The Role of Extracellular Matrix in the Development of Experimental Cardiac Hypertrophy

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Cardiac hypertrophy is accompanied by remodelling of the extracellular matrix (ECM) like in catecholamine induced left ventricular (LV) hypertrophy. The development of norepinephrine (NE) induced LV hypertrophy included a remodelling of ECM with increased expression of the profibrotic cytokine TGF-β in myocytes, with elevated collagen type I and III mRNA expression maximal after 4 days of treatment and elevated MMP-2 activity maximal after 3 days of treatment. This led to an increased accumulation of collagen after 14 days of treatment which was accompanied by an elevated myocyte diameter. The attenuation of ECM remodelling with the broad spectrum MMP inhibitor doxycycline led to an elevated NE-induced accumulation of collagen and a prevention of the enlargement of myocytes. Therefore we have to conclude that the remodelling of ECM is necessary for the enlargement of myocytes and the remodelling of ECM is necessary for cardiac hypertrophy.

Keywords adrenergic agonists; fibrosis; hemodynamic; cardiac remodelling

1. Introduction

Structural alterations in the left ventricle have in recent years been referred to as "left ventricular remodelling". This remodelling process originates as an adaptive mechanism, enabling the heart to compensate for acute changes in hemodynamic load. Over time, however, remodelling becomes maladaptive and is associated with adverse clinical events and worsened survival prognosis [1]. While remodelling is often viewed as a gross structural event involving the size and shape of the left ventricle (LV), it is predominantly a cellular process involving myocytes and the interstitium in a coordinated process that accounts for the gross structural changes observed [2].

The process of LV remodelling and the progression of heart failure was originally believed to arise from alterations in intrinsic properties of the cardiomyocytes themselves, and indeed a multitude of phenotypic changes are observed in the remodelled myocardium. The number and type of cells in the heart have been evaluated initially by separation of cells into two populations: myocytes or myosin positive cells and nonmyocytes (myosin negative) populations. These initial studies determined that the ratio of fibroblasts to myocytes was approximately 70:30 [3]. As has been pointed out in numerous studies, myocytes make up the largest volume of the heart, but little quantitative information has been done on both myocytes and other cell types. The analysis by flow-assisted cell sorting (FACS) has revealed that the number and type of cells in the heart vary greatly depending on the developmental stage, as well as with potential pathophysiological conditions [4]. While the number of myocytes appears to be fairly constant, the other cell types vary with the physiological condition of the heart. This reflects an active process during the remodelling of the myocardium regulating the composition of the extracellular space, which is largely filled by an intricate network of macromolecules constituting the extracellular matrix (ECM). While the ECM was once thought to serve as a relatively inert scaffold to stabilise the physical structure of tissues, it is now clear that the matrix is a dynamic entity, which has a far more active and complex role. Evidence accumulated in recent years provides compelling affirmation for the importance of the matrix in the pathophysiologic expressions of various cardiovascular diseases, including the development of cardiac hypertrophy.

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2. The myocardial extracellular matrix

The myocardial ECM consists of macromolecules, primarily produced locally by fibroblasts, and includes a fibrillar collagen network, a basement membrane and proteoglycans. The fibrillar collagen network strengthens the matrix and ensures the structural integrity of adjoining myocytes. It provides the means by which myocyte shortening is translated into overall ventricular pump function and contributes to myocardial diastolic stiffness [5]. In the heart, collagen type I, a fibrillar collagen with the tensile strength of steel, and collagen type III, also a fibrillar collagen, are the most abundant phenotypes [6]. The basement membrane surrounds the myocyte and is attached to the sarcolemma as well as to the fibrillar collagen network. It is postulated that myocyte adherence to basement membrane may be a major determinant in maintenance of cell shape and positional integrity within the ventricular wall [4]. Proteoglycans are composed of a protein core to which polysaccharide chains called glycosaminoglycans are covalently bound. These negatively charged molecules possess significant osmotic activity and therefore attract water and cations [7, 8]. The proteoglycan molecules in connective tissue thus form a highly hydrated, gel-like "ground substance" in which the fibrous proteins are embedded. The polysaccharide gel resists compressive forces on the matrix while permitting the rapid diffusion of nutrients, metabolites, and hormones between the blood and the tissue cells [8]. Qualitatively, the composition of the ECM is similar in all tissues. Quantitatively, however, it is unique, and reflects the physiology of that particular tissue.

