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REVIEW

Apoptosis As an Instrument in Cardiovascular Development

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Cell death as a phenomenon in embryonic development was first described over 100 years ago. Approximately 30 years ago the process was named apoptosis, and its involvement is now recognized in many life processes, in virtually every animal species, and from fertilization to the death of an organism. In cardiovascular development, it coincides with major developmental processes in specific time windows. Both intrinsic (controlled by mitochondrial activity) and extrinsic (starting with death receptors) apoptotic pathways co-regulate developmental mechanisms. During cardiac development, many cell populations are recruited to the heart, where they differentiate into cardiomyocytes, fibroblasts, smooth muscle cells, endocardial and endothelial cells lining the inner surfaces, and epicardial cells lining the outer contours. In particular, neural crest-derived cell populations, which migrate to specific locations in the heart, are prone to apoptosis. During the complex geometric changes that occur in the primary heart tube and connected vessel segments, proper interaction of the respective cell populations guarantees the ensuing steps of differentiation. Growth factors, including endothelin, VEGF, and TGF-β, as well as other factors, such as FasL, play dominant roles in these phases. Transgenic and knockout studies have provided strong evidence for aberrant patterns of apoptosis resulting in congenital malformations and syndromic malformations, including septation anomalies, interrupted aortic arch segments, coronary anomalies, and DiGeorge syndrome. Embryonic remodeling of the arterial system, including the coronary arteries, is accompanied by apoptosis patterns, the disruption of which results in severe malformations. It is interesting to note that hemodynamic factors, such as flow-driven shear stress, regulate the expression of genes that are important for signaling molecules such as endothelin and NO-synthase. In general, high shear stress protects against apoptosis, thus preventing the onset of disease processes in the fully-grown vasculature, and regulating the remodeling of the vascular system in the embryo. **Birth Defects Research (Part C) 75:305–313, 2005.** © 2006 Wiley-Liss, Inc.

INTRODUCTION

The ability to regulate cell number is a crucial property of tissues and organs during embryonic development. The increase from the one-cell zygote to the many billions of cells in an adult human is tightly regulated and is the consequence of the balance between proliferation and programmed cell death or apoptosis. Dysregulation of apoptosis (both increases and decreases) can lead to severe congenital syndromes.

Spontaneous cell death as a phenomenon in embryonic development was first recognized more than a century ago (Loos, 1898, as cited by Pexieder, 1975), and was analyzed in later years in more detail (Ernst, 1926; Glücksmann, 1930, 1951, 1966). The latter studies related patterns of cell death to differentiation processes that occur at various hierarchical levels, such as histogenetic, morphogenetic, and phylogenetic cell death. Saunders (1966) was the first to analyze the regulation of cell death during important morphogenetic events. Nearly 30 years ago, cell death as an important phenomenon in heart development was described (Pexieder, 1975), and more than 30 locations and time frames were determined. The term "apoptosis" to describe programmed cell death in pathology was introduced by Kerr et al. (1972) and later expanded by Wyllie et al. (1980). More recently, van den Hoff et al. (2000) presented an overview of methods of detection and mechanisms in apoptosis, and Poelmann et al. (2000) reviewed patterns of apoptosis in the developing heart and attempted to relate them to morphogenetic events evoked by specific growth factors and cell populations. Cheng et al. (2002) described a spatiotemporal and tissue specific pattern of apoptosis in the embryonic chick heart.

APOPTOTIC PATHWAYS

Apoptosis is a cellular disintegration process that differs greatly from necrosis, which is a cytolytic mechanism whereby cells expel their contents into the surrounding microenvironment. In contrast, programmed cell death results in cellular collapse as part of a complex mechanism involving DNA fragmentation, cytoskeletal disruption, and metabolic breakdown in-

