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THE UNDERLYING ROLES OF MACROPHAGE POPULATIONS IN MYOCARDIAL RBROSIS

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Myocardial fibrosis is one of the main structural changes following cardiomyocyte injury such as infarction. Macrophages play a central role in the development offibrotic lesions. In myocardial fibrosis, three different populations of macrophages are recognized: exudate macrophages, resident macrophages, and interstitial dendritic cells. Exudate macrophages, which are derived from blood monocytes and recruited into injured areas through chemoattractants and cell adhesion molecules, release various fibrogenic growth factors in early stages of the fibrosis. Transforming growth factor- f! and platelet-derived growth factors are proposed as the most probable growth factors produced by exudate macrophages to induce the modulation of fibroblasts towards myofibroblasts. Emerging evidence shows that macrophage-secreted nerve growth factor may also be involved in that process. The myofibroblasts are capable of producing extracellular matrix proteins which contribute to myocardial fibrosis. The exudate macrophages also participate in the induction ofapoptosis in injured myocytes and myofibroblasts in the fibrotic lesions. These apoptotic cells are phagocytized by exudate macrophages, and the macrophages themselves also disappear by apoptosis. The decrease in cellularity by apoptosis leads to the evolution of fibrous granulation tissues into scar tissues (reparative fibrosis). The resident macrophages particiapte exclusively in the late stages of the myocardial fibrosis, when their mitotic activity increases in the lesions; they are presumed to have the same roles as the exudate macrophages. In addition, the resident macrophages and interstitial dendritic cells both act as immune mediators to recruit T-lymphocytes into the lesions. In contrast to hepatic, pulmonary, and renal fibrosis, the roles of macrophage populations in myocardial fibrosis has not yet been extensively investigated. ***BiomedRev 1999; 10:89-105.***

INTRODUCTION

Fibrosis is a common sequel following diverse insults in a variety of parenchymal tissues. The change is a part of the tissue repair process and contributes to the tissue remodeling (1-3). However, if it occurs progressively or repeatedly in major organs such as liver, lungs, kidneys, and heart, it results in serious functional insufficiency in these organs, leading in some cases to death. The fibrosis is evoked through complicated processes after tissue injuries (4,5), and the mechanisms have yet to be fully characterized. In the last decade, there have been significant advances in cellular and molecular pathology, which have contributed to our understanding of the roles of cells and factors responsible for the development of fibrosis. These advances have shown that macrophages (M0) play a pivotal role in fibrogenesis and consequent tissue remodeling (6-9).

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Our laboratory has been investigating the kinetics of M0 and fibrogenic cells in rat fibrosis models, in order to shed some light on the roles of M0 in the development of fibrosis. These models include hepatic (10,11), renal (12-15) and cardiac (16) fibrosis. In this review article, general mechanisms of fibrosis will be discussed, together with the current notion on the derivation and differentiation of M0 populations. Then, we will highlight recent insights on the roles of M0 in myocardial fibrosis by referring to those in fibrotic lesions in the liver, lungs and kidneys as well as skin wound healing. With this knowledge, the possible therapeutic strategies to limit progression of fibrosis are briefly introduced.

THE PATHOLOGY OF FIBROSIS

Animal models

Many fibrosis models have been established in rats and mice by various experimental strategies. These models have contributed significantly to the elucidation of cellular and molecular mechanisms responsible for the fibrogenesis (Table 1).

The fibrogenic processes

The development of fibrosis requires the participation of various soluble factors capable of mediating cell-cell and cell-extracellular matrix (ECM) interactions, inflammatory responses, and abnormal accumulation of ECM proteins (2,4,5). Based on results obtained in fibrosis models, several common and general features have been ascertained at present, although the fibrogenic processes certainly vary from model to model and from organ to organ. The fibrogenic processes consist generally of three phases (Table 2). The firstphase represents the induction of conditions necessary for the establishment of active fibrosis and the functional elaboration of reactive cells including M0. The second phase is composed of the development of fibrogenic cells and subsequent deposition of ECM proteins. The third phase provides the resolution towards healing and tissue remodeling. However, one should notice that each phase is sequential, and that the fibrotic lesions will become more complicated depending on the degree and extent of injured tissues as well as the underlying causes.

The firstphase of fibrogenic process

In this phase, the recruitment of inflammatory cells from blood or surrounding tissues is a prerequisite for the development of fibrosis. In response-to-injury, vascular endothelial cells, parenchymal and/or inflammatory cells produce factors to attract and activate M0, neutrophils, lymphocytes, and mast cells, using para- and autocrine pathways. There are several classes of chemoattractants/chemokines and cell adhesion molecules (CAM) (7,17-22). CAM such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), lymphocyte function-associated antigen-1 (LFA-1), (32-integrin-macrophage-1 antigen (Mac-1), very late antigen-4 (VLA-4), and osteopontin are important factors for the adhesion of blood monocytes to vascular endothelial cells at inflammatory sites (17,20,22,23). In this migration process, chemoattractants such as monocyte chemoattractant protein-1 (MCP-1), macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF-cc), and transforming growth

Table 1. Experimental fibrosis models in rats and mice

Hepatic fibrosis	Carbon tetrachloride (10,29,45)* Thioacetamide (11), Galactosamine (139) Alcohol (111), Bile duct ligation (46,56,110) Granuloma induced by zymosan (27) or glucan (26)
Pulmonary fibrosis	Bleomycin (40,49,52,62,136) Silica (32,114)
Renal interstitial fibrosis	Puromycin aminonucleoside (60,94,95) Adriamycin (38) *Cis* -diaminedichloroplatinum (cisplatin) (12,13) Ureteral ligation (7,14,34,51) Rat chronic progressive nephropathy (15)
Myocardial fibrosis	Isoproterenol(16) Coronary artery ligation (1,59,80,98) Renovascular hypertensive rats (19,78,84) Spontaneously hypertensive rats (58,77) *v-fps* carrying transgenic mice (82)

*In parentheses, representative references are indicated.

factors-beta (TGF-p) are capable of inducing monocyte chemotaxis (7,21,23-27). In particular, MCP-1 has a high potential for monocytes, stimulating their infiltration into inflammatory sites (7,21,22,28). During the first stage, recruited M0 release a number of cell growth factors such as TGF-p, platelet-derived growth factor (PDGF), TNF-oc, basic fibroblast growth factor (bFGF), and colony stimulating factors (CSF) (7,24,25,29-39). Among these factors, TGF-P and PDGF strongly modulate fibroblast phenotype and ECM production both *in vitro* and *in vivo* (7,29,30,31,33, 36,37,39,40,41), and endothelin (42) also induce activation of fibrogenic cells. It is worth mentioning that most of the immune/inflammatory cells including human M0 (43,44) are source of and target for nerve growth factor (NGF). These findings suggest that NGF, in addition to its neurotrophic function, is an important para- and autocrine immunoregulatory factor, and hence implicated in a variety of pathological processes in inflammatory-fibroproliferative diseases, such as systemic sclerosis, skin wounds, and atherosclerosis (see this volume of *Biomedical Reviews).* In addition, recent evidence shows that the upregulation of cardiomyocyte myostatin, a member of the superfamily of TGF-P, after myocardial infarction could play an important role in cardiac pathophysiol-ogy (44a).

