	15q14	Actin	Olson et al. [90]

Cont.

Cont. Table 3.

Disease	Locus	Gene	Reference
Conduction defect and dilated cardiomyopathy	1p1-1q1	?	Kass et al. [88]
LQTS (Romano Ward type)	3p21-p23	SCN5A	Wang et al. [131]
	4q25-q27	?	Schott et al. [135]
	7q35-q36	HERG	Curran et al. [132]
	11p15.5	KvLQT1	Wang et al. [133]
	21q22.1-q22	MinK	Splawski et al. [134]
LQTS (Jervell and Lange-Nielsen type)	11p15.5	KvLQT1	Neyroud et al. [136] Splawski et al. [137]
	21q22.1-q22	MinK	Schultze-Bahr et al. [138]
PFHB-I	19q13.2-q13.2	?	Brink et al. [97] De Meeus et al. [98]
Supravalvular aortic stenosis	7q11.23	Elastin	Ewart et al. [109] Curran et al. [110]
Marfan syndrome	15q15-q21.3	Fibrillin-1	Kainulainen et al. [145] Dietz et al. [146]

* associated with Wolff-Parkinson-White Syndrome

heavy chain on chromosome 14, cardiac essential myosin light chain on chromosome 3, cardiac regulatory myosin light chain on chromosome 12, cardiac troponin T on chromosome 1, cardiac troponin I on chromosome 19, α-tropomyosin on chromosome 15 and cardiac myosin-binding protein C on chromosome 11 [44-53]. Available data suggest that mutations in β-myosin heavy chain and myosin-binding protein C are more common than the others. Moreover, beside locus heterogeneity, there is marked allelic heterogeneity for all the identified genes and more than 80 different mutations have been reported, the majority being missense mutations. Mutations in different components of the sarcomere appear to produce the same phenotype expression. From the functional point of view, sarcomeric contractile performance becomes depressed, suggesting that myocyte hypertrophy could be a compensatory response. Some mutations have been reported to carry a benign significance, with a low risk for SD, whereas others are associated with a poor prognosis, thus explaining the existence of subgroups of families with a malignant history. Although data on genotype-phenotype correlations are still preliminary, it seems that the phenotype varies not only with the type of mutation but also within individuals carrying the same mutation. For instance, the arginine-to-glutamine mutation in 403 codon of β-myosin is associated with a poor prognosis, whereas the arginine-to-tryptophan mutation appears more benign [51, 52]. Moreover, the knowledge that myosin-binding protein C mutations appear to be associated with age-related penetrance in adulthood would have consequences for genetic counseling [53, 54]. As to the phenotype caused by troponin

T mutation, it appears to be associated with no or mild hypertrophy, up to 50% of nonpenetrance and a high risk of SD, even in the absence of severe left ventricular hypertrophy [55, 56].

Arrhythmogenic right ventricular cardiomyopathy. Also known as right ventricular dysplasia, this is one of the leading causes of SD in the young in our series [57-59]. Arrhythmogenic right ventricular cardiomyopathy may be a concealed abnormality in apparently healthy subjects. In terms of contractility, heart performance may be preserved since the left ventricle is usually spared. This explains why the disease is observed even in sports champions, manifesting only with minor symptoms like palpitations or lipothymia, and why the diagnosis is frequently missed in preparticipation screenings [38]. In these subjects the presence of ECG abnormalities, like an inverted T wave in the right precordial leads (V1-V3), increased QRS duration >110 ms, late potentials detected by high resolution electrocardiography and ventricular arrhythmias, even in the shape of single premature ventricular beats with left bundle branch block morphology, should raise suspicion of the disease and lead to further investigation. Imaging procedures, whether non-invasive or invasive, are useful in detecting structural and functional abnormalities of the right ventricle, such as bulging, wall motion abnormalities and dilatation [60]. Nuclear magnetic resonance, furthermore, is a very effective tool for tissue characterization and may help detect the fatty myocardial infiltration [60]. The disease is characterized pathologically by a peculiar myocardial atrophy with fibro-fatty substitution of the right ventricular free wall in an apparently normal heart. Histology reveals the disappearance (atrophy) of the right ventricular myocardium and the fibro-fatty or fatty replacement, with a wave-front extension from the epicardium towards the endocardium [59, 61]. The intraventricular conduction delay, resulting from the fibro-fatty replacement is a source of electrical instability, due to reentrant phenomena, in the shape of ventricular arrhythmias (premature ventricular beats, non-sustained or sustained ventricular tachycardia) with left bundle branch block morphology, indicating a right ventricular origin. Evidence of acquired, progressive cell death rules out a congenital heart disease. The disease is now listed among cardiomyopathies in the WHO revised classification [62].