2.1 Structural and Functional Roles of Myocardial Fibrillar Collagen

The myocardial fibrillar collagen weave forms the scaffold upon which myocytes are arranged, thus fibrillar collagen is the primary determinant of tissue architecture and ventricular size and shape. In the normal heart, the number and location of intermyocyte connections prevents slippage of adjacent myocytes, either laterally or longitudinally [2]. It has been consistently demonstrated that degradation of collagen leads to ventricular dilatation [9].

In addition, because of its anatomic relation to the cardiac myocytes, myofibrils and muscle fibres and bundles, it has been hypothesised that the fibrillar collagen matrix coordinates the transmission of force generated by myocytes to the ventricular chamber. Collagen holds myocytes in a given region at essentially the same length at the end of diastole thereby imparting an equivalent preload, ensuring homogenous contraction of cells [2]. Furthermore, Baicu et al. demonstrated that systolic performance is impaired in isolated papillary muscles, but not isolated individual cardiomyocytes, following plasmin-induced collagen degradation [10]. Increases in interstitial collagen, a relatively inelastic material, will result in the myocardium becoming stiffer. A positive correlation between ventricular stiffness and collagen content has been established in nonhuman primates with experimental hypertension, rats with genetic, perinephretic or renovascular hypertension, and rats with myocardial fibrosis secondary to perinephritis and/or isoproterenol administration [9]. To exclude the possible contribution of myocardial hypertrophy often observed concurrently with fibrosis, Narayan et al. demonstrated that prevention of myocyte hypertrophy in spontaneously hypertensive rats with hydralazine did not reduce the abnormal accumulation of collagen, or the elevated passive myocardial stiffness [11]. A comparison of diastolic function in hypertensive patients and trained athletes provides further support that excess myocardial collagen and not hypertrophy is responsible for increased ventricular stiffness, as diastolic function is normal or even enhanced in the athlete, despite a significant increase in LV mass.

2.2 Alterations in Myocardial Fibrillar Collagen

It is now well recognised that myocardial fibrillar collagen plays an important role in determining the size and shape of the cardiac chambers, as well as ventricular diastolic and systolic function. Abnormal

modifications of the collagen matrix, therefore, will alter myocardial mechanical properties and ventricular function. In general, the matrix is altered by either a degradation of collagen, producing a reduction in collagen concentration characterised by a disruption and disappearance of fibrillar collagen, or an increase in collagen concentration because of reparative fibrosis and/or reactive fibrosis. Reparative fibrosis, or replacement fibrosis, is scarring that preserves the structural integrity of the tissue following myocyte necrosis. Reactive fibrosis is the synthesis of interstitial collagen fibres and the thickening of existing fibres at sites distant to myocyte cell loss.

Alterations in the fibrillar collagen network differ between the various cardiac pathologies. Pressure overload hypertrophy, for example, is associated with a rise in collagen synthesis in proportion to the increase in myocardial mass [12]. Direct and indirect evidence of enhanced collagen degradation have also been observed [13], however the rate of collagen degradation is not comparable to the increment in collagen synthesis and the result is an interstitial fibrosis. Decompensated volume overload hypertrophy, on the other hand, is characterised by grossly dilated, compliant cardiac chambers, with a high accumulation of interstitial collagen. It has been hypothesised that these changes are the result of increased collagen degradation and disruption of the collagen weave, accompanied by increased collagen deposition that is inadequately developed and poorly crosslinked and thus unable to provide the necessary structural and functional support [14].