The second phase of fibrogenic process

In the second phase, M0-secreted growth factors give rise to the phenotypical conversion of fibroblasts into myofibroblasts.

Myofibroblast development (fibrosing tissue)

Table 2. Pathology of fibrosis

Phase 1		
Recruitment of inflammatory cells including exudate macrophages		
	Cell-adhesion molecules	Vascular cell adhesion molecule-1 Intercellular adhesion molecule-1 Lymphocyte function-associated antigen Macrophage-1 antigen Very late antigen-4 Osteopontin
	Chemoattractants	Monocyte-chemoattractant protein-1 Macrophage-colony stimulating faictor (M-CSF) Granulocyte-macrophge colony stimulating factor (GM-CSF) Tumor necrosis factor-a (TNF-a) Transforming growth factor-P (TGF-P)
	Production of fibrogenic growth factors by recruited/resident macrophages	TGF-pl-3 Platelet-derived growth factor (PDGF-AA, -BB) Basic fibroblast growth factor TNF-a M-CSF, GM-CSF
T-lymphocyte induction by antigen-presenting cells (resident macrophages/interstitial dendritic cells)		
Phase 2		
	Phenotypical modulation of myofibroblasts (expression of vimentin, desmin, and/or a-smooth muscle actin)	
	Extracellular matrix protein deposition	CollgentypesI,rn,IV Fibronectin, Laminin
Phase 3		
Resolution of fibrotic lesion		
Matrix metalloproteinases: collagenases, gelatinases, stromelysins, macrophage-metalloelastase, matrilysin		
Tissue inhibitors of metalloproteinases-1-4		
Apoptosis mediated by macrophge-produced factors		
Phagocytosis of apoptotic cells by macrophages		

In hepatic fibrosis, perisinusoidal cells (Ito cells, fat-storing cells or hepatic stellate cells) can modulate into myofibroblastic cells (10,45,46). The myofibroblasts are intermediate in nature between fibroblasts and smooth muscle cells (SMC), and thus are identified immunocytochemically by detection of SMC alpha-actin (oc-SMA) (4,33,38-41,42,47-51). Through the secretion of inflammatory and antiinflammatory cytokines, chemokines, and growth factors, as well as ECM proteins and proteases, myofibroblasts play an important role in organogenesis, inflammation, repair, and fibrosis in most organs and tissues (reviewed in 52). In fibrotic lesions, it is known that cytoskeletal features change in the development of myofibro-blasts from stromal fibroblasts (47,50,52-54). The myofibroblast spectrum is divided into the following four main phenotypes: expressing of vimentin (V cells), coexpressing vimentin and desmin (VD cells), coexpressing vimentin and oc-SMA (VA cells), and coexpressing vimentin, a-SMA, and desmin (VAD cells). Such phenotypic modulations have been well established in fibroblastic cells appearing in skin wound healing, hypertrophic scar, superficial fibroma-tosis, and sclerodermal (47) and neoplastic (55) lesions. The major components of abnormally accumulated ECM proteins in fibrotic areas consist of collagen type I and type III, basal lamina-associated collagen type IV, laminin, and fibronectin (5,56). The phenotypically-modulated myofibroblasts produce mainly collagen types I and III, and fibronectin (56). Besides the production by myofibroblasts, basal lamina-related proteins may be produced by injured or regenerating parenchymal cells, for example, tubular epithelial cells in renal interstitial fibrosis and hepatocytes in liver fibrosis (4,5). Reactive cells in fibrotic lesions may express the major histocompatibility (MHC) class II molecules on cell surface, providing a mechanism for the direct involvement of antigen presentation to T-lymphocytes with subsequent production of cytokines that modulate the production of matrix proteins (4,57-59).

The third phase offibrogenic process

The third phase represents the resolution of fibrotic lesions, or further deposition of ECM proteins may occur. The abnormal accumulation of matrix proteins can be stimulated by altered balance between matrix metalloproteinases (MMP) including interstitial collagenase (MMP-1), gelatinase (MMP-2), stromelysin (MMP-3), M0-metalloelastase, and matrilysin and the tissue inhibitors of MMP (TIMP-1-4) (7,60-62). M0-secreted factors including NGF may influence the synthesis and activity of MMP (62-64). Another important event for the resolution of fibrotic lesions is the disappearance of cells that end their roles. The mechanism is due likely to apoptosis (2,9).

MACROPHAGES

Macrophage populations and their derivation

M0 have been known to be present in fibrotic areas for a long time, indicating important roles for them in the fibrogenesis (4,5,7,8). In the 1970's, a theory of the "mononuclear phagocyte system (MPS)" was proposed (reviewed in 65). According to this theory, all M0, including infiltrating M0 in inflammatory lesions, and fixed M0 (so-called histiocytes) existing in various tissues, are derived from blood monocytes, which differentiate *via* promonocytes/monoblasts originating from bone marrow stem cells. These M0 were considered to be short-lived, nondividing terminal cells, although M0 in proliferative or regenerative lesions possess a proliferative capacity (66,67). Recently, it has been shown that M0 are not homogeneous cell populations. They differ in morphology, immunophenotypes, and functions (67-69). In adult life, three major M0 populations can be recognized: exudate M0, resident (fixed) M0, and dendritic cells (defined here as M0-related cells) (65,68,70). The development, differentiation, and maturation of these M0 are shown in Figure 1.