A familial character has been demonstrated in nearly 50% of cases, with an autosomal dominant inheritance [63]. Even though a defective gene has not been identified so far, 5 different gene loci have been described, 2 of which are located in close proximity on chromosome 14 (14q23-q24 and 14q12-q22), a third locus located on chromosome 1 (1q42-q43), a fourth on chromosome 2 (2q32.1-q32.2) and the fifth on chromosome 3 (3p23) [64-68]. An autosomal recessive variant of arrhythmogenic right ventricular cardiomyopathy (ARVC) that is associated with woolly hair and palmoplantar keratoderma has been reported from the island of Naxos in Greece and linked to chromosome 17 (17q21), within the gene encoding a keratin, a reasonable candidate for this entity [69]. In the experience collected by Nava et al. in Padua, no linkage was found to the known chromosomal loci in 50% of families [70]. Thus, several genes seem to be involved, suggesting genetic

heterogeneity. A genetically determined atrophy might explain this cardiomyopathy, which might then be considered a myocardial dystrophy. The histological similarities with some skeletal muscular dystrophies, like Duchenne and Becker, favor this hypothesis.

Recently, apoptosis (genetically determined cell death) has been postulated to account for cell death [71, 72]. Evidence supporting this view has been collected both at autopsy and from biopsy material (Fig. 1). Interestingly, the presence of

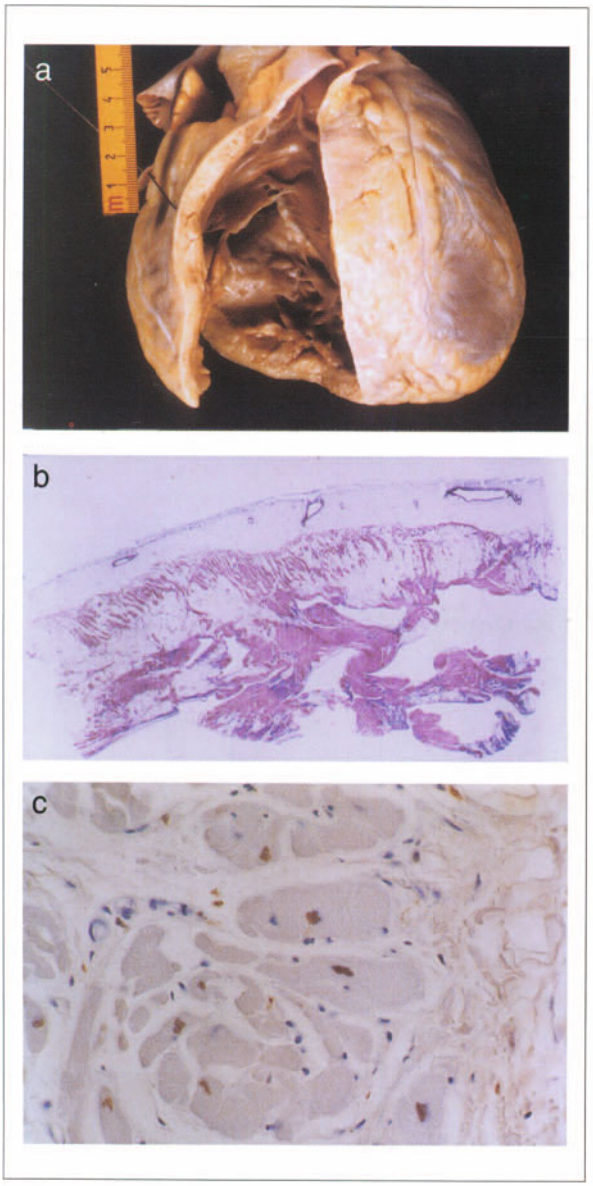

Fig. 1a-c. Sudden death due to arrhythmogenic right ventricular cardiomyopathy in a 28-year-old man with a previous history of syncopal episodes. **a.** Gross view: note the yellow appearance of the right ventricular free wall. **b.** Histology of the right ventricular freewall revealing massive fatty replacement. Azan Mallory, x 3. **c.** Photographs of TUNEL-stained myocardium: intensely dark stained nuclei of myocytes adjacent to adipose tissue. Original magnification x 300

apoptosis was found in the early and acute symptomatic phase of the disease [72].

Focal myocarditis with myocyte death was observed in all cases with fibrofatty variant: whether inflammation is primary or secondary to cell death remains to be established [59, 73]. Recent analysis using nested PCR for enterovirus failed to detect any viral genome in biopsies of affected patients with both recent and chronic clinical onset of the disease (Fig. 2). Focal, progressive cell death may lead to either fibrous or fatty replacement, with adipocytes taking the place of dying myocytes. Focal myocarditis, bouts of apoptosis, right ventricular aneurysms and left ventricular involvement most probably worsen ventricular electrical vulnerability and lower the ventricular fibrillation threshold [74].