3. Regulation of myocardial fibrillar collagen: the matrix metalloproteinases

The amount of myocardial collagen depends on the balance between collagen degradation and deposition. While the turnover of fibrillar collagen in the normal heart is a relatively slow process (the half-life of collagen type I is ~100 days [15]), turnover of collagen in cardiac disease is greatly upregulated. One of the major enzyme systems involved in the regulation of collagen turnover is the matrix metalloproteinases, or MMPs.

MMPs have a high specificity for components of the ECM, such as fibrillar collagen, and the degradative functions of the MMPs are thought to play a role in a number of disease processes. Increased MMP expression has been identified in pathological processes such as tumour angiogenesis and metastasis, rheumatoid arthritis and atheroma formation [16]. The MMPs constitute a family of zinc-dependent enzymes that currently number over 20 species [17, 18]. There are two principal types of MMPs: the membrane-bound type and those secreted into the extracellular space. The secreted MMPs comprise the majority of known MMP species and are released into the extracellular space in a latent or proenzyme state. Activation of these latent MMPs is required for proteolytic activity. MMPs, both latent and active, bind with a second class of biological molecules, the tissue inhibitors of matrix metalloproteinases (TIMPs). Therefore, overall MMP activity is determined by three important mechanisms: transcription, activation, and inhibition.

Increased MMP zymographic activity has been reported in myocardial samples from patients with end-stage chronic heart failure [19, 20]. Several studies have demonstrated increased MMP expression and abundance in experimental models of chronic heart failure and with end-stage cardiomyopathic disease in humans [21]. Recently it was shown that increased MMP-2 expression in patients with dilated cardiomyopathy is associated with elevated plasma norepinephrine level [22].

A clear cause-effect relationship between MMPs and the LV remodelling process has been demonstrated through the use of transgenic models or pharmacological MMP inhibitors [23-26]. A loss of MMP inhibitory control through TIMP-1 gene deletion has been shown to cause LV dilatation in mice [27]. The deletion of the MMP-9 gene in mice alters the course of LV remodelling post myocardial infarction (MI) [23]. Pharmacological MMP inhibition has been used in several animal models of LV dysfunction [25, 26]. For example, MMP inhibitor treatment with chronic rapid pacing attenuated the degree of LV dilatation that invariably occurs in this model [26]. In the spontaneously hypertensive heart failure rat model, MMP inhibition resulted in attenuation of LV dilatation [24]. In the mouse MI model, MMP inhibition has also been shown to reduce the degree of post MI LV dilatation [25]. Taken together,

animal models of LV dysfunction have provided compelling evidence to implicate MMPs in the myocardial remodelling process.

4. Alteration of the Extracellular Matrix during catecholamine induced hypertrophy

4.1. Norepinephrine elevated collagen expression in rat heart

Norepinephrine (NE) has been shown to induce LV hypertrophy in rats, as a result of an enlargement of myocytes [13, 28, 29]. This enlargement requires remodelling of the ECM, with degradation and increased synthesis of collagen for scaffold re-organization. This model should be used to present the importance of ECM remodelling on the development of cardiac hypertrophy. NE disturbs the balance between formation and degradation of ECM. It elevated the expression of collagen I and III, with consecutive fibrosis after 14 days [13]. These changes are important in the modulation of cardiac performance and the eventual development of heart failure.

NE induced the expression of TGF-β isoform mRNAs differentially and in a time-dependent manner. TGF-β1 has been reported to be present in cardiac myocytes and fibroblasts [30, 31]. It has been implicated in cardiac myocyte growth [30], fibrosis [32, 33], and in the re-expression of the fetal isoforms of myofibrillar protein genes [34]. In our study the mRNA-expression of TGF-β1 started to be elevated after NE treatment after 2 days, without biphasic characteristics [35], which was reported by Bhambi and Eghbali [36]. The elevation of cardiac TGF-β1 was the result of an increase of mRNA expression in myocytes and nonmyocytes (Fig. 1). This was also described by Takahashi et al. [30].