Exudate and resident macrophages

Peroxidase (PO) is one of the most important enzymes involved in M0 functions (70,71), and differences in cytochemical localization patterns of PO activity between exudate and resident M0 (65,70) have been noted. The exudate M0 show PO activity only in lysosomes. Analogous activity patterns are seen in blood monocytes. Therefore, the exudate M0 have been considered to be included in the monocytic lineage [macrophage-colony forming unit (M-CFU), monoblasts, promonoblasts and monocytes] originating from hematopoietic stem cells. In contrast, the resident M0 reveal PO activity patterns in the nuclear envelope and rough endoplasmic reticulum, and lack PO-posi-tive lysosomes. The resident M0 may also derived from hematopoietic stem cells including the M0 precursors (granulocyte/macrophage-colony forming unit, GM-CFU) (65,67). However, they develop from PO-negative immature M0 through a differentiation pathway different from that of exudate M0. Ontogenetically, the M0 precursors migrate from the bone. marrow into connective tissues, then fix as PO-negative immature M0, and differentiate into resident M0 under conditions of inflammation. Monocyte-derived exudate M0 no longer have proliferative potential and are short-lived, whereas the resident M0 are long-lived in inactive steady state conditions in tissues, and can be sustained by self-renewal (65). In fact, Kupffer cells, a representative resident M0, show proliferative activity in diseased livers (45,46,66).

According to cytoplasmic PO activity patterns, the exudate-resident M0 population was distinguished. These cells show PO activity in the nuclear envelope, rough endoplasmic reticulum as well as lysosomes (70). Although the immunocytochemical studies indicated that the exudate-resident M0 constitute a subtype of blood monocytic exudate M0 (28,65), it is likely that they are a transitional or intermediate form between the exudate andresidentM0(67,70). As shown in Figure 1, the PO activity disappears in both exudate and resident M0 in the achieved state of fibrotic process (65).

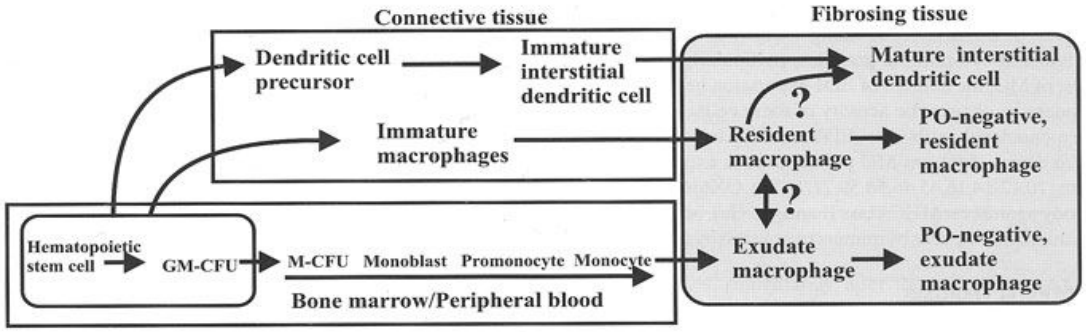

Figure 1. The development, differentiation and maturation of macrophage populations. The exudate macrophages are included in the monocytic lineage [macrophage colony forming unit (M-CFU), monoblasts, promonoblasts and monocytes] originating from hematopoietic stem cells. The resident macrophages are derived from hematopoietic stem cells including macrophage precursors (granulocyte/macrophage colony forming unit, GM-CFU); they develop from immature macrophages through differentiation pathway different from, that of the exudate macrophages; the immature macrophages migrate from the bone marrow, and then fix in the connective tissues. The exudate and resident macrophages are fundamentally distinguishable according to differences in cytochemical localization patterns ofperoxidase (PO) activity in the cytoplasm (see Text). However, there is a transitional or intermediate form somewhere between the exudate and resident macrophages. The PO activity disappears in both exudate and resident macrophages in the advanced stages of fibrosis. The interstitial dendritic cells develop from the hematopoietic stem cells including GM-CFU, and the precursor cells spread over the connective tissues. The differentiation pathway of the interstitial dendritic cells is distinct from that of the exudate or resident macrophages. However, some types of the interstitial dendritic cells may be formed from blood monocytes, and the relationship between the resident macrophages and interstitial dendritic cells in the fibrotic lesion remains unclear. The interstitial dendritic cells originally develop from MHC class II-negative precursors, and then give rise to low MHC class II-positive immature cells. Finally, the mature type of the interstitial dendritic cells upregulate the expression of MHC class II molecules in thefibrosing tissue.

Dendritic cells

Dendritic cells develop from the hematopoietic stem cells including GM-CFU, and the precursor cells at the immature situation spread overthe tissues (58,59,65,72).The differentiation pathway of dendritic cells is distinct from that of the exudate or resident M0 (65) (Fig. 1). However, it is considered that some types of dendritic cells are formed from blood monocytes (73). The dendritic cells include interstitial dendritic cells and interdigitatingfollicular cells of afferent lymphoid tissues such as the spleen, lymph nodes and Peyer's patches, and Langerhans cells of the epidermis. The interstitial dendritic cells are widely distributed in the connective tissues of most nonlymphoid organs, including the heart (58,59,73). Morphologically, the exudate and resident M0 have spherical lysosomal structures, while the interstitial dendritic cells are characterized by the presence of tubulovesicular systems consisting of numerous vesicles, sacs, and tubules (65,73). Dendritic cells have an important function in the initiation of immune responses, because they are specialized antigen-presenting cells whose primary functions are to capture, process, and present antigens to unprimed T cells (73). In the maturation process, dendritic cells originally develop from MHC class II-negative precursors, and then give rise to low MHC class II-positive cells (immature type). Finally, immature dendritic cells can further upregulate the expression of MHC class II molecules under stimulated conditions (73,74).

Immunocytochemical detection of rat macrophage populations

Detection of M0 populations in rat fibrosis models is essential to investigate their roles. As indicated above, M0 are a heterogeneous cell population (6,28,68,75). In the last 15 years, useful monoclonal antibodies recognizing rat M0 have been generated. The characteristcs and reactivity of anti-rat M0 monoclonal antibodies, which have been widely used, are shown in Table 3. EDS and TRPM-3 are known to recognize the

epitopes of antigens present on exudate M0 and certain M0 populations in lymphoid organs. ED2 and Ki-M2R are reactive for resident M0 (28,68,70). EDI recognizes many M0 populations, particularly, blood monocytes and exudate M0. However, since EDI-recognizing antigen is mainly on the membrane of cytoplasmic granules, particularly, phagolysosomes, of M0, the amount of EDI expression in a cell maybe dependent on phagocytic activity of these cells (76). Among these monoclonal antibodies, EDI and ED2 have been widely used to detect different M0 populations in experimental rat fibrosis(10,12-14,16,45,46,58,59,72,77,78). OX6is amonoclonal antibody against rat MHC class II antigen (la), and recognizes the mature dendritic cells by immunocyochemistry (58,59,72,78).