Myocarditis. SD caused by myocarditis is not rare, particularly in the young [75]. The strongest evidence that subclinical myocarditis can be a cause of ventricular fibrillation comes from an autopsy series on USA army recruits in which 40% of those who died suddenly had histological evidence of myocarditis [76]. Myocarditis usually presents with signs of pump failure and ventricular dilatation. Nonetheless, ventricular arrhythmias have been described in patients with myocarditis and apparently normal heart [77, 78]. SD may occur in either the active or the heale phases as a consequence of life-threatening ventricular arrhythmias that mostly develop in the setting of an unstable myocardial substrate, namely inflammatory infiltrate, interstitial edema, myocardial necrosis and fibrosis. Previous symptoms may consist of flu-like illness a few days before death,

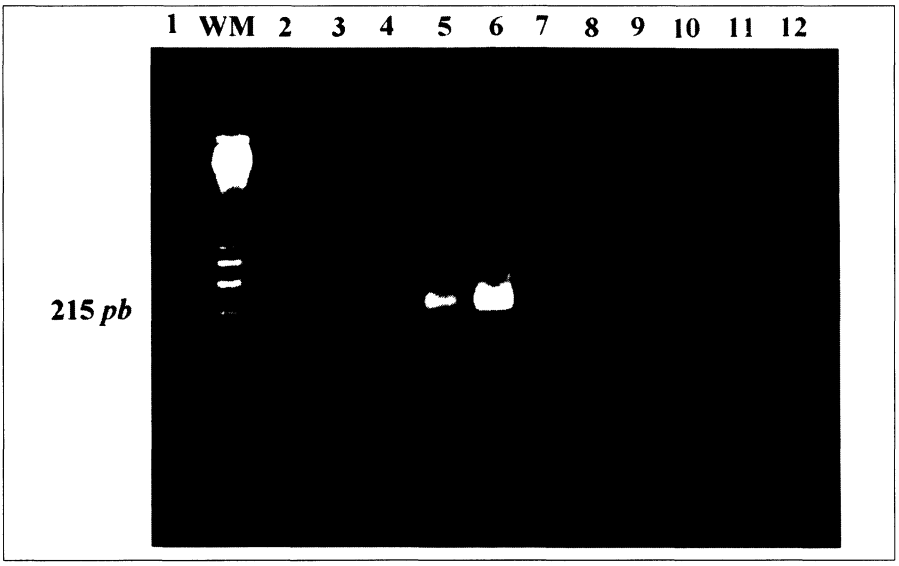

Fig. 2. Results of nested RT-PCR of endomyocardial biopsy tissues obtained from ARVC/D patients. Line 2,4,7-12: nine ARVC/D patients; line 5: coxsackievirus B3 lymphocytic myocarditis (positive control); line 6: coxsackievirus B3 infected cells (positive control); line 1: uninfected cells. (From [74] with permission)

syncopal episodes and premature ventricular beats. The gross appearance of the heart is not distinctive and its weight may be within normal values. Histology invariably discloses a patchy inflammatory infiltrate, sometimes no more than three foci at magnification x 6 and not necessarily associated with myocardial necrosis. The inflammatory infiltrate is usually polymorphous and less frequently purely lymphocytic. This subtle substrate, together with possible inflammatory involvement of the conduction system, is highly arrhythmogenic, accounting for unexpected arrhythmic cardiac arrest. Myocardial infection, whether bacterial or viral, has rarely been investigated. Noteworthy is the report of an increased sudden cardiac death rate among young Swedish elite orienteers with histopathological evidence of myocarditis and serologic demonstration of antibodies to *Chlamydia pneumoniae* [79].

Nonetheless, viral infections are the most plausible cause. Molecular biology techniques with PCR are now an essential tool and the gold standard for an etiological diagnosis. Application of gene amplification techniques is particularly useful in detecting viral nucleic acids in biopsies, especially when characteristic cytopathic changes cannot be observed on light microscopy, a rather frequent condition in acute fatal forms causing SD. Although enteroviruses are the most important causative agent in the pathogenesis of myocarditis, several studies have shown that various other viruses, such as adenovirus, herpesvirus (cytomegalovirus, herpes simplex virus, Epstein-Barr virus), parvovirus, influenza virus A and B, and hepatitis C virus can be involved in myocardial infective disease, particularly in the pediatric population (Fig. 3) [10, 80].