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side a structurally intact cell membrane. The caspase family of proteinases fulfills a critical role. The caspases are subdivided into two major groups based on their function. The proteolytic caspases (in mammals, caspase-3, -6, and -7) belong to the effector group that cleaves specific substrates. The activities of these effectors are initiated by the activator caspases (caspase-8, -9, and -10). Among species, tissues and pathologies overlap in substrate specificity, and interactions exist between many caspases; nevertheless, a common pattern of activation and inhibition can be found (Fischer and Schulze-Osthoff, 2005). Unchecked apoptosis is prevented by members of the inhibitor of apoptosis (IAP) family, which serve as endogenous suppressors of caspase activity (Salvesen and Duckett, 2002) by binding to caspase-3, -7, and -9. The many apoptotic stimuli converge into one of two major pathways (extrinsic or intrinsic). The intrinsic apoptotic program is largely controlled by mitochondrial activity, in which, for example, Bcl-2 is harbored as an inhibitor, and cytochrome c is harbored as a promoter of apoptosis. The mitochondria may release cytochrome c into the cytoplasm, which will bind to Apaf-1, forming the apoptosome complex. This complex supports the catalytic activation of caspase-9, activating the effector caspase-3, and finally resulting in the degradation of specific substrates. In addition, the mitochondrial proteins SMAC/DIABLO and Omi/HtrA2 are released and bind to the IAP family member XIAP, in a manner comparable to the binding to caspases. The caspase-inhibiting function of XIAP is countered, and thus apoptosis is promoted (Fischer and Schulze-Osthoff, 2005). The extrinsic death program usually does not require mitochondrial interference, but rather employs death receptors that harbor the death domain, including CD95, TNF-R1, and TRAIL. Upon activation by ligands (such as TNFα, TGFβ, etc.), binding is facilitated to adapter proteins, such as FADD. These are able to bind the activator caspase-8 or -10, thereby forming the death-inducing signaling complex (DISC). Subsequently, caspase-8 is activated, and it then cleaves and activates caspase-3. This is usually sufficient to execute the cell death program, although additional support from the intrinsic mitochondrial pathway, through caspase-8-mediated Bid cleavage (a pro-apoptotic member of the Bcl-2 family), appears to be required sometimes. Truncated Bid translocates to the mitochondria, where it induces the release of the above-mentioned mitochondrial substrates, thereby increasing the net effect (Fischer and Schulze-Osthoff, 2005).

CARDIAC DEVELOPMENT

Cell Interactions

To better elucidate the temporospatial pattern and the function of apoptosis and ensuing events in the mechanisms of cardiac development, a brief overview is presented regarding the formation of the heart.

The bilateral anterior tips of the unsegmented splanchnic mesoderm become defined as the cardiogenic plates, mainly by the interaction of the gene products of *GATA 4/5/6* (Laverriere et al., 1994) and *NKx2.5* (Jiang et al., 1999). After the cardiogenic precursors fuse in the midline, they differentiate into cardiomyocytes and endocardial cells. The continued addition of cardiomyocytes to the arterial pole takes place by activity of the adjacent splanchnic mesoderm, the secondary heart field (Mjaatvedt et al., 2001; Abu-Issa et al., 2002; Kelly, 2005). Furthermore, additional endocardial cells are recruited from the arterial pole endothelium (Noden, 1990). The cardiomyocytes become the contracting part of the heart, whereas the endocardium forms the inner lining of the heart tube. The space between the myocyte sleeve and the endocardium will be occupied by cardiac jelly. The endocardium gives rise to endocardial cushion cells (Ramsdell and Markwald, 1997; Person et al., 2005) by a process known as epithelial-mesenchymal transformation (EMT) (Hay, 2005), and as a consequence the cardiac jelly transforms into the extracellular matrix of the endocardial cushions. This is a complex process that requires many growth factors and various transcription factors. Some of these are involved in the apoptosis machinery, as can be concluded from knockout and overexpression studies. These include *TGFβ-2* (Bartram et al., 2001), *BMP-2* and *–4* (Keyes et al., 2003), and *Msx-2* (Abdelwahid et al., 2001). Keyes and Sanders (2002) studied the involvement of Bcl-2 family members and caspase-9 in the endocardial cushions of the developing chick embryo. They concluded that some of the dying cells derive from the neural crest (see below), as demonstrated previously (Poelmann et al., 1998; Poelmann and Gittenberger-de Groot, 1999), after they marked NCCs were marked with a *LacZ* reporter construct.