MYOCARDIAL FIBROSIS

Animal models for myocardial fibrosis

Our laboratory has investigated the kinetics of MO and myofibroblasts in myocardial fibrosis induced in rats by isoproterenol, an p-adrenergic stimulant (16). The isoproterenol-induced myocardial injury is characterized by multifocal necrotic lesions in the subendocardial portions of the left ventricle, followed by fibrous granulation and scar tissues. The pathological events in the model are similar to those seen in human myocardial infarctions and rat infarction models by coronary arterial ligation (1,59,79,80). Pcrivascular fibrosis in the left ventricle has also been reported in spontaneously hypertensive rats or reno vascular hypertensive rats induced by clipping renal artery (19,58,77,78). This fibrosis is caused by the decrease in left ventricular compliance and the increase in arrhythmogenic risk associated with cardiac overload (77,81). Transgenic mice expressing protein-tyrosine kinase were introduced as a myocardial fibrosis model (82).

Myofibroblasts in myocardial fibrosis

In rat myocardial infarction, myofibroblasts appeared between 2 and 4 days (80). Similarly, on days 3-7 after isoproterenol injection, there was a dramatic increase in the number of myofib roblasts (16). In human myocardial fibrosis, myofibroblastic cells appear within 4 to 6 days after the infarction (50). The myofibroblasts produce ECM proteins, mainly collagen types I and III (1,83,84). Cardiac myofibroblasts can alter their cytoskeletal proteins. Willems *et al* (50) compared the chara cteristics of myofibroblasts in human myocardial fibrosis with those in rat cardiac infarction (85) and in rat dermal wounds (86). Myofibroblasts in all studies expressed both vimentin and a-SMA (VA cells). In contrast to dermal wounds that possess VA, DVD and VAD cells, desmin expression in cardiac myofibroblasts appears to be weak or lacking. The most interesting feature of myofibroblasts appearing in myocardial fibrosis is their parallel orientation to surviving adjacent cardiomyocytes (16,50,80). Myofibroblasts persist in fibrotic lesions in the myocardium for a long time, whereas they are only transiently present in dermal wounds (2,50,86). The persistence of myofibroblasts and their preferential alignment in myocardial fibrosis may be explained by the continuous mechanical stress caused by the ongoing contraction andrelaxation of the surrounding viable myocardium (16,50). The expression of the cytoskeletal a-SMA filaments provide the myocardial fibrotic cells with the possibility to contract and counteract the mechanical forces (reparative fibrosis) (50,85). Recently, cardiac myofibroblasts were shown to express a homologue of *Drosophila* tissue polarity gene frizzled (/z2) when migrating into the granulation tissues, and the expression is decreased after the cells have aligned. The/z2 may be involved in the spatial control of cardiac wound repair after infarction (87). However, the precise origin of cardiac myofib roblasts remains unclear. ^,

*Table 3. Characteristics and reactivity of anti-rat macrophage monoclonal antibodies**

Antibody	*Isotype*	*Site of recognition*	*Reactive cell*
EDI	IsGl	Membrane of cytoplasmic granules	Monocytes, most macrophages (particularly exudate macrophages)
ED2	IgG2a	Cell membrane	Resident macrophages
ED3	IgG2a	Cell membrane	Exudate macrophages, certain macrophage populations in lymphoid organs
TRPM-3	IgG2a	Cell membrane	Exudate macrophages, certain macrophage populations in lymphoid organs
Ki-M2R	IgA	Cell membrane	Resident macrophages
OX6	IgGl	Cell membrane	la antigen-presenting macrophages, dendritic cells, B-lymphocytes

This table is made by referring to reference 28.

Table 4. *Characteristics of interstitial dendritic cells and resident macrophages in rat myocardium**

Cell type	*MHC class II sensitivity*	*ED2 antigen*	*Radiation antigens*	*Phagocytosis*
Interstitial dendritic cells	+++	-	Yes	
Resident macrophages		++	*No*	++

*This table is made by referring to reference 72.

MACROPHAGE POPULATIONS IN NORMAL MYOCARDIUM

M0 are normally present in the cardiac tissues. By using CD68 pan M0 marker, the immunopositive cells are seen in the interstitium of normal human hearts, often close to blood vessels. The cardiac M0 are more frequently seen in the ventricles (88). However, CD68-positiveM0 were not identified. In rats, ED2-immunolabelled, resident M0 are observed in the interstitium of the myocardium; the numbers of ED2-positive cells per 0.1 mm^2 in the normal myocardium was 6.3+7-2.5(16). The distribution of ED2-positive cells forms a regular network of the interstitial cells throughout the cardiac tissues. OX6-immunopositive interstitial dendritic cells are also distributed diffusely in normal rat heart, their number being 8.2+7-1.0/0.1 mm^2 (59). Apparently, the distribution pattern of the interstitial dendritic cells is similar to that of the the resident M0. Spencer and Fabre (72) showed some differences between interstitial dendritic cells and resident M0 in rat hearts (Table 4). The interstitial dendritic cells constituti vely express large quantities of MHC class II antigens, and they are ED2-negative. In contrast, ED2-positive resident M0 do not reveal MHC class II antigens. Radiation administration experiments using ^{137}Cs suggest that the turnover of the resident M0 is much slower than that of the interstitial dendritic cells. Besides these differences, the interstitial dendritic cells have no phagocytic activity (89). These studies indicate that the interstitial dendritic cells and resident M0 represent two distinct populations in rat hearts. However, interferon gamma (IFN-y) induces MHC class II antigens on the resident M0 (72). Interstitial dendritic cells at immature stages, which are normally existing in connective tissues, do not exhibit MHC class II antigens at a high level (73). It was recently shown that dendritic cells have phagocytic activity, like resident and exudate M0 (90). The relationship between these cells still remains to be evaluated (Fig. 1).