Little is known about the function of TGF-β2. All TGF-β isoforms bind to TGF-β I and II receptors [37]. Different functions are considered, since the TGF-β isoforms show different tissue distributions and affect different cell types differently. Moreover, it is known, that the proportion of TGF-β isoforms influences the biologic effects. Therefore, the shift in the ratio of TGF-β1:TGF-β2:TGF-β3 from 22:1:4 in myocytes from control animals to 7:1:1 after 1 day of *in vivo* NE-treatment suggests that TGF-β2 may play an important role in myocardial remodelling [35]. The expression of TGF-β2 increased earlier and to a higher extent than that of the other two isoforms only in the myocyte fraction (Fig. 1). A NE-induced isoform shift from TGF-β1 to TGF-β2 was also detected by Fisher and Absher [38]. Analysis of TGF-β2 null mice has revealed that TGF-β2 is most commonly involved in epithelial-mesenchymal interactions, cell growth, ECM production, and tissue remodelling. Moreover, it plays an essential role in the development of the heart [39].

The expression of TGF-β3 was increased later than the other two isoforms after NE treatment only in myocytes (Fig. 1). TGF-β3 was the TGF-β isoform which was increased predominantly in the infarct area after myocardial infarction [40]. It was proposed that TGF-β3 affects wound healing [41]. The NE-induced elevation of expression of all TGF-β mRNA isoforms was inhibited by α-adrenoceptor blocker (Fig. 1) [35].

Bhambi and Eghbali have shown that NE induced accumulation of collagen in the myocardium [36]. Our results indicate that this accumulation occurs predominantly in the LV [28]. This has been shown for the two main components of the extracellular matrix, collagen I and III. We also examined the level of colligin. The colligin mRNA level was increased for the first time after 12 h NE-infusion, followed by collagen I and III after 24 h (Fig. 1) [28]. This can easily be explained by the regulatory function of colligin as a chaperone of different collagens [42]. The elevation of both type I and type III collagen mRNA expression after NE-treatment was accompanied by consecutive collagen accumulation [13].

Interestingly the NE-induced elevated collagen accumulation was accompanied by an elevated MMP-2 mRNA expression and an increase of the MMP-2 activity with a maximum after 3 d of stimulation (Fig. 1) [13, 35]. TIMP-2 mRNA expression which occurred parallel to the elevated MMP-2 expression seems to be necessary to reduce the MMP-2 activity after an early phase of a higher level of ECM remodelling after NE treatment (Fig. 1) [13]. The protein expression of TIMP-2 was elevated after 4 d of stimulation.

This was accompanied with decreased MMP-2 activity (Fig. 1) [13]. The elevation of the MMP-2 activity is accompanied by remodelling of ECM [29].

The NE-induced increased expression of collagen mRNAs was a result of α- and β-adrenoceptor stimulation, since the expression was reduced by α-adrenoceptor as well as by β-adrenoceptor blocker [35]. The NE-induced elevated TGF-β expression was inhibited only by α-adrenoceptor blocker. This result supports the hypothesis that α-adrenoceptor stimulation and not β-adrenoceptor stimulation may induce collagen accumulation with a signal transduction including TGF-β isoforms. Moreover, there is an additional signalling through the β-adrenoceptor, which not includes the TGF-β pathway.

