REPUBLIC OF TURKEY
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INVOLVEMENT OF SEROTONIN IN THE DEVELOPMENT OF CARDIAC REMODELING

MSc. Samiye YABANOĞLU

Program of Biochemistry
DOCTOR OF PHILOSOPHY THESIS

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To the Director's Office of the Institute of Health Sciences of Hacettepe University

This study has been accepted and approved as a Ph. D. thesis in the program of biochemistry by the examining committee whose members listed below.

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ABSTRACT

Yabanoğlu, S., Involvement of serotonin in the development of cardiac remodeling. Hacettepe University Institute of Health Sciences, Doctor of Philosophy Thesis in Biochemistry, Ankara 2008.

Platelet activation occurs in different acute and chronic heart diseases including myocardial infarction, obstructive hypertrophic cardiomyopathy and valve stenosis. Recent studies suggested that some factors secreted by activated platelets may participate in inflammation and cardiac remodeling observed during cardiac injury. In the present study it was investigated whether platelets and platelet-released serotonin (5-HT) are directly involved in the functional regulation of cardiac fibroblasts.

Treatment of neonatal rat cardiac fibroblasts with platelet lysate, 5-HT and the 5-HT_{2A} receptor agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) increased the expression of alpha-smooth muscle actin (α-SMA) protein, a marker of fibroblast differentiation into myofibroblasts. Platelet lysate (PL), 5-HT and DOI also induced a time-dependent stimulation of cardiac fibroblast migration that was inhibited by the 5-HT_{2A} receptor antagonist ketanserin. Incubation of cardiac fibroblasts with platelet lysate or 5-HT enhanced secretion of transforming growth factor beta-1 (TGF-β1) and expression of matrix metalloproteinase-3 (MMP-3) and matrix metalloproteinase-13 (MMP-13). As observed for fibroblast migration, these effects were prevented by ketanserin.

The effect of PL and 5-HT on proinflammatory cytokines synthesis was determined in cardiac fibroblasts. Both platelet lysate and 5-HT stimulated interleukin-6 (IL-6) protein secretion and mRNA expression by cardiac fibroblasts in a time and concentration-dependent manner. Ketanserin inhibited this stimulatory effect of PL and 5-HT. Furthermore, PL and 5-HT upregulated tumor necrosis factor-α (TNF-α) and Granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion by CFs both in protein and mRNA level. Ketanserin abrogated this induction of TNF-α and GM-CSF secretion and expression. The induction of monocyte chemotactic protein-1 (MCP-1) mRNA expression by 5-HT and platelet lysate was time-dependent. The levels of MCP-1 released by CFs exposed to PL or 5-HT were 1.5- and 10-fold higher than that of control, respectively. Ketanserin totally inhibited the effect of 5-HT on MCP-1 protein secretion whereas this effect was partial when CFs were stimulated with platelet lysate.

These results demonstrated for the first time that factors released from platelet directly regulate cardiac fibroblasts by enhancing the secretion of TGF-β and MMPs and promoting their migration, differentiation and proliferation. Besides, in the site of injury serotonin released from platelets leads to cytokine and chemokine secretion from cardiac fibroblasts. Serotonin released by platelets appears to be a major contributor of platelet effects which are mediated through 5-HT_{2A} receptors.

Keywords: Serotonin, Platelet, Fibroblast, Inflammation, Cardiac Remodeling

ÖZET

Yabanoğlu, S., Kardiyak yeniden düzenlenimde serotoninin rolü. Hacettepe Üniversitesi, Sağlık Bilimleri Enstitüsü Biyokimya Programı Doktora Tezi, Ankara 2008.

Miyokard enfarktüsü, obstrüktif hipertrofik kardiyomiyopati ve valf stenozu gibi çeşitli akut ve kronik kalp hastalıklarında platelet aktivasyonu gözlenir. Son yıllarda yapılan çalışmalarda aktive olmuş plateletlerden salınan bazı faktörlerin kardiyak hasar sırasında gözlenen enflamasyonda ve kardiyak yeniden düzenlenimde rol aldıkları ileri sürülmüştür. Bu çalışmada plateletlerin ve plateletlerce salınan serotoninin (5-HT) kardiyak fibroblastların fonksiyonel regulasyonuna direkt etkileri incelenmiştir. Neonatal rat kardiyak fibroblastlarının platelet lizatı (PL), 5-HT ve 5-HT_{2A} reseptör agonisti 1-(2,5-dimetoksi-4-iyodofenil)-2-aminopropan (DOI) ile stimülasyonu sonucu fibroblastların miyofibroblastlara dönüştüğünü gösteren belirteç olan alfa-düz kas aktin (α-SMA) protein ekspresyonunun arttığı saptanmıştır. PL, 5-HT ve DOI ile stimülasyonun ayrıca zamana bağımlı olarak fibroblastların migrasyonunu arttırdığı ve bu artışın bir 5-HT_{2A} reseptör antagonisti ketanserin ile inhibe olduğu saptanmıştır. Kardiyak fibroblastların PL veya 5-HT ile inkübasyonu sonucu transforme edici büyüme faktörü-beta1 (TGF-β1) sekresyonunun ve matriks metallo proteinaz-3 (MMP-3) ile MMP-13 ekspresyonunun arttığı belirlenmiştir. Fibroblastların migrasyonunda olduğu gibi bu etkilerin ketanserin ile inhibe olduğu saptanmıştır. PL ve 5-HT her ikisi de, zaman ve konsantrasyon bağımlı olarak kardiyak fibroblastlarda interlökin-6 (IL-6) protein sekresyonunu ve mRNA ekspresyonunu uyarmıştır. Ketanserin bu etkileri de inhibe etmiştir. Bunlara ek olarak PL ve 5-HT kardiyak fibroblastlarda tümör nekrozu faktörü (TNF-α) ve granülosit makrofaj-koloni stimüle edici faktör (GM-CSF) sekresyonunu hem protein hem de mRNA düzeyinde stimule etmişlerdir. Ketanserin, PL'nin ve 5-HT'nin TNF-α ve GM-CSF sekresyonu ve ekspresyonu üzerindeki tetikleyici etkilerini ortadan kaldırmıştır. 5-HT ve PL ile gözlenen monosit kemoatraktan protein-1 (MCP-1) mRNA ekspresyonundaki artış zamana bağımlı olarak izlenmiştir. MCP-1 salınımının PL ve 5-HT ile uyarımı takiben kontrole oranla sırasıyla 1.5 ve 10 kat fazla olduğu saptanmıştır. Ketanserin 5-HT'nin bu etkisini tamamen inhibe etmiş olmasına karşın PL'nin etkisi üzerindeki inhibisyonunun kısmi olduğu belirlenmiştir.

Bu çalışma ile ilk kez plateletlerce salınan faktörlerin hem TGF-β ve MMPs salınımını arttırarak hem de migrasyonu, diferansiasyonu ve proliferasyonu tetikleyerek direkt olarak kardiyak fibroblastları regüle ettiği ortaya konmuştur. Bununla beraber hasarlı bölgede plateletlerce salınan serotoninin kardiyak fibroblastlardan sitokin ve kemokin salınımını arttırdığı tespit edilmiştir. Plateletlerden salınan serotoninin platelet stimülasyonu ile gözlenen etkilerin çoğunluğundan sorumlu olduğu ve bu etkilerin 5-HT_{2A} reseptörleri aracılığıyla oluştuğu ortaya konmuştur.