EXUDATE MACROPHAGES IN MYOCARDIAL FIBROSIS

In isoproterenol-induced rat myocardial fibrosis, a markedly increased number of EDI-positive M0 was seen within the affected tissues immediately after the injection, reaching a peak on day 3 (16). Thereafter, the number decreased gradually, but remained abundant in the granulation tissues. ED 1 -positive cells appearing in injured tissues are usually regarded to be a result of migration into the injured site from circulating system (19,68). Marked infiltration of M0 have been reported in human myocardial infarction (91,92), but M0 populations related to the infarction and subsequent fibrosis have not been identified. The increased expression of LFA-1, Mac-1, VLA-4, and ICAM-1 was detected on monocytes in human and rat myocardial infarctions (19,22,93). These molecules contribute to adhesion of monocytes to endothelia in the ischemic territory. The findings that LFA-1, Mac-1,1C AM-1, and VLA-4 were simultaneously expressed on monocytes indicate that monocytes can form microaggregates in the cardiac lesions (22). Estradiol treatment blunted the increased immunostaining of ICAM-1 in the injured rat myocardium, resulting in decrease in cardiac leukocyte accumulation (93). Expression of osteopontin on infiltrating M0 has been confirmed in rat and human myocardial necrotic lesions; it was increased in the early stages and dramatically downregulated as healing proceeds (17). These findings indicate that several adhesion molecules take part in both extravasation of monocytes and cellular adhesion of exudate M0 in myocardial fibrosis. In cardiac lesions, plasma renin activity and blood pressure affect M0 density through stimulating CAM (78).

Relationship between exudate macrophages and myofibrob lasts

Figure 2 shows the kinetics of M0 populations and myofibroblasts in rat myocardial fibrosis. Exudate M0 play an important role in development of fibrosis by producing various cell growth factors (7-9,33). In isoproterenol-induced rat myocardial fibrosis, after the maximal emergence of ED1-positive M0, the myofibroblast number was significantly increased on days 3-7 when fibrous granulation tissues began to be formed (16). In spontaneously/renovasucular hypertensive rats, many EDI-positive M0 were seen in perivascular myocardial fibrosis (77,78). These findings suggest that exu-

date M0 influx from the blood in response to tissue injury is important in mediating myofibroblast induction and proliferation through products released by the M0. In chemically-and cholestatic-induced rat liver fibrosis, the number of my-ofibroblastic perisinusoidal cells markedly increased following an expansion of EDl-positive M0 (10,45). Intriguingly, the expression of leptin, an obese *(pb)* gene product, was reported in hepatic stellate cells and circulating leptin level elevated in patients with alcoholic cirrhosis (94), suggesting a link between the cell biology of fibrosis and adipose tissue (94,94a). In cisplatin-induced rat renal interstitial fibrosis, EDl-positive M0 influx was related to myofibroblast involvement in the early stages (12,13). Essential fatty acid-deficient diet or protein-restricted diet inhibits M0 infiltration, and the administration of such a diet to rats results in a reduction of interstitial fibrosis in puromycin aminonucleo-side-induced nephrosis (60,95). The administration of suble-thal X irradiation to ureteral obstructed rats also resulted in a decrease in both macrophage number and development of myofibroblasts in early stages of the fibrosis (7). There is a close relationship between M0 influx and myofibroblast involvement in fibrogenic lesions. Fibrogenic cell growth factors and exudate M0 and PDGF produced by M0 are the most often implicated factors for the induction of myofibroblast development in fibrogenic lesions. In hepatic and renal fibrosis, increased expression of $mRNA^{TGF\_lM}$ has been well documented, and expression of TGF-p was found on macro-phages by immunostaining or hybridization methods (7,29,34,38,60,95).

TGF-J31 is present in the developing and adult heart (96,97). Its immunoreactivity disappeared in the central necrotic part of rat myocardial infarctions, but a strong immunoreactivity and increased mRNA amounts were found mostly in myocytes at the margins of the infarcted areas (98). Increased production of collagen types I and III at the mRNA and protein levels was confirmed in rat infarcted myocardium, and the source in the myocardial fibrosis was myofibroblasts (1,84). Collagen production by cardiac fibroblasts was stimulated by TGF-pl (99-101). Moreover, cultured cardiac fibroblasts were shown to secrete TGF-J31 which can be stimulated by angiotensin II, suggesting that myofibroblastic cells in myocardial fibrosis act as an autocrine/paracrine stimulus to collagen formation (102). These observations strongly indicate thatTGF-(3l is important for development of myocardial fibrosis. Besides cardiomyocytes and myofibroblasts, exudate M0 can produce TGF-[3 demonstrated in hepatic, renal and pulmonary fibrosis (5,7,29). However, there has been no direct demonstration of TGF-P production of exudate M0 in myocardial fibrosis (19).

The exogenous administration of PDGF-BB to rats induces development of myofibroblasts and accumulation of collagen type III within the kidney interstitium (37). In renal interstitial fibrosis and glomerulosclerosis, a possible origin of PDGF is infiltrating M0 and platelets as well as regenerating renal tubular epithelia (4,8,15). Increased expression of $mRNA^{PDGF\sim AA}$ was reported in the heart of renovascular hypertensive rats (19). PDGF-AA is a potent mitogen for cardiac fibroblasts (103), and stimulates collagen production by cardiac fibroblasts (101). However, the contribution of PDGF-AA and -BB to myocardial fibrosis remains uncertain (19).

TNF-cc is thought to act as an early trigger for the fibrotic cascade (25,104). However, unlike TGF-(3 and PDGF that serve as direct growth factors mediating myofibroblast development and ECM production, TNF-a has weak or negative effects on fibroblast proliferation and matrix production, and, by itself, is unable to induce the myofibroblastic phenotype *in vitro* or *in vivo* (25,105). Cardiac M0 were reported to produce TNF-a (104), but its association with myocardial fibrosis has not yet been investigated. Rather, M0-produced TNF-a contributes to myocardial dysfunction and cardiomyocyte death (apoptosis) in ischemic and chronic heart failure and cardiac allograft rejection (104). Note that mast cell chymase induces both cardiomyocyte apoptosis and myocardial fibroblast proliferation, thus contributing to the progression of heart failure (106). GM-CSF and bFGF have been reported to induce fibrotic responses with accumulation of myofibroblasts (24,35,108,109). The mechanisms underlying GM-CSF- or bFGF-related myofibroblast phenotypes await clarification. GM-CSF and bFGF production by macrophages has not yet been studied in myocardial fibrosis. In short, although cell growth factors such as TGF-J3, PDGF, TNF-a, GM-CSF, bFGF, and NGF have been suggested to be important in the induction of fibrogenic cells and ECM production (106,107) and the M0 infiltrating the fibrosing lesions are seen as possible source of such growth factors, the contribution of s"uch growth factors to myocardial fibrosis has not sufficiently been evaluated.