The atrioventricular cushions develop in the transition zone of the primitive atrium and ventricle (the so-called "A-V canal"), whereas the outflow tract (OFT) cushions develop in the conotruncal transition between the ventricle and the aortic sac. In the inner curvature of the heart, both cushion systems are in close proximity to each other. The OFT cushions will partly transform into the semilunar valve leaflets, but are also important in septation of the OFT into the aorta and the pulmonary trunk. The AV cushions partly transform into the mitral and tricuspid AV valves, and by heterol-

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ogous fusion with the interventricular or primary fold and the intra-atrial spina vestibuli, will play an important role in AV septation (Wenink et al., 1986). Apoptosis patterns are encountered in both the myocardium and the endocardial cushions at various time points in development, as discussed below.

Two other extracardiac cell populations—the cardiac neural crest and the pro-epicardial organ (PEO)—are very important in cardiogenesis. In both of these primordia the original epithelium transforms by EMT into migratory mesenchyme. Cardiac neural crest cells (NCCs) migrate from the border zone of the neural plate during early embryogenesis (in a chicken embryo, between Hamburger Hamilton stages 9 and 12) when the neural folds have not yet fused. The section of the neural crest dedicated to migrate to the heart is anteriorly bound by the otic placode and posteriorly by the third somite, i.e., deriving from the caudal rhombencephalon (Kirby et al., 1983). This was most prominently demonstrated by microsurgery and chicken-quail chimerization (Hutson and Kirby, 2003), and more recently in transgenic mice harboring a neural crest reporter (Waldo et al., 1999; Epstein et al., 2000; Jiang et al., 2000). The boundaries, however, have not been reliably established in a mammalian model. Subpopulations of NCCs migrate into the spinal ganglia, the pharyngeal arches (to form, e.g., the arterial vessel wall, which consists of adventitial fibroblasts and smooth muscle cells), the OFT cushions, the AV cushions, and the subepicardial ganglia and nerve cells. Apoptosis occurs frequently in the pharyngeal arches and the cushions in specific time windows.

The PEO starts as a small grape-like structure extending from the inner lining of the body cavity (the splanchnic mesoderm) and is located close to the liver primordium. It expands and migrates as a confluent epithelium over the outer surface of the initially nude myocardium (Virágh et al., 1993; Vrancken Peeters et al., 1995). After the heart tube is completely covered, part of the epicardium transforms into mesenchyme and the cells migrate into the heart wall to take up positions in various locations, such as the AV cushions, and throughout the cardiac wall to differentiate into cardiac fibroblasts and the smooth muscle cells and adventitial fibroblasts of the coronary vessels (Gittenberger-de Groot et al., 1998). In studies of the NCC reporter mouse, the possibility was raised that NCCs may form part of the coronary vessel wall (Poelmann et al., 2002, 2004). Poelmann et al. (1993) proposed that the endothelial cells of the coronary vasculature derive from existing microvasculature in the liver region, although others claimed that endothelial cells may also derive from the PEO (Perez-Pomares et al., 2002; Munoz-Chapuli et al., 2005). The coronary arteries connect to the aorta in a relatively late phase of cardiogenesis (Bogers et al., 1989), and apoptosis is encountered specifically in this area (Eralp et al., 2005).

Looping and Chamber Formation

The geometrical changes that occur during cardiogenesis are striking. A single, almost straight tube can transform into a double pump containing atria, ventricles, valves, and a conduction system. Processes such as looping of the primary tube, wedging of the OFT between the atria, ballooning of the chambers, muscularization of the fused endocardial cushions, atrial and ventricular septum formation, induction of the central and peripheral conduction systems, valve differentiation, and formation of the coronary vasculature are some of the more important highlights. During many of these processes, apoptosis is part of the differentiation mechanism, as will be described in detail below.

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A thorough description of the morphological changes that are important in terms of congenital anomalies was recently provided by Gittenberger-de Groot et al. (2005), and the genomic coding for chamber formation was reviewed by Moorman and Christoffels (2003).