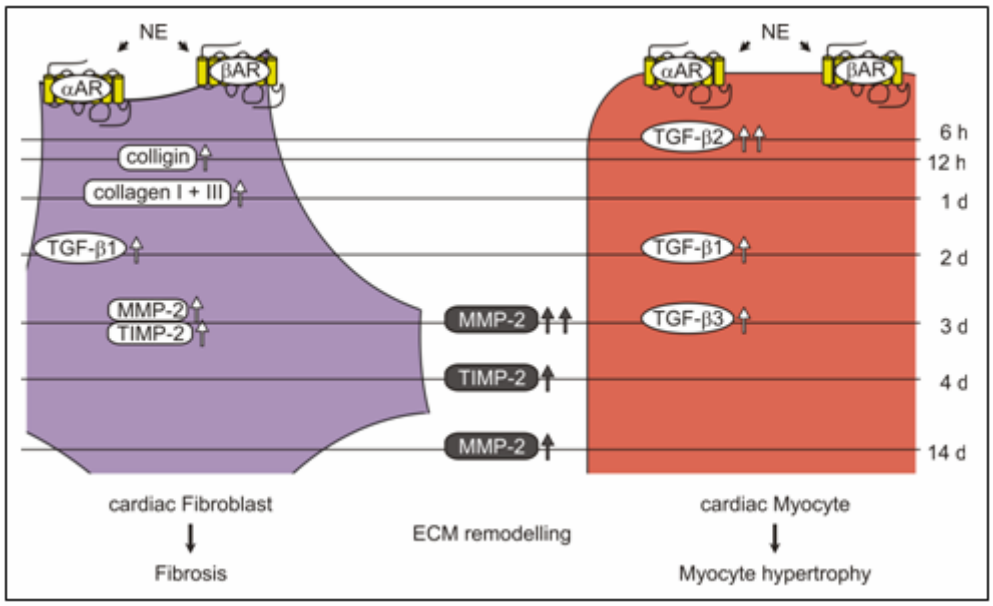

Fig. 1 The results of in vivo norepinephrine (NE) stimulation in rats are arranged in a time-order of the start of their stimulation and separated by the expression origin, in myocytes or nonmyocytes (among them are fibroblasts) and by their receptor specificity [35]. It is well accepted that the main source for the cardiac collagen production are fibroblasts. Therefore, the results from nonmyocytes are summarized as results from fibroblasts. Both cell fractions were separated after in vivo stimulation. The expression of TGF-β2 was elevated first after 6 h in myocytes, followed by colligin and both types of collagen in nonmyocytes. Than the expression of TGF-β1 was elevated in both cell fractions which was followed by an elevation of the mRNA expression of MMP-2 and its inhibitor. The activity of MMP-2 was elevated at the same time point. The reduction of this activity could be explained by elevated TIMP-2 protein expression after 4 days. There was an elevated MMP-2 activity only in the LV, which represented an ongoing remodelling process of the ECM.

4.2. Inhibition of ECM remodelling inhibit the NE-induced enlargement of myocytes

What is happen in the hypertrophying heart if the remodelling of the ECM is blocked? To answer this question the MMP activity was inhibited with doxycycline during NE treatment in rats [29]. Tetracyclines are known as antibiotics [43], but recent work has shown that doxycycline, and other derivates of tetracycline, are potent broad-spectrum MMP inhibitors [44, 45]. The only MMP inhibitor so far approved as a drug, Collagenex's Periostat, is the oral tetracycline, doxycycline, for the treatment of peridontitis [46]. Clinical data for the effect of tetracyclines are well established [44]. Their broad spectrum of activity makes them a useful tool for MMP inhibition. This is the reason why we have used doxycycline to inhibit MMP activity during NE-stimulation in rats.

The inhibition of MMPs by doxycycline in NE-treated rats led to an additional increase in the NE-induced collagen accumulation in the LV [29]. MMP-inhibition could reduce or elevate myocardial

fibrosis depending on the species studied. Treatment of spontaneously hypertensive heart failure (SHHF) rats with the MMP-inhibitor PD 166793 prevented cardiac dilatation, preserved contractility, and reduced myocardial fibrosis compared with untreated SHHF controls [26]. This suggested that the beneficial effects of MMP inhibition are mediated by limiting cardiac remodelling, thereby slowing the progression to heart failure. Similar MMP inhibition studies by Spinale et al. [26] found that concomitant treatment with PD 166793 in pigs undergoing rapid pacing attenuated the degree of LV dilatation, but was associated with a qualitative increase in interstitial collagen and an abnormal increase in myocardial stiffness. They concluded that the increase in ventricular stiffness was due to a greater amount of fibrillar collagen in the hearts of treated animals, suggesting that MMP inhibition might also have negative effects by inhibiting normal collagen turnover. This increase of collagen fraction was seen after combined treatment with NE and doxycycline too [29].