RESIDENT MACROPHAGES AND INTERSTITIAL DENDRITIC CELLS IN MYOCARDIAL FIBROSIS

ED2-positive resident M0 also participate in the development of rat myocardial fibrosis (16,58,59). In isoproterenol-induced rat myocardial fibrosis, however, the peak of ED2-positive cell number was different from that of EDl-positive M0, in that ED2-positive cell number was maximal on day 14 in the late stages (16) (Fig. 2). TheED2-positive cells showed proliferative activity, demonstrable by the double-labelling methods with bromo-deoxyuridine antibody (16). An increased number of OX6-positive interstitial dendritic cells was maximal at the border zones between normal and necrotic areas 7-14 days after coronary ligation (59). The time of appearance and the distribution of resident M0 correspond to those of the interstitial dendritic cells (16) (Fig. 2). It thus is considered that in the fibrotic lesions the roles of resident M0 are similar in part to those of the interstitial dendritic cells. The roles of resident M0 in myocardial fibrosis are poorly understood. However, it is possible to postulate their roles on the basis of

studies on resident M0 in other organs or on the interstitial dendritic cells in the myocardium.

Kupffer cells in hepatic fibrosis and granulomas

Kupffer cells are a well known resident M0 (6). In CCl_4-induced rat hepatic fibrosis, ED2-positive Kupffer cells are markedly increased in injured areas following the expansion of ED1-positiveM0;proliferative activity of Kupffer cells was confirmed (10,45). After the increased in the number of EDI- and ED2-positive M0, myofibroblastic perisinusoidal cells increased in the affected areas. $mRNA^{TGF\sim pl}$ levels also increased dramatically following CCl_4-induced hepatic injury (29). Themostimportant profibrogenic factor in hepatic fibrosis is TGF-(31, produced by ED 1 - and ED2-positi ve M0 (5,29). Gadolinium chloride blocks phagocytosis by Kupffer cells and selectively eliminates activated Kupffer cells (69,110). The treatment with gadolinium chloride reduced liver injury and subsequent fibrosis in rats receivingchronicethanoladministrationorCCl_4treatment(l 11). Infiltrating M0 and Kupffer cells also take a part in the formation of hepatic granulomas. Liposome-encapsulated dichloro-methylene diphosphonate given intravenously is ingested by M0 and kills them by inducing apoptosis (112). Using this model, it was recently reported that the number and size of the granulomas induced by zymosan were reduced in the Kupffer cell-depleted mice, compared with those of untreated mice (27). The finding that M-CSM and its receptor *c-fms* in M0 are involved in zymosan-induced granuloma formation is similar to that seen with glucan-induced granuloma formation in the Kupffer cell-deficient M-CSF null mutant *op/op* mice (26). The preexisting Kupffer cells form a microen vironment important for M0-related granuloma formation accompanied with accumulation of my ofibroblasts. Activated Kupffer cells are known

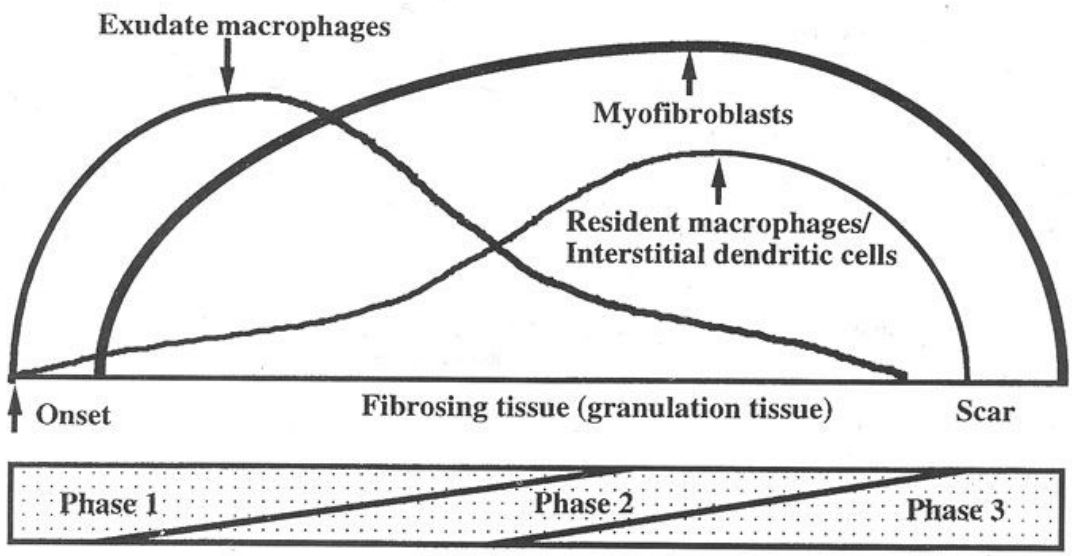

Figure 2. The kinetics of macrophage populations and my ofibroblasts in experimental rat myocardialfibrosis. In the early stages of the fibrosis, the exudate macrophages which are derived from blood monocytes quickely increase. They can induce the development of myofibroblasts by producing cell growth factors such as TGF-fi and PDGF. The resident macrophages gradually increase by dividing and participate in the advance of myocardial fibrosis by releasing cell growth factors similar to those released by the exudate macrophages. The interstitial dendritic cells have a similar appearance and distribution patterns to the resident macrophages. The resident macrophages and interstitial dendritc cells are important in immune-mediated T cell induction in the advanced stages of the myocardialfibrosis. Thefibrosing tissues consist of my ofibroblasts and extracellular matrix proteins such as collagen types I and III. The myofibroblasts contribute to the reparative fibrosis in the myocardium. As the scar tissues develop, the cell components in thefibrosing tissues are decreased by apoptosis, which may be mediated by tnacrophage-produced factors such as TNF-CL and NO. Apoptotic cells are phagocytized by the exudate and resident macrophages. The major events in phases 1-3 of the fibrogenesis are shown in Table 2.

to secrete a number of growth factors including TGF-fS and CSF (5,6,27). In hepatic fibrosis and granulomas, Kupffer cells contribute, as do exudate M0, to the production of cell growth factors capable of inducing myofibroblastic cell development.