After the two cardiogenic plates fuse in the ventral midline of the embryo, the heart primordium becomes more three-dimensional by folding into a hollow heart tube that remains connected to the dorsal body wall by the mesenchymal mesocardium. The mesocardium is disrupted locally, but will remain intact at the arterial and venous poles. At the venous pole, the longitudinal folding appears incomplete and the dorsal mesocardium protrudes into the atrial segment. This spina vestibuli is important for atrial segmentation. The cardiac tube, which is never completely straight, will loop in a complex three-dimensional curve, keeping the venous and arterial poles in close proximity, but allowing for longitudinal expansion of the tube and subsequent ballooning of the cardiac chambers. It must be kept in mind that longitudinal growth is also brought about by activity of the secondary or anterior heart field (Kelly, 2005), which adds cells particularly to the OFT region. The inner curve of the heart, where the OFT and AV cushions meet, is usually very tight, which is important for normal septation. An open curvature is associated with ventricular septal defects found in the double outlet right ventricle and double inlet left ventricle (Gittenberger-de Groot et al., 1995, 1996, 1997, 2001; Gittenberger-de Groot and Poelmann, 2000). Septation of the OFT requires the coordinated interactions of the OFT cushions, with the subpopulation of NCCs homing in to this region and the OFT cardiomyocyte sleeve. Apoptosis of NCCs (Poelmann et al., 1998; Poelmann and Gittenberger-de Groot, 1999) and cardiomyocytes (Rothenberg et al., 2002) is very prominent. AV canal septation requires fusion of the AV cushions, including the homed NCCs, matching with

the OFT septum, with the primary muscular fold, and with the spina vestibule for proper septation. Cardiomyocytes penetrate parts of the cushions as a final differentiation step in septum formation. Septation of the atria is also complex, but it does not involve apoptosis and therefore is not discussed in this review.

Differentiation of the OFT semilunar valves, and AV mitral and tricuspid valves and their tendinous apparatus, involves coordinated interactions of the endocardial cushion cells with migrated NCCs, and probably also invasion of the epicardial-derived cells (EPDCs) in conjunction with the surrounding cardiomyocytes (Gittenberger-de Groot et al., 2000). Contractions of the heart tube start as a peristaltic movement (de Jong et al., 1992), but soon change to a cyclic base-to-apex-contraction, which in time is followed by an apex-to-base constriction (Chuck et al., 1997; Rentschler et al., 2001; Sedmera et al., 2004) in a time-specific manner. In a certain time window, which remains to be established, these alterations in contraction mode are paralleled by differentiation of the myocardial entity, which acquires fast and slow conduction properties (Christoffels et al., 2000). The formation of the conduction system probably requires the involvement of NCCs (central conduction system) and EPDCs (peripheral Purkinje fibers) (Gittenberger-de Groot et al., 1998), as well as signaling molecules such as endothelin (Hall et al., 2004), which is shear stress dependent (Groenendijk et al., 2005). It is of the utmost importance to realize that cardiac performance includes both physical and hemodynamic forces, and that high shear stress protects against apoptosis (Li et al., 2005), whereas low and turbulent flows promote apoptosis.

PATTERNING THE ARTERIAL TREE

Pharyngeal Arch Arteries

Cardiac output is delivered to the arterial pole connected to the aortic sac. This part of the vascular system is subjected to complex remodeling during embryonic development. A number of arterial segments appear in an orchestrated series of events involving NCCs (Le Lièvre and Le Douarin, 1975; Bergwerff et al., 1998), but many of these disappear in subsequent stages. Apoptosis is an important mechanism in the breakdown of specific segments, and involves not only the vascular wall proper but also the surrounding mesenchyme (Molin et al., 2002).