Anahtar Kelimeler: Serotonin, Platelet, Fibroblast, Enflamasyon, Kardiyak yeniden düzenlenim

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ABBREVIATIONS

HF	Heart failure
CF(s)	Cardiac fibroblast(s)
5-HT	Serotonin, 5-hydroxytryptamine
PL	Platelet lysate
CNS	Central nervous system
CVS	Cardiovascular system
SERT	5-HT transporter
CXCL 4	CXC chemokine ligand 4, platelet factor 4
vWF	von Willebrand factor
MAO	Monoamine oxidase
5-HIAA	5-hydroxyindole acetic acid
LV	Left ventricular
ECM	Extracellular matrix
TGF-β	Transforming growth factor beta
MMP	Matrix metalloproteinase
MT-MMP	Membrane type matrix metalloproteinase
TIMP	Tissue inhibitors of metalloproteinase
PDGF	Platelet-derived growth factor
EGF	Epidermal growth factor
bFGF	Basic fibroblast growth factor
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted (CCL 5)
IL	Interleukin
PAI-1	Plasminogen activator inhibitor-1
MCP-1	Monocyte chemotactic protein-1, CCL2
TNF-α	Tumor necrosis factor-α
GM-CSF	Granulocyte macrophage colony stimulating factor
α-SMA	Alpha smooth muscle actine

PBS	Phosphate buffered saline
FBS	Fetal bovine serum
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
DAPI	4',6-diamidino-2-phenylindole
EGTA	Ethylene glycol tetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
TBS	Tris-Buffered Saline
PVDF	Polyvinylidene fluoride
HRP	Horseradish peroxidase
RT-PCR	Reverse transcription polymerase chain reaction
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
PET	Polyethylene terephthalate
DMEM	Dulbecco's Modified Eagle's Medium

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1. INTRODUCTION

In the developed countries heart failure (HF) is one of the main factors leading to death. Among the different types of cardiomyopathies which lead to cardiac insufficiency, ischemic cardiomyopathies represent 60 to 70 % of the cases. These pathologies are induced by a breakdown in cardiac oxygenation which leads to myocardial infarct. Acute coronary occlusion induces cardiac tissue necrosis, inflammation and a complex remodeling that occurs both in the infarcted and the non infarcted myocardium.

Platelet activation is a primary physiological response to limit bleeding after acute vascular insults. Factors released by activated platelets participate in the pro-thrombotic cascade and in the local vasoconstriction to stem the flow of blood from the injury site. Platelets influence leucocyte function via direct cell-cell contact and/or soluble mediators. During the last years, several studies showed that platelets are also activated in different acute and chronic diseases. In the heart, platelet activation occurs in acute situations such as myocardial infarction or chronically, during obstructive hypertrophic cardiomyopathy and valve stenosis. In these cases, it has been suggested that factors secreted by activated platelets participate in cardiac remodeling through the regulation of endothelial and vascular smooth muscle cells. Cardiac fibroblasts (CFs) may also represent an additional target of platelet-released factors.

The role of fibroblasts in cardiac homeostasis is complex: on one hand, they play a critical role in cardiac repair after acute myocardial injury; on the other hand, they participate in pathological ventricular remodeling leading to reactive fibrosis and HF. Previous studies showed that some cytokines found in platelets behave as fibroblast mitogen factors in different tissues. However, the impact of platelet released factors on phenotype and function of CFs is still not defined.

Among factors released by platelets, this study was focused on the biogenic amine serotonin (5-hydroxytryptamine, 5-HT). 5-HT has been reported as one of the mediator potentially involved in cardiac remodeling. Indeed, excessive blood 5-HT concentration has been associated with various cardiac dysfunctions in human and is suspected to actively participate in the pathogenesis of heart diseases secondary to

carcinoid tumors. Moreover, 5-HT infusions in the rat or genetic deletion of 5-HT transporter in mice induce valvulopathy with cardiac fibrosis and ventricular dysfunction.

In the present study, the effects of platelet released 5-HT on regulation of CF functions were investigated. To fulfill that, the effects of 5-HT and platelet lysate (PL) on CFs in the aspect of fibrosis and development of inflammation were analyzed.

2. GENERAL INFORMATION

2.1. 5-HT

5-HT, is a biogenic monoamine that is widely distributed in the body. It was first identified in the blood as a vasoconstrictor of large vessels (1) and subsequently identified as a neurotransmitter synthesized in the central nervous system (CNS), where it modulates a variety of behavioral functions, including the regulation of sleep or wakefulness, appetite, nociception, mood, stress and maternal or sexual behaviour (2). In addition, 5-HT participates in numerous other physiological and homeostatic processes, such as gastrointestinal peristalsis, blood coagulation and the maintenance of blood pressure (3–5). The altered regulation of 5-HT in humans has been implicated in a wide range of psychiatric conditions, including obsessive–compulsive disorder, depression, anxiety, eating disorders, substance abuse and dependence (6, 7). It has also been implicated in cerebrovascular conditions (such as migraine (8), gastroenteric diseases (including irritable bowel syndrome (9) and cardiac arrhythmia, conduction block or valvular fibroplasia (10).

2.1.1. Synthesis and storage of 5-HT

The essential amino acid tryptophan is the precursor for 5-HT. Over 95% of 5-HT in the body is synthesized in the enterochromaffin cells of the intestine, with the remainder synthesized in the raphe nuclei of the brain, neuroendothelial cells that line the lung and a few other discrete sites including the cardiovascular system (CVS) (11). 5-HT synthesis depends on the specific action and rate-limiting step of the enzyme tryptophan hydroxylase, which transfers a hydroxyl group to the benzyl ring of tryptophan. Subsequent decarboxylation by amino acid decarboxylases results in the formation of 5-HT (fig. 2.1). Tryptophan hydroxylase is encoded by two genes: the well characterized tph1 gene (12) and a recently identified tph2 gene. Whereas the expression of tph2 is neuronal, that of tph1 is non-neuronal (13, 14). This allows for distinct sources of peripheral and central 5-HT.

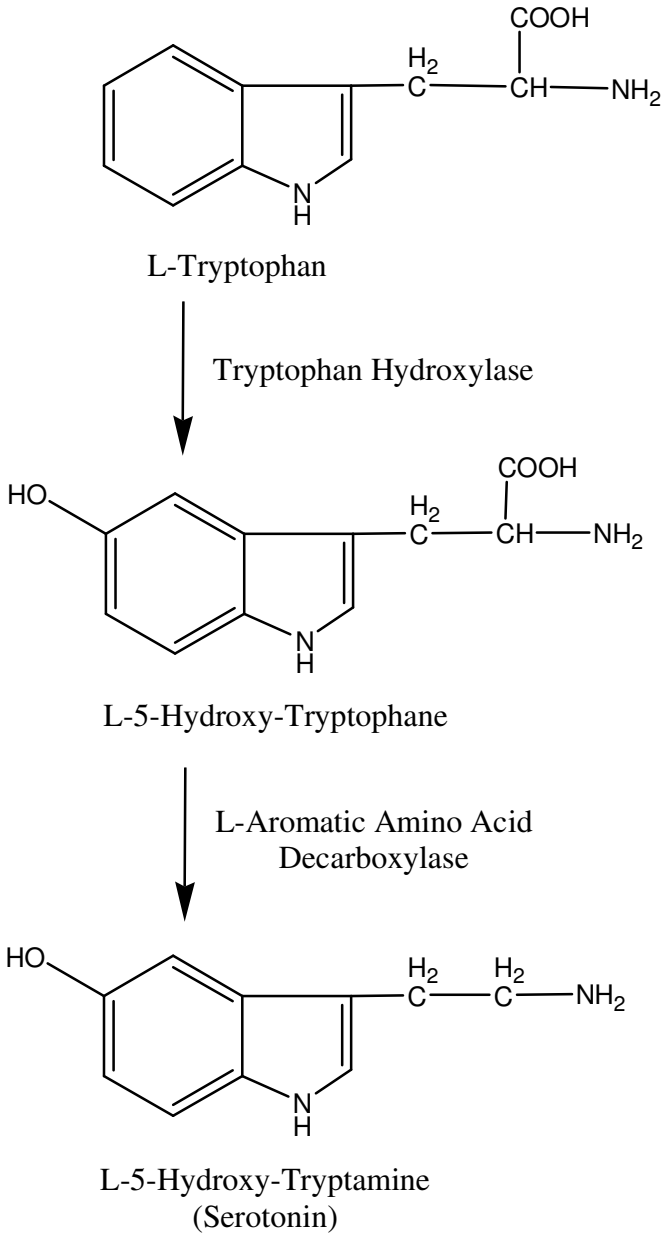


Figure 2.1: Conversion of tryptophan to 5-HT.