Interstitial resident macrophages in pulmonary fibrosis

In rat lungs, alveolar M0 are positive for ED 1, but not for ED2 (68). The alveolar M0 are probably derived from blood monocytes(l 13). Onthecontrary,mostinterstitialM0areED2-positiveresidentM0(68). In the generation of mouse pulmonary fibrosis after silica injection, it was found that the interaction between particles and M0 within the interstitium is more important than that occurring in the alveolar space in stimulating the fibrotic process (114). The close contact between M0 and fibroblasts within the interstitium may permit a more efficient transfer of M0-derived growth factors to preexisting fibroblasts. Interstitial macrophages secrete various cell growth factors including bFGF, TGF-(3 andTNF-a. Such growth factors have been demonstrated to be involved in development of various types of pulmonary fibrosis (24,40,105,114).

Resident macrophages in renal interstitial fibrosis

In experimental tubulointerstitial fibrosis in rats, ED 1 -positive exudate M0 contribute to the induction of myofibroblasts (12-14,51,60,95). In contrast, ED2-positive M0 were not detected in the affected areas in the renal fibrosis, although such cells are present in the vicinity of the arcuate and interlobular arteritis (12,14). It thus appears that the resident M0 do not participate in rat renal fibrosis. However, tissue M0 exist in the normal kidneys. Locally-produced factors may inhibit the maturation of these cells and their participation in the formation of fibrotic lesions in the kidneys (72).

Interstitial dendritic cells in myocardial fibrosis

Appearance of OX6-positive interstitial dendritic cells has been reported in rat myocardial fibrosis (19,58). The dendritic cells are probably mobilized from bone marrow in response to tissue injury (Fig. 1), and the interstitial dendritic cells differentiate under the influence of locally produced growth factors such as GM-CSF,TNF-oc, andinterleukin(IL)-ll3(58,59,73). Activated interstitial dendritic cells up-regulate MHC class II molecules, and the interstitial dendritic cells are major antigen-presenting cells (73). They have several mechanims for antigen internalization. The best characterized pathways are phagocytosis and pinocytosis, in addition to mannose receptor-mediated endocytosis (74,90,115). It is thought that, after myocardial necrosis, the interstitial dendritic cells present heart-derived antigenic components to helper T-lymphocytes. Indeed, the appearance of CD4-positive helper T-lymphocytes in rat hearts after infarction was shown to be associated with the formation of clusters consisting of interstitial dendritic cells (57-59). T cells primed by the interstitial dendritic cells produce IL-1 ,-2, and cytokines involved in Thl/Th2 lymphocyte polarization are produced locally by the interstitial dendritic cells (59,73,116).

Roles of resident macrophages in myocardial fibrosis

Resident M0 reside in connective tissues throughout the body are stimulated to proliferate after the onset of injury (65,68,72) (Fig. 1). In fibrotic lesions, like exudate M0, the resident M0 show phagocytic activity, and produce a variety of cell growth factors including TGF-p, PDGF, TNF-oc, bFGF, and CSF which can induce myofibroblast activation directly or indirectly. In addition, the resident M0 may play an important role in the presentation of highly immunogenic antigen to the T-lymphocyte system as antigen-presenting cells, like the interstitial dendritic cells. However, the repertoire of growth factors secreted by the resident M0, which can contribute to the development of myocardial fibrosis and the regulation of the healing process, remains to be clarified. Yet, a question remains as to whether an increase in M0 number is causally related to or associated with the development of fibrosis (see 117 for mast cells in fibrosis induced in *Ws/Ws* mast cell-deficient rats).

Anitschkow myocytes in myocardial fibrosis

Anitschkow myocytes, which have been observed frequently at the border of the healing infarct (79) and in tissues of cardiac tumors (118), are characterized by caterpillar-like configuration of nuclear chromatin. The possible origin of these cells is still unknown, but myocytes, nerves, histiocytes, endothelial cells and fibroblasts have been proposed as possible precursors (79). In rat myocardial fibrosis, such cells are ED2 positive. This indicates that Anitschkow myocytes are resident M0-derived cells. Similar cells are also seen in rheumatic fever in humans and animals in association with Aschoff bodies. The functional roles of such cell types remain to be evaluated.

APOPTOSIS AND MACROPHAGES IN MYOCARDIAL FIBROSIS

Apoptosis is amechanism of programmed cell death that occurs in a range of physiological processes such as embryonic differentiation and development (reviewed in 119). In the last few years, it has been shown that apoptosis plays a major role in promoting resolution of inflammatory responses (2,9). Apoptosis is also the mechanism for the evolution of granulation tissues into scar tissues with a striking decrease in cellularity (2). In skin wound healing, many myofibroblasts showed changes compatible with apoptosis (2). Polunovsky *et al* (120) have reported *a* role for mesenchymal cell death in lung remodeling after acute injury. In hepatic fibrosis, myofibroblastic peri-sinusoidal cells underwent apoptosis during the repair (3). We investigated the appearance of apoptotic cells in isoproterenol-induced myocardial fibrosis by the terminal deoxyribonucleotide transferase (TdT)-mediated dUTP nick