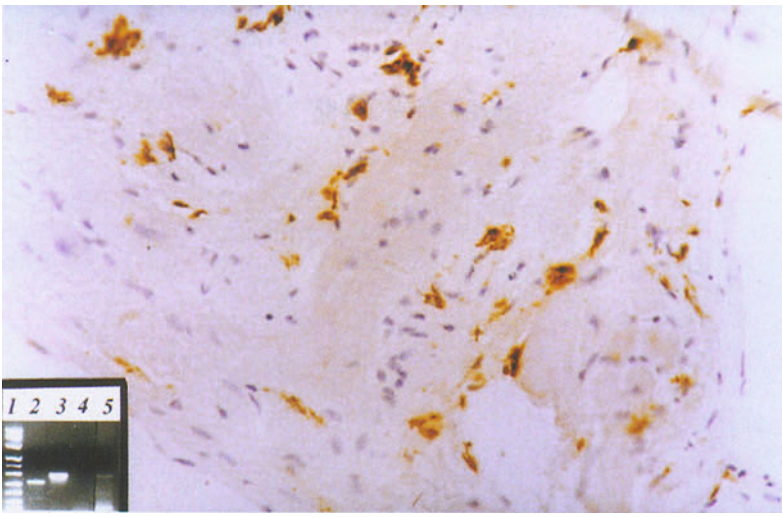

Fig. 3. Myocarditis in pediatric age. Numerous inflammatory cells stained strongly for UCHL-1 (CD45RO), demonstrating that the lymphocytes of the infiltrate are primarily T cells. *Insert:* PCR analysis for adenovirus: lane 1: molecular size marker, lane 2: β-globin amplimer ("housekeeping" gene), lane 3: adenovirus positive control, lane 4: negative control, lane 5: myocarditis

Dilated cardiomyopathy. Dilated cardiomyopathy is a genetically and clinically heterogeneous disease. The natural history demonstrates that death occurs not only due to progressive congestive heart failure or as a complication of thromboembolism, but also abruptly due to arrhythmic cardiac arrest. In these circumstances death is obviously expected and, according to definition, it should not be considered strictly as a true SD. However, in a few cases of dilated cardiomyopathy arrhythmic SD may be the first manifestation of the disease and the diagnosis achieved only at postmortem by observing a heavy heart with dilated ventricles and no inflammatory or coronary artery disease.

As to the molecular basis of dilated cardiomyopathy, at least 30% of cases are inherited, with a significant percentage of the remaining cases being acquired (i.e., myocarditis, autoimmune, etc.). Inherited forms may have autosomal dominant, autosomal recessive, X-linked, or mitochondrial transmission, with evident genetic heterogeneity [83].

Genes for the autosomal dominant form have been mapped to six different loci. Four of them are associated with "pure" dilated cardiomyopathy: 1q32, 2p31, 9q13, and 10q21-23 [84-87]; whereas the remaining two loci refer to dilated cardiomyopathy with conduction defects: 1p1-1q1 and 3p22-3p25 [88, 89]. Quite recently, cardiac actin gene mutation on chromosome 15q14 has been identified [90].

The genes for two X-linked cardiomyopathies have been identified and multiple mutations of both have been reported as well. They are the dystrophin gene (Xp.21.2), which is also responsible for Duchenne and Becker muscular dystrophy [91, 92], and the G 4.5 gene (Xq28) in the Barth syndrome [93]. Whereas the G 4.5 gene function is still unknown, dystrophin is a large cytoskeletal protein of the inner face of the sarcolemma, attaching itself to F-actin in the matrix and to the dystrophin-associated glycoprotein (DAG) complex, which is a transmembrane protein.

The persistence of a viral infection is thought to have a pathogenetic role in different chronic myocardial diseases of unknown etiology [94, 95]. Different molecular techniques have produced controversial results with respect to the rate of enteroviral positivity in myocardial samples from patients with dilated cardiomyopathy. Several studies have showed no or a very low percentage of enteroviral PCR positivity in patients suffering from the end stage of dilated cardiomyopathy (Fig. 4) [96]. Viral clearance or the involvement of other potentially cardiotropic viruses, such as human cytomegalovirus, coronavirus, adenovirus and hepatitis C virus, could explain the predominantly negative findings.

Conduction System Diseases

Progressive familial heart block. This is an autosomal dominant disease characterized by progression from a normal conduction pattern to bundle branch block and subsequently to complete heart block with wide QRS complexes [97]. Typical manifestations of the disease are syncope, SD and Morgagni-Stokes-Adams attacks. Treatment with pacemaker implantation is required. A study of 86 family members of three pedigrees, in which 34 members were affected, led to the