5-HT can be stored in intracellular organelles present in nerve endings, mast cells, adrenal medullary cells, and platelets. Other than playing a crucial role in 5-HT synthesis, enterochromaffin cells are also a storage site for 5-HT. In the periphery, platelets are the major 5-HT storage site. Platelets themselves do not synthesize 5-HT, but they take up 5-HT from the gut through 5-HT transporter (SERT) and function as a buffer, keeping the free circulating 5-HT at low levels. Excluding

platelets, the free circulating levels of 5-HT in plasma (15–120 nM) are lower than the levels of 5-HT in whole blood (μM range). As the carrier and storage site of 5-HT, the platelets store 5-HT in dense electron-opaque granules. Storage of 5-HT in platelet granules requires active uptake of 5-HT from the cytoplasm by vesicular monoamine transporter 2. Different forms of 5-HT storage may exist, as suggested by the findings in enterochromaffin cells, showing that a small part of 5-HT remains outside the secretory granules. 5-HT could also be covalently bound to proteins that are to be stored in platelets (15). 5-HT is released by platelets during specific platelet activation.

Upon platelet activation, platelets lose their discoid shape (fig. 2.2a), become spherical, and extend long, spiky pseudopods (fig. 2.2b). The organelles are contracted towards the platelet center and are enclosed by a tight-fitting ring of reassembled microtubules and microfilaments (16).

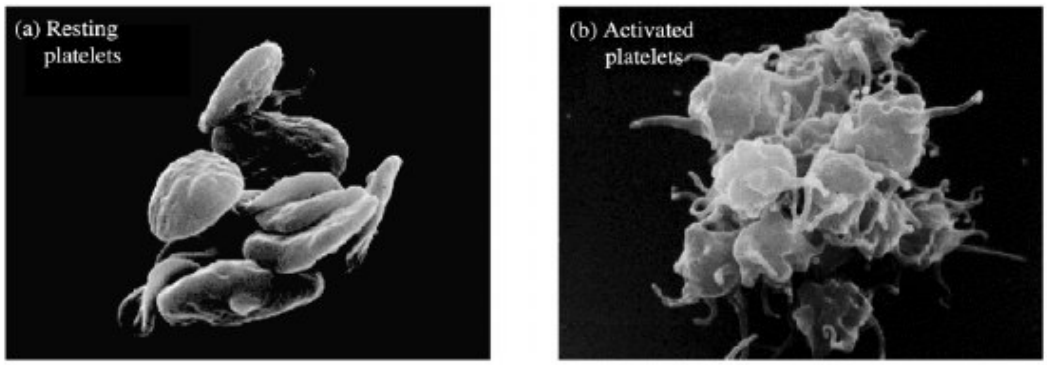


Figure 2.2: Resting (a) and activated (b) platelets (16).

Platelets contain a plasma membrane, internal membranes (open canalicular and dense tubular systems), a cytoskeleton (microtubules and microfilaments), mitochondria, glycogen granules, storage granules (α-granules and dense granules), lysosomes, and peroxisomes.

α-Granules and dense granules are platelet-specific storage granules. α-Granules contain mainly proteins, such as platelet factor 4 (CXCL 4: CXC chemokine ligand 4), β-thromboglobulin, platelet-derived growth factor, fibrinogen, fibronectin, thrombospondin, plasminogen activator inhibitor-1, and von Willebrand

factor (vWF). Dense granules are rich in 5-HT, adenosine diphosphate (ADP) and calcium. Like other contents of dense granules; 5-HT is also released into the local microenvironment when platelets are activated. Exact contributions of this secreted 5-HT are not yet fully understood.

2.1.2. Metabolism of 5-HT

Metabolism of 5-HT primarily occurs by deamination via mitochondrial monoamine oxidase (MAO) to form 5-hydroxyindole acetaldehyde, which in turn is oxidized by aldehyde dehydrogenase to produce 5-hydroxyindole acetic acid (5-HIAA) (fig. 2.3). MAOs are degradative enzymes of monamines, such as norepinephrine, 5-HT, and dopamine. There are two izoforms of MAO, MAO-A and MAO-B, based on their selectivity to substrate and inhibitors. Whereas MAO-A preferentially metabolizes norepinephrine and 5-HT and is inhibited by clorgyline, MAO-B acts on dopamine and is inhibited by L-deprenyl. Pargyline and iproniazid inhibit both MAO-A and MAO-B. Tissues or cells that contribute significantly to 5-HT metabolism include the lung, intestine, and vascular smooth muscle cells. Over 90% endogenous 5-HT is cleared in the pulmonary circulation of the lung, but any cell that can take up 5-HT and possesses MAO has the potential to metabolize 5-HT (11).

The byproducts generated during oxidation of biogenic amines include hydrogen peroxide (H_2O_2), a potential intracellular messenger mediating different cellular functions like proliferation, hypertrophy, differentiation, apoptosis and necrosis. This original mechanism of action of biogenic amines has been validated in numerous models such as smooth muscle cells, kidney cells, neurons and cardiomyocytes (17).

It was previously demonstrated that 5-HT behaves as a proapoptotic factor in cardiomyocytes and that its effect occurs independently of receptor stimulation. This mechanism of action of 5-HT that authors have described requires 5-HT uptake into cardiac cells, its degradation by MAO-A, and H_2O_2 production. It was also demonstrated that H_2O_2 production by MAO-A plays a critical role in post-I/R events, leading to cardiac damage (18).

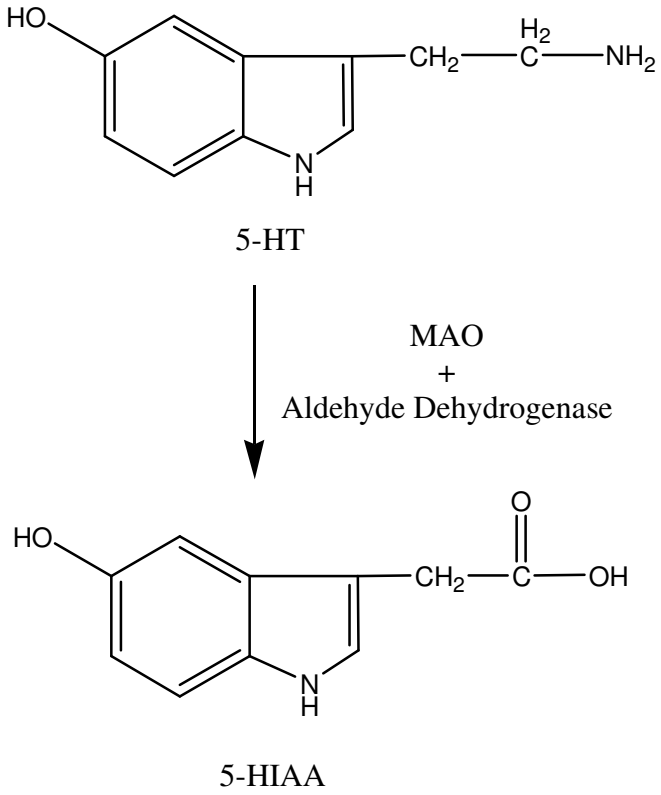


Figure 2.3. Metabolism of 5-HT by MAO and aldehyde dehydrogenase

2.1.3. Uptake of 5HT

5-HT is a protonated molecule that, under physiological conditions, is not capable of crossing the membrane lipid bilayer. SERT, a bidirectional transporter, is the major protein responsible for uptake of 5-HT. SERT, cloned in rat, human, and mouse, is an integral protein that decreases the function of 5-HT by removing 5-HT from the site of action and bringing it into the cell for metabolism and storage (11).