end labeling method; only a few myofibroblasts showing DNA fragmentation were found (unpublished data). The number of apoptotic cells was much smaller than that we expected, given the active apoptosis of myofibroblasts that occurs in rat tubulointerstitial fibrosis induced by PDGF-BB injection (37). Generally, it is thought that apoptotic processes are an extremely rapid means of cell deletion; the development of morphological changes, uptake by phagocytosis, and degradation of the ingested apoptotic cells beyond histological recognition are completed in 30 minutes to 3 hours (37,121). The lower number of detectable apoptotic cells may be due to these rapid processes. In myocardial fibrosis, apoptosis in myofibroblasts and affected myocytes may be one biological process involved in tissue remodeling after myocyte injury. Several proteins or antigens have been known to regulate apoptosis. Among them Fas antigen, p53, and c-myc protein stimulte apoptosis, whereas bcl-2 protein inhibits apoptosis (3,122,123). *C-myc* and interleukin-1 p-converting enzyme have been shown to induce apoptosis in fibroblasts (124,125), and fibroblasts lack bcl-2 expression as assessed by the antibody staining (126). These situations may be involved in apoptosis in myofibroblasts, because myofibroblasts are considered terminally differentiated fibroblasts (2,47). Cytokines such as TNF-oc and TGF-P have been shown to induce apoptosis in hepatocytes *in vitro* and *in vivo* (119,127). These cytokines are present in the fibrotic tissues, suggesting that apoptosis in the fibrotic lesions is cy tokine-dependent (2,128). The main sources of these factors are exudate and activated resident M0. The increased activity of inducible nitric oxide synthase (iNOS), which catalyzes the reaction of L-arginine to L-citrulline and nitric oxide (NO), is also related to the induction of apoptosis in myocytes in myocardial infarction and allograft rejection (129,130). The immunohistochemical localization revealedM0 as amajorsourceof iNOS expression (129-131). In inflammatory sites, clearance is accomplished mostly by M0 engulfment of cells undergoing apoptosis (2,9,63). The strongest candidate for the M0 receptors responsible for recognition of apoptotic cells is the phosphatidylserine receptors (PSR), which has yet to be characterized, although candidates for PSR function include several types of scavengerreceptors (9). In inflammatory resolution, M0 engulfing cell debris appear to leave the inflamed site *via* lymphatics, and die by apoptosis in adjacent lymph nodes (132). Recently, it was reported in experimentally-induced rat crescent!c glomerulonephritis that M0 themselves undergo apoptosis at inflamed sites (64). Apoptotic M0 have been found at border zones adjacent to the infarcted myocytes in rabbit models (131,133) and in isoproterenol-induced rat myocardial fibrosis (unpublished data). Terminally differentiated dendritic cells appear to die locally by apoptosis (73). Myo-fibroblast and myocyte apoptosis, mediated by M0-produced factors, the subsequent uptake by M0 and the apoptosis of the M0 themselves are the major method of inflammatory clearance and subsequent tissue remodeling in myocardial fibrosis.

THERAPEUTIC IMPLICATIONS: CONTROL OF MACROPHAGES AND MACROPHAGE-PRODUCED FACTORS

For the normal healing of fibrosis, the availability of the signaling substances must be optimal, precise, and synchronized. Inhibition, interruption or excess expression of these signals seems to be responsible for failure in healing, which is manifested by either impairment (nonhealing) or excess tissue formation (134). M0 and their secretory products play an important role in the fibrogenesis and resolution. The regulation and removal of M0 and/or M0-secreted growth factors/ cytokines are avenues of therapeutic potential for fibrotic lesions, including myocardial fibrosis. Indeed, treatments designed to inhibit M0 infiltration could reduce the degree of fibrotic lesions in experimental fibrosis models of the liver and kidneys (see above Sections: "Relationship between exudate macrophages and myofibroblasts" and "Kupffer cells in hepatic fibrosis and granulomas").

Furthermore, accumulating findings demonstrate that micro-tubule-disassembling (antitubulin) agents, particularly, col-chicine, may inhibit (;') the release of certain proinflammatory/ fibrogenic cytokines (135) and MMP (136), and *(ii)* the intracellular secretory pathway in various fibrogenic cells (reviewed in 137). It is noteworthy that colchicine and other antitubulins upregulate the expression of $mRNA^{NGF}$ in neuronal cells and also in skin fibroblasts (13 8). And NGF treatment may improve the healing process in various tissues (107,139-141).

Another therapeutic approach may include the antibodies neutralizing cell growth factors, as demonstrated in experimental fibrosis models. For example, antibodies to TGF- P reduce hepatic and pulmonary fibrosis, and glomerulosclerosis (29,142), whereas TNF-a antibodies ameliorate pulmonary fibrosis (32,143). Alpha 2-macroglobulin (oc2M) is aprotein thatregulates the distribution and activity of many growth factors such as TGF- p, TNF-a, and PDGF, and hence may also be a therapeutic target for fibrosis (reviewed in 144). Strategies to interfere with TGF-p activity, such as through the administration of decorin (leucine-rich proteoglycan), have been successful in limiting disease in experimental fibrotic models (145). Recently, the usage of antisense oligonucelotides against cell growth factors has been reported in growth factor-mediated disease states. For example, the administration of antisense oligonucleotides to TGF-p 1 effectively blocked M0 expression of TGF-P 1, resulting in the prevention of progressive fibrosis (134). Adenosine, which reduces TNF-a production by M0, decreases cardiac TNF-a level and improves postischemic myocardial function (107). M0 are a major source of iNOS production, which is related to the induction of apoptosis in cardiomyocytes. The preferential inhibition of iNOS by S-methylisothiourea sulfate resulted in a significant improvement of left ventricular performance and increased regional myocardial blood flow in rabbit cardiac infarction models (129). The selective inhibition of iNOS (116,129) and MMP (146) activity provides a therapeutic strategy

in cardiovascular fibrosis. However, the efficacy and safety of these therapeutic strategies for regulating M0 and M0-produced soluble factors have not yet been established in human myocardial fibrosis.

CONCLUSION

M0 are heterogeneous cell populations and show various roles in the fibrotic lesions. In myocardial fibrosis, exduate and resident M0 and interstitial dendritic cells are involved. In the early stages, exudate M0 derived from blood monocytes play a major role in induction of myofibroblasts to develop fibrous granulation tissues by producing cell growth factors such as TGF-P, PDGF, TNF-a, bFGF, NGF, and CSF. Thereafter, the resident MO gradually increase by dividing and participate in the advance of myocardial fibrosis by releasing cell growth factors similar to those released by exudate M0. Although the relationship between residentM0 and interstitial dendritic cells has to be further investigated, these cells appear to be important in immune-mediated T cell induction in the advanced stages of myocardial fibrosis. The repair of myocardial cell death terminates with the establishment of scar tissue consisting of myofibroblasts, small vessels and ECM components such as collagen types I and III (reparative fibrosis). As the scar evolves, the cell components are decreased by apoptosis, which may be mediated by M0-secreted factors such as TNF-a and iNOS. Apoptotic cells arc phagocytized by the exudate and resident M0. The regulation of M0 and M0-produced factors may provide novel therapeutic strategies in clinical trials of myocardial fibrosis.

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