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In an early stage of development, only one pair of pharyngeal arteries can be found connecting the heart to the developing microvascular network of the head region of the embryo. Subsequently, the next pairs of arteries come into existence; however, during development of the fourth pair, the first pair starts to disintegrate. The fourth pair is very special because it will form part of the aortic arch and the subclavian artery. Its unique morphology is associated with common anomalies, such as type B interruption and arteria lusoria (Bergwerff et al., 1999). The fifth pair does not develop in mammals and birds (DeRuiter et al., 1993). During development of the sixth pair, the second one starts to disintegrate. The consequence is that the third, fourth, and sixth pairs of the pharyngeal arch arteries form in a nearly symmetrical fashion. The adult stage is reached in the next stage of remodeling. The left fourth artery (in mammals) or the right fourth artery (in birds) turns into the aortic arch. The contralateral arterial segment becomes incorporated into the subclavian artery. For this, the right-sided alpha-segment of the dorsal aorta has to disappear (Molin et al., 2002). In mammals, the left sixth artery persists to give rise to the ductus arteriosus and the pulmonary arteries. The right one disappears long before birth by apoptosis (Molin et al., 2002). In birds, both sixth arteries persist until hatching (Bergwerff et al., 1999). Shortly after birth/hatching, the ductus arteriosus contracts and closes to allow the pulmonary circulation to achieve full function. A remaining arterial ligament is still present in adulthood. Depending on the species, closure may be accompanied by endothelial/intimal cushion formation and fusion (DeReeder et al., 1988; DeReeder, 1989; Slomp et al., 1997). It is interesting to realize that apoptosis in the adult vascular wall may present clues as to what is happening in embryonic life during normal remodeling. Von Wnuck (Lipinski et al. 2005) described the function of matrix metalloproteinases (MMPs) in apoptosis of human vascular smooth muscle cells in vitro. They concluded that degraded collagen induces calpain-mediated inactivation of anti-apoptotic proteins such as x-IAP, propagating apoptosis of smooth muscle cells by relieving the anti-apoptotic block. The components (including the extracellular matrix) of the various arterial segments differ greatly (Bergwerff et al., 1996), which is also the case for the activity of growth factors of the *TGFβ* pathway (Molin et al., 2003). Molin et al. (2002) reported that various malformations occur during the remodeling process of the pharyngeal arch artery system (PAAS), including aortic arch interruption type B

Growth factor-driven apoptosis is therefore included in the remodeling mechanism of the arterial tree.