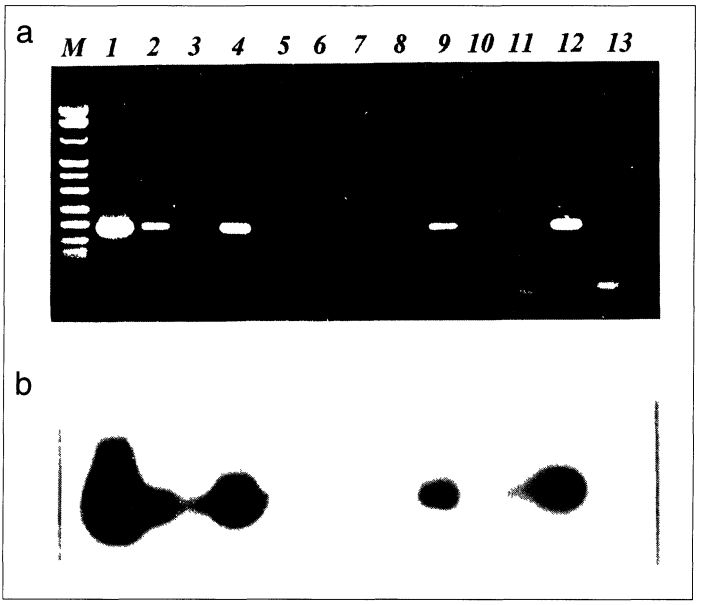

Fig. 4a,b. RT-PCR for enterovirus in native hearts of cardiac transplanted patients. Agarose gel electrophoresis (**a**) and its southern hybridization (**b**). M: molecular size marker Line 1: Coxsackievirus B3 infected cells (positive control), lane 2: case of DC (enterovirus +), lane 4: case of lymphocitic myocarditis (enterovirus +), lane 9 and 12: cases of lymphocitic myocarditis (coxsackievirus B3 + ,positive controls), lane3, 5-8 and 10,11 are cases of ischemic cardiopathy, dilated cardiomyopathy and valvular disease (enterovirus -), lane 13: uninfected cells (negative control). (From [96] with permission)

demonstration of genetic linkage to chromosome 19 (19q13.2-q13.3) and the gene was localized to within 10cM of the kallikrein locus [98].

Familial Wolff-Parkinson-White syndrome. Wolff-Parkinson-White syndrome is a disorder in which an aberrant myocardial fascicle joins the atria to the ventricles, beyond the specialized AV junction [99]. Known by the eponym of Kent's bundle, it consists of a 200- to 400-micron-thick structure, directly connecting the atrial with the ventricular musculature [100]. It is the smallest common congenital heart disease and affects 0.5-1‰ of live births [101]. Usually located in the lateral rings, especially the left AV one related to the attachment of the mural mitral leaflet, it consists of ordinary myocardium which does not possess decremental properties of specialized AV nodal conducting tissues. Due to the regular delay through the specialized AV junction tissue, the atrial impulse excites the ventricles earlier through the accessory pathway. The risk of SD in patients is low and mainly related to the occurrence of atrial fibrillation, which may convert into ventricular fibrillation due to the short refractoriness of the AV accessory pathway, which allows transmission of more than 300 impulses per minute to the ventricles [102]. In most of the cases, the disease is not hereditary, although some familial cases of Wolff-Parkinson-White have been reported, suggesting autoso-

mal dominant inheritance. In a single pedigree of 25 living members with either Wolff-Parkinson-White syndrome or hypertrophic cardiomyopathy, or both, the disorder was linked to chromosome 7 (7q3) [50]. It is unknown whether a single defect is responsible for both clinical pictures or whether two genes are located very close to each other (contiguous gene syndrome), thus frequently cosegregating. However, other associations of familial hypertrophic cardiomyopathy and Wolff-Parkinson-White syndrome have been identified, like that described in the cardiac troponin I gene on chromosome 19 [47], thus suggesting that Wolff-Parkinson-White syndrome may have several different pathogeneses.

Valve Diseases

Aortic stenosis. SD occurs in up to 20% of patients with aortic stenosis [103]. The risk of SD is usually confined to patients with a left ventricular/aortic gradient above 50 mmHg. Nowadays aortic stenosis is an uncommon cause of SD, because of improved identification of patients at risk, sport restriction and timely surgical intervention. Elevated ventricular systolic pressure and increased myocardial mass may both account for raised oxygen consumption and reduced coronary reserve and provide a substrate for myocardial ischemia, particularly in the subendocardium, even in the absence of coronary artery disease. Pathologic study of these SD cases usually discloses subendocardial ischemia in terms of myocytolysis and scarring, both well-known arrhythmogenic substrates. Exercise increases oxygen demand and the blood perfusion discrepancy, with the onset of lethal ventricular tachyarrhythmias. Whereas in the elderly aortic stenosis is usually the result of senile dystrophic calcification of the aortic valve or of a calcified bicuspid valve [104, 105], in the young it is mostly related to congenital valve malformations, like unicuspid or bicuspid conditions with dysplastic cusp stiffness [106].