SERT is widely distributed in the CNS and a target of antidepressant drugs, such as fluoxetine, fluvoxamine, citalopram, and paroxetine, and the anorexigen (+)-fenfluramine. SERT is also found in the peripheral sympathetic nervous system, as well as in platelets, gastrointestinal system, and lung.

SERT abnormalities are strongly associated with pulmonary hypertension. The classic concept that 5-HT function is terminated once inside the cell has been

challenged by the findings that intracellular 5-HT mediates pulmonary arterial smooth muscle proliferation after uptake by SERT (19).

2.1.4. SERT Polymorphysim

Polymorphic alterations of the SERT of platelets are associated with a higher risk of myocardial infarction (20). The SERT is encoded by a single gene expressed in neurons, platelets, as well as endothelial, vascular and other cells. Human SERT expression is determined genetically by an insertion/deletion polymorphism in the promoter region of the gene with long (L) and short (S) forms (21). The L allele doubles or triples the rate of SERT gene transcription compared to the S allele (22), reflected by higher SERT mRNA and protein levels and increase in 5-HT uptake into lymphoblasts with the LL genotype compared to the SS and LS genotypes (21). Individuals with the LL and LS genotypes had significantly higher blood 5-HT than did those with the SS genotype (23).

The lower SERT expression in platelets results in lower 5-HT uptake and less 5-HT available for release, thereby reducing the risk of platelet aggregation, thrombus formation and vascular spasm. As expected, patients with the SS SERT genotype appear to have a lower risk of myocardial infarction, delaying the age of onset, in particular among smokers (24). Antidepressants which are inhibitors of 5-HT uptake by SERT, thereby depleting platelet 5-HT storage and reducing function (25), also reduce the incidence of myocardial infarction; increasing transporter affinity correlates with greater protection (26). Selective inhibitors of 5-HT uptake by SERT further inhibit platelet function in patients with congestive HF already on aspirin (27).

Conversely, because of greater 5-HT platelet storage, platelet activation is greater in a group of clinically depressed patients with the SERT LL genotype than with other genotypes and this may facilitate ischemic heart disease (28). Similar conclusions were made from a study with Japanese individuals in which the L allele of the SERT was associated with coronary heart disease in smokers (29).

2.1.5. Role of 5-HT in the Heart

5-HT is found mainly in three areas of the body: the intestinal wall (where it causes increased gastrointestinal motility), blood vessels (where it causes constriction of large vessels), and CNS in human. The effects and functions of 5-HT have been mostly studied in CNS. The effects of 5-HT in the CVS are complex, and the functional evidence is not compelling. 5-HT receptor antagonists have been shown to affect cardiovascular regulation in conflicting ways: for example, their cardiovascular effects are associated with bradycardia or tachycardia, hypotension or hypertension and vasodilatation or vasoconstriction (30).

Based mostly on work with murine myocardium, it is plausible that 5-HT has a physiological role in human cardiac embryogenesis as it appears to have in mice. Recently, studies using a tph1$^{-/-}$ mouse have provided clear evidence for the importance of a crucial level of blood 5-HT in the CVS. Mice lacking tph1 displayed a drop in peripheral 5-HT, most notably in the blood (with a residual 5-HT level of 4–8% of normal values), heart, lungs and spleen (13). These animals developed progressive alteration of cardiac function, with a wide range in the severity of the cardiopathic phenotype, and they ultimately died from HF. Côté et al. (13) postulated that the lack of 5-HT was deleterious to heart function.

Harmful effects of 5-HT are due to platelet-released 5-HT which can produce thrombogenesis, vascular spasm, mitogenesis and proliferation of vascular smooth muscle cells. The endocardium protects the myocardium by preventing platelet aggregation, but when the endocardium is damaged as for example in HF or I/R platelet aggregation and secretion is facilitated (31) and 5-HT can exert harmful effects on myocardial cells (32). Plasma levels of 5-HT, originated from enterochomaffin cells, appear to be elevated in patients with congestive HF (33, 34). In addition, the human heart itself stores and perhaps synthesises 5-HT. Human left ventricular (LV) myocardium contains ~400 ng 5-HT g^{-1} (35) but its histological localisation was unknown.

Although some of the effects exerted by 5-HT, such as pulmonary arterial smooth muscle proliferation, are mediated through receptor-independent signaling mechanisms (19), most of its effects on cardiac tissue is mediated by receptors.

2.1.6. The 5-HT Receptors in the Human CVS

The 14 different 5-HT receptors are divided into 7 groups (5-HT_1 through 5-HT_7) based on molecular structure, signal transduction properties and pharmacological properties (36-37).

2.1.6.1. The 5-HT_1 group of receptors

The 5-HT1 group consists of 5 different receptors, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}. These receptors all couple via the G protein Gi with inhibition of adenylyl cyclase as their primary signaling mechanism (38).

The 5-HT_{1A} receptor is mainly located in the CNS, and except for a possible role in rat renal vasorelaxation (39), there is no evidence for direct cardiovascular effects mediated through this receptor. Furthermore, evidence for localisation of this receptor in the heart or blood vessels is also lacking. In contrast, the 5-HT_{1B} receptor is widely distributed in the CVS. The 5-HT_{1B} receptor, also previously known as 5-$HT_{1D\beta}$ (37, 40, 41) is found on both endothelial cells and smooth muscle cells of several human vessels (42) including coronary and pulmonary artery (Fig. 2). The 5-HT_{1B} receptor is mainly implicated in vasoconstriction (43). The cardiovascular role of the 5-HT_{1D} receptor is less clear.

The 5-HT_{1E} receptor will not be further discussed as there is no evidence of cardiovascular location or effects of this receptor. The 5-HT_{1F} receptor on the other hand may potentially be involved in the anti-migraine effect of at least some of the triptans. However, this postulated effect is believed to result from inhibition of neurogenic inflammation but not from vasoconstriction (44).

Recent evidence suggests that the molecularly elusive so called 5-HT_{1P} receptor found in the gut (45) may represent an oligomeric form of the 5-HT_{1B} receptor through interaction with either the D2 dopamine receptor (heterooligomers) or other 5-HT_{1B} receptors (46). As yet, there is no evidence for this receptor in the human CVS.

2.1.6.2. The 5-HT_2 group of receptors

This receptor class contains 3 members, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} (36, 37). These receptors couple primarily via the G proteins Gq or G11 to activation of phospholipase C.

The 5-HT_{2A} receptor is widespread in the human CVS. It is present on arterial smooth muscle (42) and mediates vasoconstriction (43), whereas it is absent on endothelial cells (42). It is also present on platelets and facilitates platelet aggregation (47, 48). The 5-HT_{2B} receptor mediates mitogenic signaling and may be involved in pulmonary hypertension as well as valvulopathy (49). The 5-HT_{2C} receptor has not been found in the CVS.