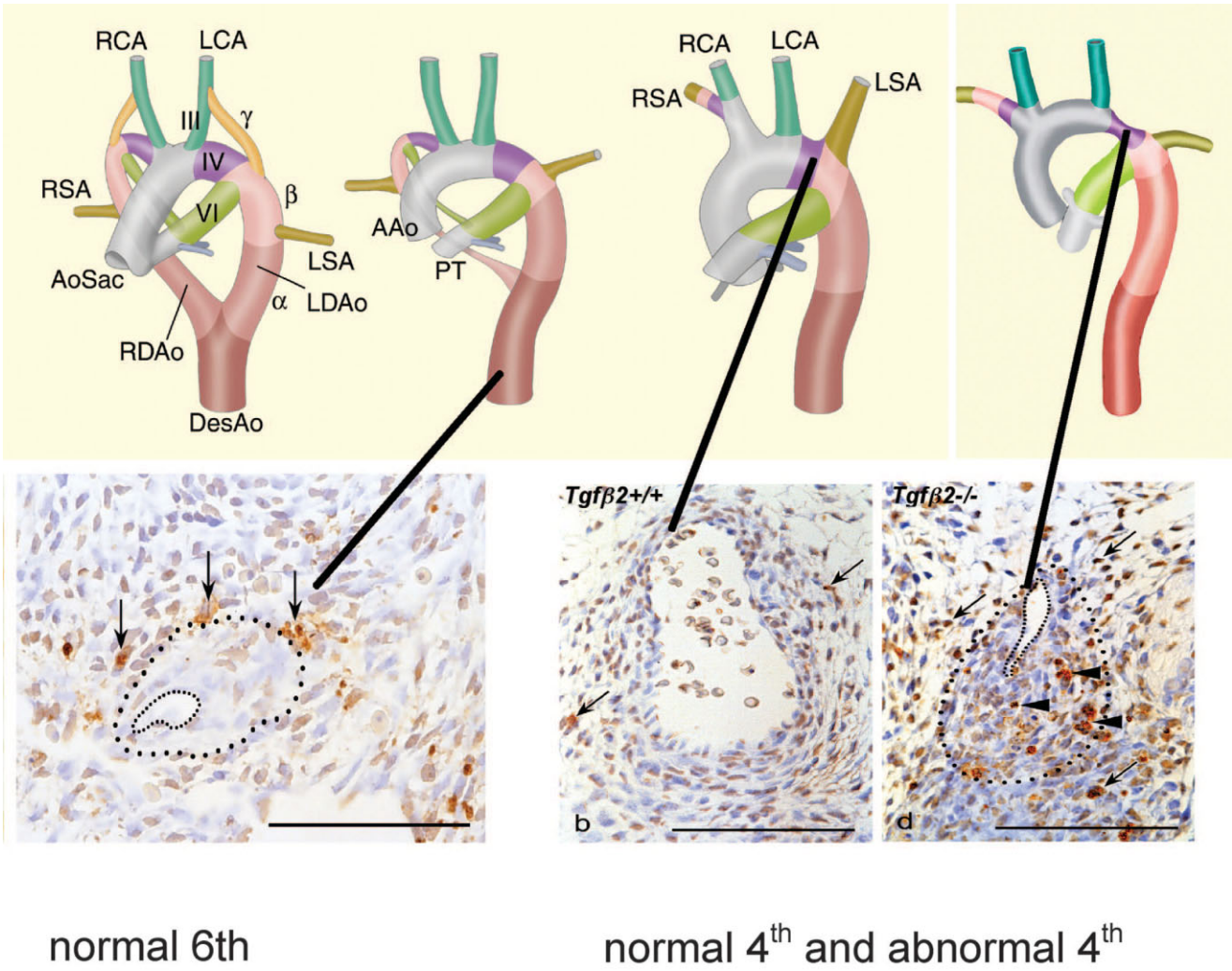

Figure 1. Three stages of normal development of the mammalian PAAS and one stage of an abnormal PAAS, demonstrating a stenotic aortic arch artery (IV). These images are based on reconstructions of serial stained sections, including TUNEL-stained sections. The sections are presented underneath and show apoptotic cells (arrows and arrowheads). Note that apoptosis accompanies normal development but is increased in the stenotic IV, as in the TGFβ2 $-/-$ (right-hand images). AAo: ascending aorta; AoSac: aortic sac; DesAo: descending aorta; LCA/RCA: left/right carotid artery; LDAo/RDAo: left/right descending aorta; LSA/RSA: left/right subclavian artery; PT: pulmonary trunk; III, IV and VI: respective pharyngeal arch arteries; α, β, γ: segments into which the embryonic dorsal aorta is subdivided. (Modified from Molin et al., 2002.)

and aberrant right subclavian artery (ARSA). They were able to compare the frequency of apoptosis in normal and *TGFβ-2−/−* mice with the occurrence of these anomalies. The normal high incidence of apoptosis in the fourth arch arteries is decreased to background levels. Growth factor-driven apoptosis is therefore included in the remodeling mechanism of the arterial tree. This is also the case with vascular endothelial growth factor (VEGF). Many important embryonic processes depend on proper VEGF signaling. Stalmans et al. (2003) analyzed this growth factor as a modifier gene in the 22q11 deletion syndrome (DiGeorge) using mutant mice as a model. DiGeorge syndrome presents with aortic arch interruption, and although it is not yet proven, aberrant apoptosis patterns may be involved.

Although the expression of *VEGF* is shear stress dependent, epigenetic and environmental factors also play a role as forces exerted by the rhythmic contractions of the heart. It is well known that many genes are regulated by shear stress, which is dependent on the fluid flow inside a blood vessel (Topper and Gimbrone, 1999). *TGFβ*, *eNOS*, and *VEGF*, as well as many other genes, are regulated by shear stress. Furthermore, pulsatile and relatively high levels of shear stress protect against apoptosis (Li et al., 2005), in which process NO produced by eNOS is a potent intermediate (Dimmeler et al., 1999; Groenendijk et al., 2005), whereas in low shear stress areas and areas with turbulent flow, apoptosis is promoted (Li et al., 2005).

Flow depends, along with other factors, on the diameter of the blood vessels (i.e., a larger diameter allows for more flow). Although the PAAS is in general terms a symmetrical construction, the diameter of the various vascular segments (in particular

right- vs. left-sided parts) varies to some degree. The vessels that are destined to disappear as a result of a high frequency of apoptosis always have a slightly smaller diameter (Molin et al., 2002). Indeed, the shear stress regulated gene *KLF-2* shows a slightly asymmetrical expression pattern in the pharyngeal arch arteries (Groenendijk et al., 2004). These findings support the hypothesis (Fisher et al., 2000) that the geometry of blood vessels and the ensuing flow/shear stress is a dominant environmental factor in regulating gene expression and, as a consequence, determining the apoptosis-associated remodeling of the vascular system.