The increased interstitial collagen fraction correlated with reduced LV contractility which is elevated by NE [29]. The deterioration of heart function by fibrosis may be due to myocardial stiffening. There are different hypotheses for the development of myocardial stiffening like mentioned earlier. Studies using the Dahl-S rat diastolic heart failure model demonstrated that the transition from the compensatory stage to the overt heart failure was associated with the progression of LV hypertrophy, fibrosis and myocardial stiffening [47]. Those results suggest a crucial role of myocardial stiffening in the development of heart failure. However, in view of the contribution of LV hypertrophy and fibrosis to myocardial stiffening, previous studies yielded different results. Narayan et al. [11] and Matsubara et al. [48] concluded that collagen accumulation, not LV hypertrophy, was responsible for myocardial stiffening. Schraeger et al. showed that LV hypertrophy, not fibrosis, is closely related to myocardial stiffening [49]. The attenuation of the NE-induced functional effect after doxycycline treatment seems to be the result of collagen accumulation and not of myocytes hypertrophy, since the diameter of LV myocytes was smaller after MMP inhibition [29].

There was not a significant effect of doxycycline on diastolic aortic pressure (DAP), LVSP and cardiac output (CO), although DAP and LVSP were slightly higher and CO lower after combined treatment with NE and doxycycline [29]. In addition there was a significant elevation of TPR. The elevated DAP could be a result of stiffer resistance vessels. The inhibition of vessel enlargement by MMP inhibition was shown in an animal model of chronic volume overload [50, 51]. It was demonstrated that flow-mediated arterial enlargement is limited by competitive MMP inhibition in a dose-dependent fashion. Furthermore, it was shown that doxycycline elevated pulmonary artery pressure in rats with 15 days of chronic hypoxic pulmonary hypertension [52]. This increased pressure was accompanied by collagen accumulation in pulmonary arteries.

There was a significant inverse relationship between LV myocyte diameter and interstitial collagen fraction [29]. The more pronounced collagen network was associated with the inhibition of the enlargement of myocytes. This may indicate that there is a cross-talk between myocytes and the ECM which was disturbed by MMP inhibition. Myocytes may need the information from the environment so that there is room for enlargement. This may be a new important function of MMP inhibition. An inhibition of ventricular hypertrophy by blocking MMP activity was seen in the TNF-α transgenic mouse model of dilated cardiomyopathy [53]. This seems to be a result of prevented enlargement of myocytes too. Additionally, an extensive remodelling of ECM by over-expression of MMP resulted in compensatory myocyte hypertrophy [54].

The NE-induced increase of collagen expression could not be explained by direct stimulation of cardiac fibroblasts, because collagen expression was not elevated in isolated cardiac fibroblasts after NE treatment [36]. NE induced the collagen expression *in vivo* by an increase of TGF-β2 expression in myocytes (Fig. 2) which was implicated by the time pattern of consecutive expression of TGF-β2, colligin and collagen after NE treatment (Fig. 1). However, there seem to be a second trigger involved which was induced by β-adrenoceptor stimulation (Fig. 2). Furthermore, myocyte hypertrophy in the LV required the remodelling of connective tissue (Fig. 2), because there was no NE-induced enlargement of myocytes detectable during NE treatment and inhibition of MMP activity by doxycycline [29].