In mice the 5-HT_{2B} receptor appears to have an obligatory role for normal cardiac embryogenesis. Genetic ablation of 5-HT_{2B} receptors causes partial lethality in midgestation and ventricular trabeculation defects (50). Midgestation mortality of the 5-HT_{2B} knock-out mice has been attributed to an impairment of the ability of the 5-HT_{2B} of wild-type mice to transduce mitogenic signals in fibroblasts (51). In addition, 5-HT_{2B} receptors have antiapoptotic properties in murine cardiocytes (52). Whether these functions of the 5-HT_{2B} receptors occur in the developing human heart is unknown.

2.1.6.3. The 5-HT_3 receptor

The 5-HT_3 receptor is the only 5-HT receptor which is not a G protein-coupled receptor, but belongs to the Cys-loop family of receptors (53). It is a pentameric ligand-gated cation channel similar to the nicotinic acetylcholine and $GABA_A$ receptors (54). So far, two human 5-HT_3 receptor subunits had been cloned, 5-HT_{3A} (55, 56) and 5-HT_{3B} (57). In the heart, the 5-HT_3 receptor is mainly believed to be located on afferent vagal and sympathetic neurons, mediating reflex bradycardia and pain, respectively. 5-HT_3 receptors could also mediate a possible reflex vasodilation in the human forearm. Blauw et al. (58) showed that infusion of 5-HT into the radial artery of healthy volunteers elicited forearm vasodilation, assessed with venous plethysmography, that was prevented by the 5-HT_3/5-HT_4

antagonist tropisetron. However, direct vasodilation through 5-HT_4 receptors has not been ruled out.

2.1.6.4. The 5-HT_4 receptor

The 5-HT_4 receptor is positively coupled to the G_s protein/adenylyl cyclase system and, thus, cAMP formation (59).

In the human CVS, 5-HT_4 receptors are present in cardiac atria (60) and ventricles (61), where they mediate increases in contractility. 5-HT_4 receptors may also be expressed on human endothelial cells (42) but their function is still unknown. The implications to cardiac function of this plethora of splice variants remain unresolved, partly because of a lack of tools to address this question.

The human 5-HT_4 receptor exists in multiple splice variants, which are identical up to Leu- 358, followed by different C-terminal tails. These are named 5-$HT_{4(a)}$, 5-$HT_{4(b)}$, etc., with the 5-$HT_{4(i)}$ splice variant as the most recent addition to the list (62).

Recent work suggests different coupling of 5-$HT_{4(b)}$ receptors, compared to 5-$HT_{4(a)}$ receptors (63) or 5-$HT_{4(d)}$ receptors (64); possible implications for cardiac arrhythmias. Other work, so far only with the mouse 5-$HT_{4(a)}$ receptor, indicates that the 5-HT_4 receptor can also activate G_{13}, resulting in activation of members of the Rho family of small GTPases (65). This may open new avenues of possible effects mediated through this receptor, perhaps also in the CVS.

Although 5-HT_4 receptors mediate human cardiostimulation, cardiac dysfunction has not been reported in 5-HT_4 knockout mice (66).

2.1.6.5. 5-HT_5 and 5-HT_6 receptors

The human 5-HT_5 receptor is still considered as a putative receptor since there isn't any evidence to confirm that this receptor is expressed in an endogenous setting (67). Concerning the 5-HT_6 receptor, there is no evidence to support a role for this receptor in the CVS.

2.1.6.6. The 5-HT_7 receptor

The 5-HT_7 receptor couples positively to Gs protein thereby activating the adenylyl cyclase.

Recombinantly expressed human 5-HT_7 receptors behave as if they are preassociated with G protein in the absence of ligand (68) and express the unusual property of apparently stealing signaling capacity from other Gs-coupled receptors (69), but the physiological and human cardiovascular significance of this is as yet unknown.

In the human CVS, mRNA for the 5-HT_7 receptor has been found in coronary arteries (70) and on vascular smooth muscle, where it may mediate relaxation (42). However evidence for human cardiac functions of the 5-HT_7 receptor is not known yet.

2.2. Cardiac Remodeling in Pathological Situations

HF is a leading cause of morbidity and mortality in developed countries. In recent years, a greater understanding of the cellular mechanisms of HF has led to new insight into its medical management. In HF increased mechanical stress, the influence of circulating and myocardial neurohormones, cytokines result in alterations in the architecture of the myocardium, a collective process known as myocardial remodeling (fig. 2.4). This process involves three primary responses at the cellular level:

(1) hypertrophy, dysfunction, and death of cardiac myocytes,
(2) increased deposition and alteration of the cardiac extracellular matrix (ECM), often described by the oversimplified term myocardial fibrosis and
(3) inflammation.

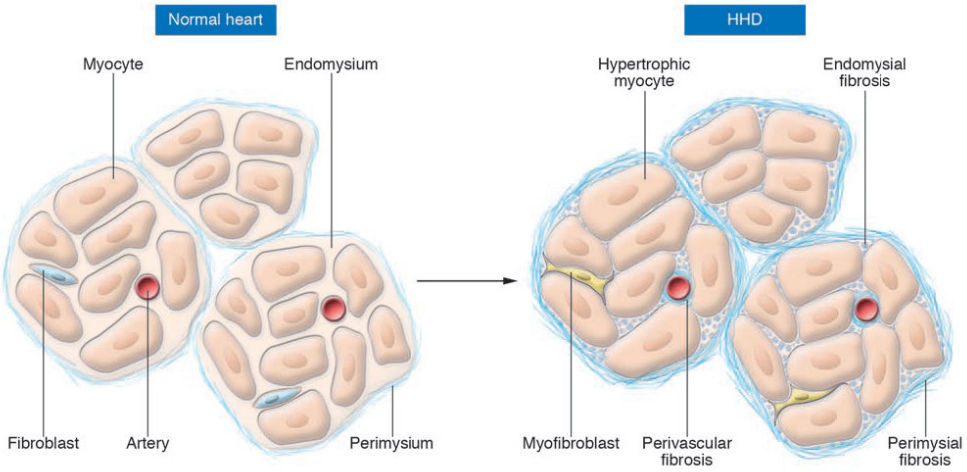


Figure 2.4. Schematic representation of changes to the collagen network in HF. In the normal heart, thin layers of perimysium and endomysium surround myocardial bundles and myocytes, respectively. The walls of the blood vessels also contain adventitial fibroblasts that create an endomysial network. In HF, there is often hypertrophy of cardiomyocytes and transition of fibroblasts to myofibroblasts. These changes are associated in early disease with increases in ECM manifest by fibrosis (71).

Remodeling is an essential process that allows the heart to adapt to changes in mechanical, chemical, and electrical signals (72-74). This is a complex process involving all of the components of the heart, cellular and extracellular. Remodeling is a normal process associated with heart growth, especially during the maturation of the heart from the neonatal period to adulthood. However, myocardial fibrosis also occurs in a number of pathological processes, most commonly hypertension. Other disease states capable of producing cardiac fibrosis include hypereosinophilia, scleroderma, sarcoidosis, radiation and drug effects, viral myocarditis and inherited genetic mutations. When the heart undergoes hypertrophy or dilation, remodeling must occur. In pathological conditions, the process of remodeling becomes detrimental to cardiac function (75-77).

2.2.1. Hypertrophy

The hypertrophy of cardiomyocytes is a well-recognized remodeling response to imposed load (78-80). Mechanical load is imposed when the heart is injured and myocardial contractile elements are dysfunctional or lost. Myocyte hypertrophy is likely an adaptive mechanism designed to improve pump function by expanding the number of contractile units in the myocardium while simultaneously reducing wall stress by increasing wall thickness. When excessive or prolonged, myocyte hypertrophy is maladaptive. Hypertrophy can directly result in chronic HF, as evidenced by patients with hypertrophic cardiomyopathy (81). In the failing heart, excessive LV hypertrophy is associated with reduced myocardial compliance and altered diastolic properties, myocardial fibrosis, and lethal arrhythmias (78, 82).