In another animal model, the streptozotocin-induced diabetic pregnant rat, an aberrant apoptosis pattern (a significant decrease in the right sixth and alpha segments, and an increased frequency in the right and left fourth arch segments) is likewise important to explain anomalies in both the OFT and the aortic arch system (Molin et al., 2004).

Coronary Arteries

The system of the coronary circulation starts relatively late in cardiac development and is preceded by coating of the myocardial heart tube by the epicardial epithelium. By EMT, a subpopulation of the epicardial cells migrates into the subepicardial space and invades the myocardial wall to form the EPDCs, which integrate into the coronary vasculature to form the smooth muscle cells of the media and the fibroblasts of the adventitial layer (Vrancken Peeters et al., 1999; Gittenberger-de Groot et al., 2000; Manner et al., 2001; Lie-Venema et al., 2003, 2005; Wessels and Perez-Pomares, 2004). Specific factors are required for proper development of the epicardium and related vessels. Among these are *VCAM-1* (Kwee et al., 1995), *alpha4 integrin* (Yang et al., 1995), and FOG-2, a cofactor of *GATA* transcription (Tevosian et al., 2000). Disruption of these factors causes extensive pericardial bleeding and embryonic death.

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Only at the time of cardiac septation and separation of the aortic and pulmonary trunk do the coronary arteries invade the aorta to establish circulation under aortic pressure (Bogers et al., 1989). This phase of vascular invasion into the aortic semilunar sinuses is accompanied by apoptosis as determined by the regulation of *FasL* (Eralp et al., 2005). Filippatos et al. (2004) studied in vitro Fas-induced apoptosis in human endothelial cells derived from coronary arteries, and Romeo et al. (2000) prevented drug-induced apoptosis in human coronary endothelial cells by modulating Fas/FasL and the caspase-3 pathway. Sallee et al. (2004) studied myocardial apoptosis after viral FasL induction in embryos; however, they concentrated on the OFT of the heart and did not mention the coronary system. It is evident that apoptosis plays an important role in vascular disease in adult patients (Kockx and Herman, 2000; McCarthy and Bennett, 2000). Finally, the vascular network interlaced in the myocardium and subepicardium will be pruned (Benjamin et al., 1998) to give rise to the definitive system (Vrancken Peeters et al., 1997).

DISCUSSION OF APOPTOSIS AND CONGENITAL MALFORMATIONS

It is evident that apoptosis is an important mechanism in embryonic development of the cardiovascular system, as indicated by the many sites and time windows of occurrence. The exact function of apoptosis is not always clear, although removal of cells is an obvious phenomenon. An example is the apoptotic events that accompany the remodeling of the PAAS. However, the impression exists that on many occasions there is more to the matter. We refer to the possibility that apoptosis is not the final phase of a developmental process, but functions in itself as a step in further signaling. Such is the case for the apoptotic processes in which NCCs are involved during septation of the OFT. When NCCs are inhibited in their function, including apoptosis, ensuing myocardialization of the OFT cushions does not occur. It is very likely that apoptosis changes the microenvironment of that area, e.g., by altering the extracellular matrix, thereby releasing signaling molecules such as TGFβ. Norris et al. (2005) described mesenchymal *TGFβ* expression in locations of dominant apoptosis. van den Hoff et al. (1999) concluded that nonmyocardial signals stimulate myocardialization in the OFT, and Nakajima et al. (2000) described a spatiotemporal pattern of *TGFβ* expression that was very reminiscent of apoptotic patterns. However, whether apoptosis precedes activation of *TGFβ* and concomitant myocardialization, or is a result of TGFβ signaling is still being debated (Kubalak et al., 2002).

apoptosis changes the microenvironment

A comparable chain of events involving apoptosis takes place dur-

ing penetration of the aortic wall by the developing coronary arteries (Eralp et al., 2005), which brings the coronary circulation under aortic pressure. Apoptosis is the accompanying mechanism and may change the microenvironment in a similar way to provide for penetration of the thick muscular layer of the aortic root.

The presence of a large amount of apoptotic cells in the myocardium of the OFT (Rothenberg et al., 2002) during recruitment of cells from the secondary or anterior heart field remains an enigma. More study is needed to determine whether apoptosis at this location is involved in further differentiation or serves as an end stage for cells that have outlived their functional life (Glücksmann, 1951).

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