Bicuspid aortic valve occurs in about 1%-2% of the general population and can be a predisposing factor for SD not only due to the increased risk of aortic dissection (see below) but also the risk of development of aortic stenosis [107]. Familial cases with autosomal dominant inheritance have been reported [108].

Supravalvular aortic stenosis occurs with a frequency of 1/25,000. A familial pattern with autosomal dominant inheritance has been reported. The disease has been linked to chromosome 7 and results from a defect in the elastin gene which causes an hour-glass obstruction of the ascending aorta and left ventricular hypertrophy [109, 110]. The phenotype is thus linked to gross DNA rearrangements in elastin. It is marked by elastosis of the aortic tunica media, intimal thickening and dysplastic cusps [111, 112]. Isolation of the coronary ostia, because of fusion of semilunar cusps leaflets with the aortic wall, as well as a stenotic intra-arterial course of the coronary arteries further aggravate the coronary ischemia in these patients [113]. Williams syndrome is an autosomal dominant disorder that is characterized by supravalvular aortic stenosis, peripheral pulmonary stenosis, obstructive coronary lesions, abnormal facies and mental retardation. This syndrome was found to be associated with deletion of a region

of chromosome 7 (7q11.23) that includes the elastin gene and is thought to be a contiguous gene disorder caused by the deletion of multiple adjacent genes.

Mitral valve prolapse. This has been reported to occur in 1% of the male and 6% of the female population. However, SD is rare, especially in people less than 20 years of age [114]. Unexpected death may rarely be a mechanical complication of valve function, with chordal rupture and pulmonary edema. More frequently it is a consequence of an abrupt electrical disorder in the form of ventricular tachycardia and fibrillation. It was postulated that elongated chordae or redundant valve leaflets, by rubbing against the ventricular endocardium, may elicit ventricular electrical instability and promote cardiac arrest. Hemodynamically significant mitral valve regurgitation, autonomic nervous system dysfunction, conduction system abnormalities as well as focal myocarditis have also been advanced as possible etiopathogenetic mechanisms [114]. Recently, histologic studies of the right ventricular myocardium disclosed significant fatty infiltration, especially at infundibular level, in a subset of patients dying suddenly [114, 115].

Familial mitral valve prolapse has been classified as an inherited connective tissue disorder and several studies suggest that the mode of inheritance is autosomal dominant. It may occur alone or in association with minor physical features such as pectus excavatum, straight back syndrome, long thin chest, long arms or joint hypermobility, but in the absence of other features of Marfan syndrome. Moreover, mitral valve prolapse is very frequent in patients affected by Marfan syndrome (see below), and it is also well recognized to occur in numerous other heritable disorders of connective tissue, such as Ehlers-Danlos syndrome, osteogenesis imperfecta and pseudoxanthoma elasticum [116, 117].

Coronary Artery Disease

The coronary artery pathology in SD adult victims consists of single, double or triple vessel atherosclerotic disease and usually includes a thrombotic occlusion of a coronary segment, which accounts for sharp interruption of the regional myocardial blood flow [31, 32]. By contrast, coronary SD in the young usually is due to a single subobstructive plaque, located at the first tract of the anterior descending coronary artery, mostly fibrocellular, devoid of atheroma, fissuring or thrombosis [36]. The preservation of the tunica media, the absence of thrombosis and the frequent occurrence of unexpected death at rest, following event of variant angina, are all features in keeping with a transient ischemic event, most probably ascribable to coronary vasospasm. In the setting of acute thrombosis, superficial erosion seems to be a peculiar mechanism precipitating plaque instability, unlike in the adult where it is mainly due to rupture of the thin fibrous cap [118]. Endothelial erosion may be the consequence of plaque inflammation and of intimal smooth muscle cell proliferation.

Several reports, using molecular hybridization assays, have shown a correlation between the incidence of atherosclerosis and the presence of infective microorganisms, like herpesviruses and *Chlamydia pneumoniae* [119-121]. Both

organisms have been identified in atheromatous lesions in coronary arteries and in other organs obtained at autopsy. Increased titers of antibodies to these organisms have been used as a predictor of further adverse events in patients who have had a myocardial infection. Atherosclerotic lesions were not reproduced experimentally in animals by injection of these microorganisms, a fact that leaves their role questionable as etiologic agents, according to Koch postulates. However, the possibility that infection, combined with other factors, may be responsible for the genesis of atherosclerotic plaques in some patients cannot be ruled out.