The study of the cellular and molecular biology of HF is mostly centered almost exclusively on myocyte hypertrophy and dysfunction. The cardiac ECM was believed to function as a relatively inert, supportive scaffold for the myocytes and blood vessels of the heart. However, it has now become increasingly clear that the cardiac ECM is a dynamic, metabolically active entity that plays an independent and important role in the progression of HF.

2.2.2.Fibrosis

2.2.2.1. Fibroblasts

Cellular compartment of cardiac tissue is composed of cardiac myocytes (cardiocytes), occupy 75% of its structural space; however, constitute only one third of the cell population (83, 84). All remaining cells are found in the cardiac interstitium. They include;

1) endothelial cells, and are known to influence the vasomotor reactivity of blood-containing vessels (85)

2) vascular smooth muscle cells, which are found in epicardial and intramyocardial coronary arteries and arterioles and, like endothelial cells, influence the reactivity and vasodilatory capacities of these vessels (86)

3) CFs, which have the responsibility to both produce and degrade the structural proteins collagen and elastin in the interstitium; and

4) macrophages and mast cells, which are defenders against invasion by foreign proteins.

Like endothelial and vascular smooth muscle cells, fibroblasts are capable of reentering the cell cycle and can therefore undergo mitosis or hyperplastic growth. On the other hand, adult cardiomyocytes are thought to be terminally differentiated and therefore do not proliferate. Unlike endothelial and vascular smooth muscle cells, which are highly specialized, fixed anatomic elements of the vascular compartment, CFs are multipotential cells that are free to move within the extracellular space but they are connected to each other, myocytes, and ECM by a variety of receptors, including integrins, cadherins, and connexins.

In addition, CFs function as a major source of cytokines, growth factors and chemokines which regulate the function of CFs, cardiomyocytes and other non-myocytic cells.

Under pathologic conditions, morphologically distinct cells termed *myofibroblasts* appear (fig. 2.5). These cells are defined by their dual functions: fibroblast-like in terms of ECM synthesis and smooth muscle myocyte–like in terms of migration. Myofibroblast-mediated collagen turnover is regulated by autocrine and paracrine factors (such as transforming growth factor beta (TGF-β) and endothelin 1) generated within the myocardium and by endocrine hormones derived from the circulation (such as Angiotensin II).

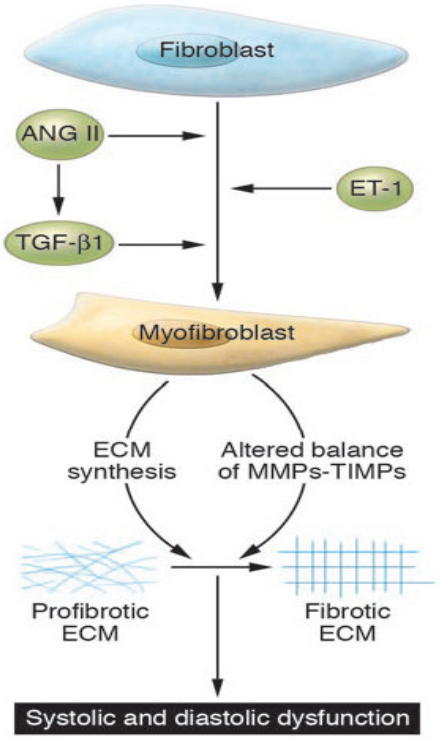


Figure 2.5. Mechanisms for transition of fibroblasts to myofibroblasts (87). Ang II:Angiotensin II; TGF-β1:Transforming Growth Factor-β1; ET-1:Endothelin-1 ECM: Extra Cellular Matrix; MMPs: Matrix metalloproteinases TIMPs: Tissue inhibitors of metalloproteinases.

2.2.2.2. Extracellular Compartment of the Cardiac Tissue

The extracellular compartment of the cardiac tissue, ECM, is an extensive network of fibrillar collagens, laminin, elastin, proteoglycans and fibronectin that surrounds the myocytes and maintains the structural integrity of the heart: fibrillar collagen is a multi-functional and essential component of the myocardium responsible for interstitial biomechanical strength. Fibrillar collagens are produced by CFs, and of the different types of collagen (I, II, III, IV, V) within the myocardium, type I and III are the most abundant structural proteins (88).

Type I collagen is the thick fibrillar collagen with tensile strength and resists tissue deformation (89). Type III collagen is more distensible and forms fine reticular networks (90). The network of collagen maintains the alignment of myocytes during

contraction and prevents myocyte slippage, facilitates the transmission of force to the ventricular chamber, contributes to relaxation and supports vessel structure (91). Since collagen fibers are critical for the structural integrity of the ventricle, any changes in its quality or quantity are crucial. The interaction of CFs, ECM and cardiac myocytes were shown in figure 2.6.

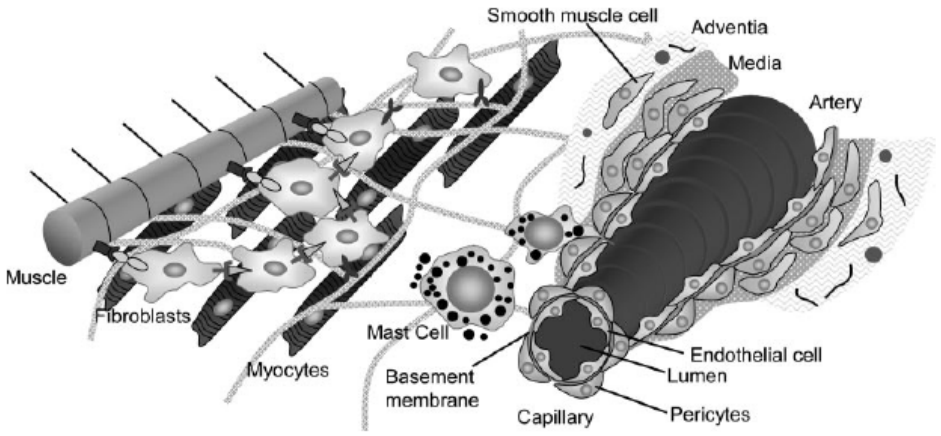


Figure 2.6. Representation of interaction of CFs, ECM and cardiac myocytes. Fibroblasts and mast cells are associated with vasculature and indicate a potential lineage with pericytes and/or blood cells. Fibroblasts are connected to each other, myocytes, and ECM by a variety of receptors, including integrins, cadherins, and connexions (92).

Net collagen concentration in the heart depends upon a balance between collagen synthesis and collagen degradation. ECM components including collagens, may be degraded by matrix metalloproteinases (MMPs), which belong to the family of Zn^{+2} and Ca^{+2} dependent-enzymes (93). At least fifteen secreted MMPs and membrane type MMPs (MT-MMP) have been identified (94). These proteins have been classified into two groups based on their substrate preference and structural features and were listed as follow: classic type including collagenase (MMP-1, MMP-8, MMP-13), stromelysins (MMP-3, MMP-7 and MMP-10), gelatinases (MMP-2 and MMP-9), elastase (MMP-12) and novel type including MMP-11, MT-MMP1-3 or MMP-14) (95). For example, rate limiting degradation of mature fibrillar collagen types I and III may be initiated by MMP-1, MMP-8 and MMP-13, whereas

MMP-2 may degrade collagen type IV. Stromelysins catalyze the disruption of various ECM members such as fibronectin, laminin, elastin, proteoglycans, collagen type III and IV (95). The activity of MMP is regulated at three levels: transcription, activation of latent proenzyme and inhibition of proteolytic activity (96).