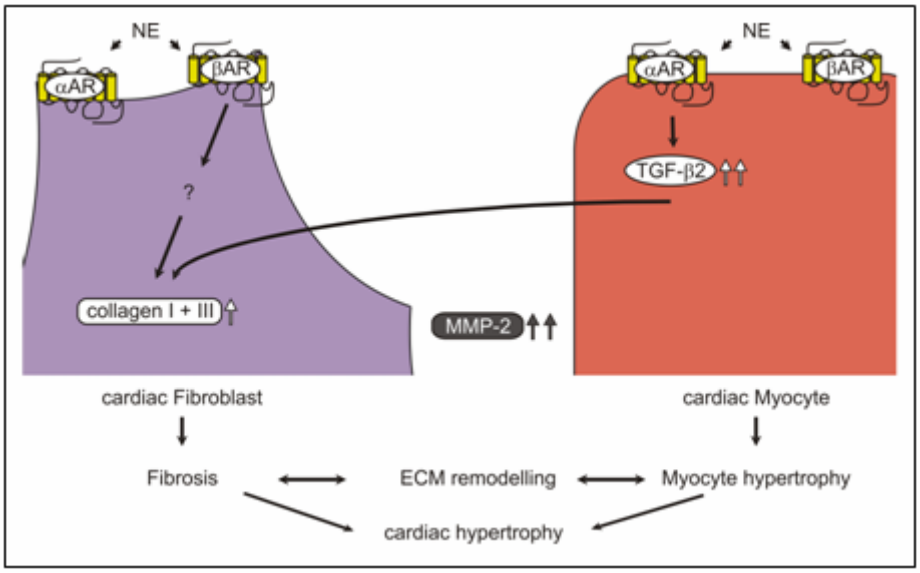

Fig. 2 The schema represents some consequences of norepinephrine (NE) treatment in rat hearts. NE induced the elevation especially of TGF-β2 by stimulation of α-adrenoceptors in myocytes. This TGF-β2 seems to be the stimulus for the NE induced elevation of collagen type I and collagen type III expression. However, there has to be an additional signal for the stimulation of collagen production since collagen expression was repressed by the β-receptor blocker too. Collagen accumulation led to fibrosis and continuous adrenergic stimulation induced an enlargement of myocytes. This enlargement requires the remodelling of ECM. The enlargement of myocytes and the fibrosis are responsible for cardiac hypertrophy.

5. Lessons from cardiac remodelling

Historically, MMPs were thought to function mainly as enzymes that degrade structural components of the ECM. However, MMP proteolysis can create space for cells to migrate, can produce specific substrate-cleavage fragments with independent biological activity, can regulate tissue architecture through effects on the ECM and intercellular junctions, and can activate, deactivate or modify the activity of signaling molecules, both directly and indirectly [18, 55]. Because cells have receptors (for example, integrins) for structural ECM components, MMPs can also affect cellular functions by regulating the ECM proteins with which the cells interact [56]. In many cases, MMP cleavage of ECM substrates generates fragments that have different biological activities from their precursors. For example, the cleavage of laminin-5 or collagen IV results in the exposure of cryptic sites that promote migration [57, 58]. Type I collagen degradation that is mediated by MMP1 is necessary for epithelial cell migration and wound healing in culture models [59]. Cleavage of ECM proteins by MMPs can also release ECM-bound growth factors, including insulin growth factors and fibroblast growth factors [60, 61]. Alternative mechanisms of action have also been observed, including functional intermolecular MMP complexes: MMP14 binds to TIMP2, which binds to pro-MMP2, thereby positioning it for activation by a second molecule of MMP14 [62]. Furthermore, human MMP11 has an alternative splice isoform that functions as an intracellular proteinase and enters the nucleus [63].

The molecular alterations that accompany LV remodelling during the development of cardiac hypertrophy are complex. It is evident that MMPs and TIMPs are involved in the remodelling process, and that a specific portfolio of these enzymes is expressed according to time in this development. The use of MMP plasma profiling in the clinical situation may assist in tracking LV remodelling during the development of cardiac hypertrophy, heart failure and post MI, however the ubiquitous expression of MMPs in the body and their involvement with diverse pathologies (such as cancer, arthritis and

atherosclerosis) may confound such measurements. Therapies targeting MMPs to prevent LV remodelling post MI have now reached clinical trial but so far have failed to demonstrate significant benefit. Further research is essential to elucidate more definitively the subtypes of MMPs and TIMPs involved in the remodelling process, and at what time point they are best targeted. The ability of MMP regulation to confer additional protection over coronary reperfusion and current standard therapy also needs consideration.

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