Genetic studies have shown that different genes are expressed in the disease and this could be an interesting factor in deciphering the complex nature of atherogenesis [122]. Because atherosclerosis is a multigenic disease, understanding the patterns of gene expression may help to explain varying susceptibility to agents causing disease as well as response to therapy. Studies in transgenic mice have revealed that Lp(a) lipoprotein, cholesterol ester transfer protein, apolipoprotein A (the principal apoprotein of high density lipoprotein), and other molecules have little effect on atherogenesis, whereas macrophage colony- stimulating factor appears to be important in the regulation of the numbers of monocytes and macrophages and in lesion formation [123, 124].

Some authors, using ISEL or DNA gel electrophoresis, have shown that apoptosis may modulate the cellularity of lesions that produce human vascular obstruction, particularly those with evidence of more extensive proliferative activity [125].

Arrhythmic Sudden Death Without Structural Heart Disease ("Idiopathic" Arrhythmic Sudden Death)

There are patients who undergo a cardiac arrest due to ventricular fibrillation without clinical identification of even a subtle structural abnormality [126]. Overall, sudden cardiac death remains unexplained in 5%-10% of cases, even after a thorough macroscopic and microscopic examination, including the conduction system and cardiac innervation [127]. In other words, no apparent organic substrate is detected by traditional investigations ("mors sine materia") and death is ascribable merely to an abrupt, functional disorder. Whether these cases are truly idiopathic or unexplained because of a clinical inability to identify pathologic substrates, remains to be elucidated. It may be that the structural abnormality resides at a molecular level.

Long QT Syndrome

The long QT syndrome (LQTS) is the best known congenital cause of arrhythmic SD in the absence of gross structural cardiac pathology [128]. It is a familial disease with high cardiac electrical instability, presenting with syncope due to ventricular tachyarrhythmias or with cardiac arrest on exercise or emotional stress, often under the age of 15. The cause of death cannot be ascertained at necropsy unless there are prior ECG data.

Genetic analysis reveals multiple abnormalities in genes related both to potassium and sodium cardiac channels. Alterations of ion pumps and current account for the lengthened action potential and prolonged QT interval on ECG, and the propensity to ventricular fibrillation. The mortality in untreated symptomatic cases exceeds 60% within 15 years. Clearly ECG screening of surviving relatives is the only way to establish the diagnosis in asymptomatic carriers [128].

On the basis of pattern of transmission, two major clinical syndromes have been described: the more common autosomal dominant form with a pure cardiac phenotype (Romano-Ward) [129] and the rarer autosomal recessive form characterized by the association with congenital deafness (Jervell and Lang-Nielsen) [130].

Five loci have been associated with the Romano-Ward LQTS and they are located on chromosome 3 (3p21-p23), encoding for the cardiac sodium channel (SCN5A); chromosome 7 (7q35-q36), encoding for the Ikr potassium channel protein (HERG); chromosome 11 (11p15.5), encoding for the α-subunit of the Iks potassium channel protein (KvLQT1); chromosome 21 (21q22.1-q22), encoding an ancillary subunit for the Iks channel complex (MinK); and chromosome 4 (4q25-q27); but the defective protein is still unknown [131-135]. Moreover, families linked to none of these genes have been described, thus suggesting the existence of other disease genes. Apart from a few mutations which are "hotspots", most of the mutations identified are missense mutations which are not confined to a single location but are frequently found at various positions within each gene in different families. It seems that this remarkable genetic heterogeneity contributes to the high variability of the clinical picture. The autosomal recessive variant of LQTS (Jervell and Lange-Nielsen) arises in patients who inherit abnormal KvLQT1 or minK alleles from both parents and expresses itself with especially long QT intervals. The abnormal allele can be the same or different ("compound heterozygosity") [136-138]. As a consequence, parents of subjects with Jervell and Lange-Nielsen variant carry long QT syndrome mutations, although most are asymptomatic.

As to the functional consequences of LQTS mutations, if mutations in KvLQT1, KCNE1 or HERG are expressed alone or with wild-type alleles in oocytes or in other lines, they exhibit "loss of function", thus resulting in a reduction of the total current carried by the defective channel complexes. On the other hand, SCN5A channel mutations cause a "gain of function" with an increased sodium current. With respect to genotype-phenotype correlations, the different time and voltage dependence of the ionic currents may in some way explain the variable phenotype [139].

Brugada Syndrome

A clinical and ECG syndrome, characterized by right bundle branch block with right precordial ST segment elevation and an apparently normal heart, has been described in cases of SD by Brugada and Brugada, unfortunately without postmortem reports [140]. These ECG characteristics may depend on exaggerated

transmural differences in action potential configuration, especially in the right ventricular outflow tract. Actually, Martini et al. [141] previously reported similar cases with apparent idiopathic ventricular fibrillation in which there was evidence of concealed right ventricular pathology. By studying a family with a case of SD, confirmation of an organic substrate was given recently by Corrado et al. [142], who reported not only fibro-fatty dystrophy in the right ventricular free wall but also involvement of the conduction system with sclerotic interruption of the right bundle branch. The coexistence of both "septal" and "parietal" right conduction defects might account for the ECG pattern of right bundle branch block and persistent ST segment elevation as well as ventricular electrical instability.