A family of naturally occurring specific factors, namely tissue inhibitors of metalloproteinases (TIMPs), tightly control the activation of MMPs (fig. 2.7). Four members of this family have been identified and referred to as TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (96, 97). TIMP-1 has relatively high affinity for the active forms of collegenase, stromelysin and gelatinase; the noncovalent binding TIMP-1 to the target MMPs is irreversible. Of the various MMPs and their inhibitors, it is known that MMP-1, MMP-2, MMP-9, TIMP-1, TIMP-3 and TIMP-4 are expressed in heart (98, 99). TIMP-4 has been found to be highly expressed in heart indicating a role in cardiac collagen remodeling (97-99).

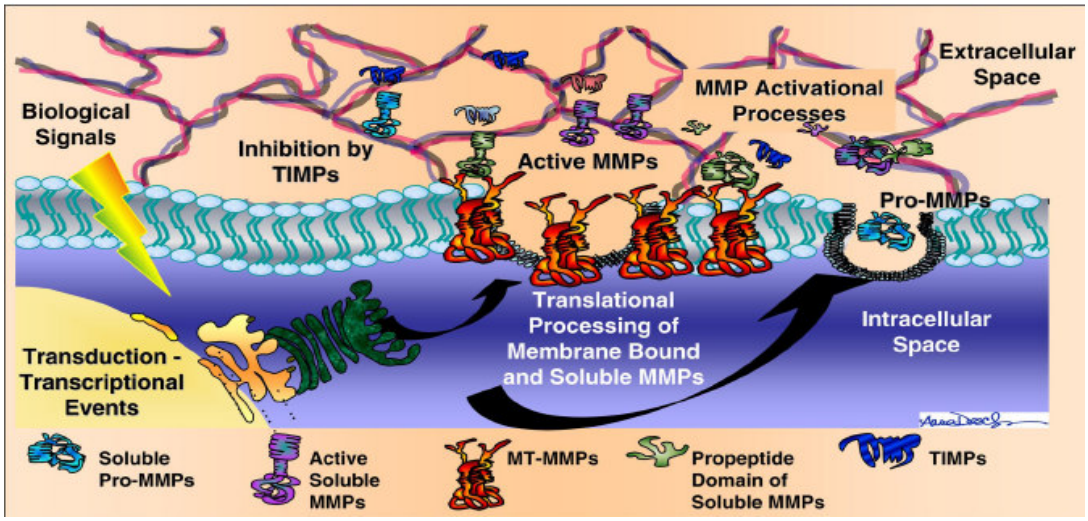


Figure 2.7. A schematic representation of the current concepts regarding the regulation of MMP activity within the myocardial interstitium. A number of biological signals that include both biochemical and mechanical can affect MMP and TIMP transcriptional activity. The activation of the soluble MMPs requires proteolytic cleavage of the prodomain, which can occur by other proteases or by the MT-MMPs. The active MMPs are inhibited by the TIMPs. The MMP and TIMP transcriptional and posttranslational events form a dynamic set of interactions that contribute to the overall structure and function of the myocardial intersitium (100).

Major feature of the myocardial remodeling process is the transition of CFs to myofibroblasts. Typically, myofibroblasts produce a different ECM than fibroblasts and modify the balance of MMPs and TIMPs. Changes in the ECM are correlated with altered physiological parameters of cardiac function. Little is known concerning the events that trigger this process. Most of the studies on remodeling have focused on the myocytes with little emphasis on other cell types.

Increases in the ECM or fibrosis may be reparative, replacing areas of myocyte loss with a structural scar, or reactive, involving increases in ECM deposition at sites distinct from the focal injury. Fibrosis causes an increase in ECM deposition which results in mechanical stiffness, and contributes to diastolic dysfunction. Progressive increase in fibrosis can also cause systolic dysfunction and LV hypertrophy. Additionally, increased collagen levels were shown to disrupt electronic communication between myocytes (100).

Perivascular fibrosis around intracoronary arterioles is reported to impair myocyte oxygen availability and exacerbates myocyte ischemia. HF is characterized by substantial differences in levels of disease severity and progression, even within comparable HF etiologies. This reflects polygenic and environmental influences in the heart disease phenotypes in a patient-to-patient manner. Thus, CFs and their fibrotic remodeling may act as the disease modifiers and can be used as predictive risk factors in HF.

2.2.2.3. Role of 5-HT in fibrosis-Carcinoid Syndrome

Certain diseases such as gastrointestinal carcinoid tumors of the mid-gut, which sometimes release large amounts of 5-HT into the blood, may produce a characteristic pattern of mostly right-sided cardiac fibrosis which can be identified at autopsy. This pathology has also been seen in certain West-African tribes who eat foods (Matoki-- a green banana) containing excess amounts of 5-HT.

The small bowel carcinoid tumors are slow-growing neuroendocrine neoplasms derived from the 5-HT-producing enterochromaffin cell. Carcinoid tumors, though relatively rare, are the most common small bowel neoplasms with

distinctive clinical and histological features. Because of their inconspicuous size and deep submucosal location, primary carcinoid tumors are rarely diagnosed before metastases have developed, and thus, patients often present with advanced disease. When these tumors manifest clinically, it is most commonly the result of extensive fibrosis around the tumor that often extends throughout the peritoneal cavity (101).

Fibrosis associated with carcinoid tumors is not limited to the peritoneum; in fact, carcinoid heart disease is known as a unique complication of carcinoid syndrome affecting 20–38% of patients (102). First reported in 1952 by Biörck et al., patients with midgut carcinoid tumors may develop pronounced endocardial fibrosis with deposition of ECM in the valves of the right heart, resulting in tricuspid insufficiency and pulmonary regurgitation (103). These subendocardial fibrotic plaques consist of fibroblasts or myofibroblasts, and a matrix-rich fibrous stroma comprised of relatively few cells (104).

The underlying biology of the relationship between carcinoid tumors and fibrosis remains still unknown. Various authors have attributed the etiology of the fibrosis to the local physical effect of the tumor itself, which could produce mechanical stress or ischemia on the surrounding tissues (105). It was previously suggested that the fibrosis associated with carcinoid syndrome was probably closely related to local 5-HT levels and recently carcinoid tumors are known to secrete 5-HT, (106).

More evidence, however, exists for 5-HT as an etiologic agent in the development of carcinoid heart disease. High concentrations of 5-HT and/or other vasoactive substances released by the tumor which enter the inferior cava, and subsequently the right side of the heart, are considered to initiate the pathological process leading to plaque formation on the downstream side of the tricuspid and pulmonary valves. The passage of blood through the pulmonary parenchymal circulation enables sufficient degradation to explain the predominance of right-sided heart valve damage (figure 2.8) (107).