Conversely, in the absence of structural heart disease, the ECG abnormalities could arise from ion current dysfunction, such as I_{to}. L-type Ca^{2+} current [$I_{Ca(L)}$] and I_{Na}. Noteworthy is that at least one variant of the Brugada syndrome is caused by mutations in cardiac sodium channels gene SCN5A [143]. This is the same gene implicated in a form of long QT syndrome (LQT3), the mutation of which causes loss of function in the Brugada syndrome and gain of function in the LQT3. However, other families with Brugada syndrome have been tested showing no defects on the cardiac sodium channel, thus suggesting genetic heterogeneity analogous to that seen in other inherited heart diseases.

Mechanical Sudden Death: Aortic Rupture

This occurs as a consequence of spontaneous laceration of the ascending aorta with hemopericardium and cardiac tamponade. The basic defect consists of elastic disruption in the the tunica media and cystic medial necrosis leading to aortic wall fragility [144]. The disease is rarely isolated in the young, being usually associated with a genetic or congenital anomaly, like Marfan syndrome, isthmal coarctation or a bicuspid aortic valve [37].

Although many cases have an equal severity of tunica media degeneration, only in *Marfan syndrome* has a genetic defect been discovered, mapping to chromosome 15q15-q21.3 [145, 146]. The disease is familial in the majority of patients, whereas 30% are sporadic. The defective gene encodes fibrillin-1, which is the major constituent of microfibrils of the extracellular matrix. The heart in Marfan patients who die suddenly because of aortic dissection (usually type I-II with rupture within the pericardial cavity) exhibits typical cardiovascular features consisting of mitral valve prolapse, annulo-aortic ectasia, with or without fusiform aneurysm of the ascending aorta, and aortic incompetence. Nonetheless, aortic dissection in Marfan syndrome may also be observed without dilatation of the aorta, so that its occurrence may be unpredictable on clinical grounds.

Familial aortic dissection, in the absence of Marfan stigmata and hypertension, has been reported rarely and no defective gene has yet been identified [147].

The association between an isolated *bicuspid aortic valve* and dissection is not incidental. Indeed, the incidence of bicuspid aortic valve among those with aortic dissection is significantly higher than in the normal population (12% vs 1%) (148-150). The rupture involves a severely degenerated ascending aorta, with

or without dilatation, in the setting of a normally pliable bileaflet valve [37]. Although dissections have been reported amongst the offspring of individuals with a bicuspid aortic valve, familial SD has not been proven. Considering the frequency of a bicuspid aortic valve amongst the general population, the risk of dissection is quite rare. Most probably, only in a subpopulation of patients with a bicuspid aortic valve is medial necrosis present. Echocardiographic monitoring of the aortic root in individuals with this anomaly may detect progressive aortic dilatation as a marker of underlying vessel wall degeneration and impending rupture, thus providing indirect evidence of aortic wall fragility [151, 152]. One may wonder whether a bicuspid aortic valve and medial necrosis are the phenotypic expressions of the same genetic disease or simply a congenital heart disease complex, in which the maldevelopment involves either the aortic valve or wall, which both derive from the neural crest [153, 154].

This seems to be the case in *isthmal coarctation* (so-called adult coarctation), which is also associated with a bicuspid aortic valve in 50% of cases and in which aortic dissection frequently occurs in the natural history. An equal severity of medial necrosis in spontaneous aortic rupture has been reported in Marfan syndrome, isolated bicuspid aortic valve or isthmal coarctation, with or without a bicuspid valve. A relationship between the development of the aortic arch and neural crest has been proven by experimental embryologists [154].

More recently, apoptosis has been demonstrated to play a role in the progressive loss of smooth muscle cells of the tunica media in patients with aortic dilatation and congenital aortic valve malformation (bicuspid aortic valve), thus suggesting that premature smooth muscle cell apoptosis in the medial layer could be a part of a genetic program underlying aortic disease in patients with aortic valve malformations [155].

Conclusions

In conclusion, the pathologist's role should not simply be to establish whether SD is due to natural or unnatural causes. A careful postmortem investigation can be the source of vital information for the community, relatives and future generations. An accurate diagnosis of the underlying morbid entity and ultimate cause of death is the prerequisite to establish whether the disease is hereditary, thus representing the starting point for a wide-ranging investigation of the family members, as well as to assess the possible role of acquired etiologic factors such as infectious agents.

Acknowledgements. Supported by Veneto Region, Venice, Italy

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