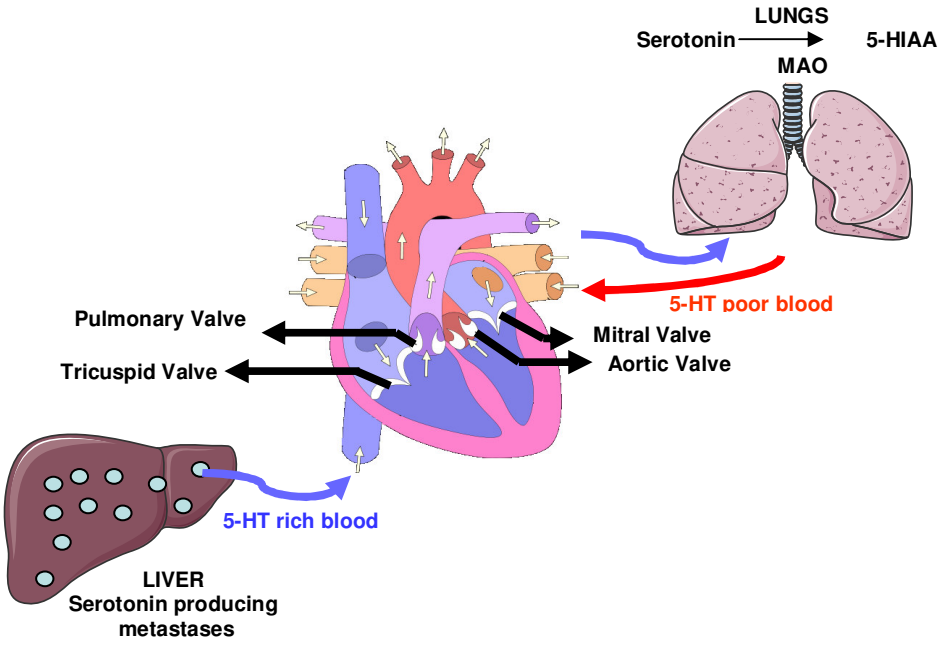


Figure 2.8. The topography of carcinoid tumors. Enterochromaffin cell carcinoid liver metastases releasing 5-HT, and other vasoactive tumor products which pass to the right heart via the blood stream. The 5-HT rich blood then passes to the lungs where MAO degrades 5-HT to 5-HIAA. When the blood enters the left side of the heart, 5-HT levels are significantly decreased, and thus less capable of inducing fibrosis.

2.2.3. Inflammation

Inflammation is a biological response to injury and serves as a principal mechanism for an organism to adapt and repair its tissues. The role of ongoing inflammation as a fundamental mechanism underlying cardiovascular disease has recently gained considerable attention (108). Persistent inflammation, involving increased levels of inflammatory cytokines, seems to play a pathogenic role in chronic HF by influencing heart contractility, inducing hypertrophy and promoting apoptosis, contributing to myocardial remodeling. The inflammatory response to any

kind of stimuli may represent a common final pathogenic pathway in HF regardless of the initial event.

Platelets can actively initiate the development of severe cardiovascular complications, such as unstable angina, acute myocardial infarction, or stent thrombosis, and influence the outcome of cardiovascular interventions, such as percutaneous interventions or bypass surgery. Consequently, effective platelet inhibition reduces major adverse cardiovascular events in acute cardiovascular syndromes and cardiovascular interventions (109).

Various mechanisms have been documented whereby platelets modulate the inflammatory response. Such mechanisms include intravascular 'priming' of leukocytes for efficient recruitment to tissue, chronic inflammatory events leading to tissue remodeling and regeneration, release of platelet-derived mediators that cause tissue damage directly, and the involvement of platelets linking the innate and adaptive immune responses (fig. 2.9).

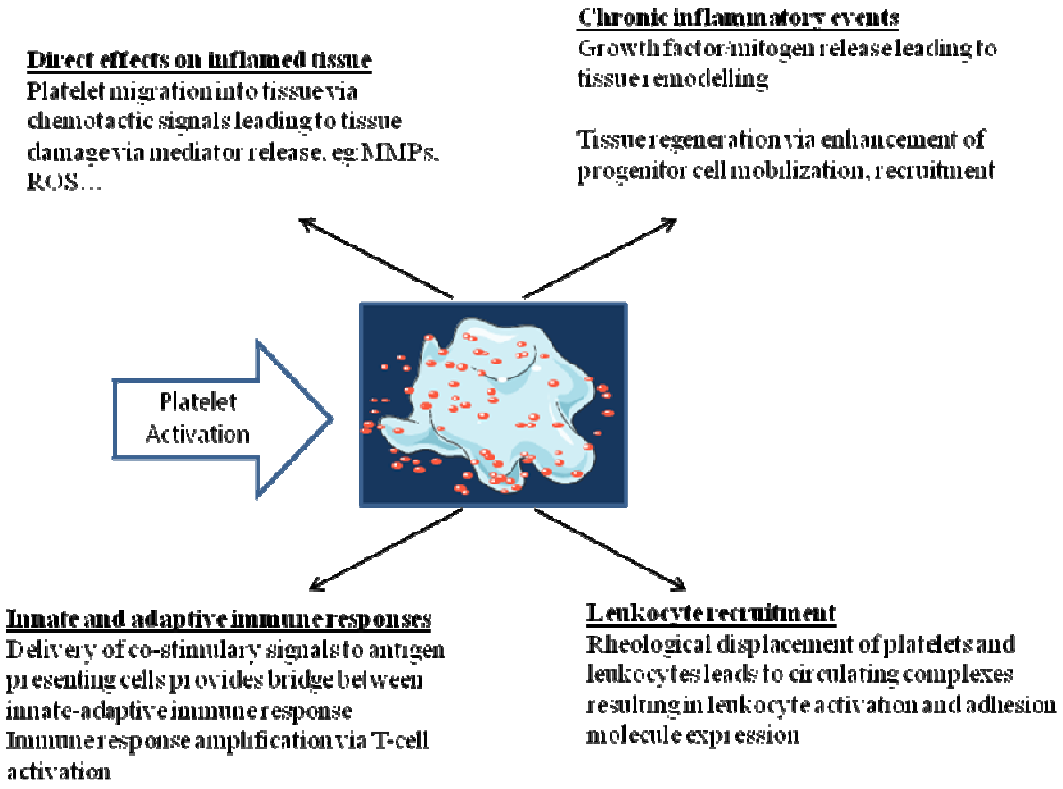


Figure 2.9. Platelet participation in inflammation (110).

Upon platelet activation platelet derived mediators are released into local microenvironment from dense granules, α-granules, lysosomes, the canalicular system, or the cytosol. These platelet derived mediators can be classified as; adhesion proteins (e.g., fibrinogen, fibronectin, vWF, thrombospondin, vitronectin, P-selectin, GPIIb/IIIa), growth factors (e.g., platelet-derived growth factor (PDGF), TGF-β, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF)), chemokines (e.g., Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES or CCL5), CXCL 4, epithelial neutrophil-activating protein 78 [CXC chemokine ligand 5]), cytokine-like factors (e.g., interleukin 1 beta (IL-1β), CD40 ligand, β-thromboglobulin), and coagulation factors (e.g., factor V, factor XI, plasminogen activator inhibitor-1 (PAI-1), plasminogen, protein S). These proteins act in a concerted and strongly regulated manner to influence the biological functions such as cell adhesion, cell aggregation, chemotaxis, cell survival and proliferation, coagulation, and proteolysis, all of which accelerate inflammatory processes and cell recruitment.

The recent information concerning 5-HT secretion during platelet activation is that; platelet derived 5-HT mediates liver regeneration and this effect of 5-HT is 5-HT_{2A} and 5-HT_{2B} receptors dependent (111). Furthermore studies on ischemia-reperfusion injury, suggests the dual role of 5HT on ischemia-reperfusion injury. During ischemia, 5HT is released endogenously, constricts coronary smooth muscles via 5-HT_{2A} receptors, and aggravates cardiac function. In contrast, application of 5-HT exogenously affects predominantly non-$5HT_{2A}$ receptors on the endothelium and induces coronary vasodilatation via endothelial NO production, which was shown to be protective against ischemia-reperfusion injury (112). However, there isn't any information about the role of 5HT on inflammation specifically yet.

Secreted cytokines (and chemokines) are reported to have special importance in progression of inflammation at the site of action by affecting either non activated platelets or other cells. The amount of IL-1β synthesized from activated platelets may induce the endothelial cells to express related genes that mediate the adhesion of leukocytes (113). IL-1β is an important mediator of the platelet-induced activation of endothelial cells, causing them to increase the release of chemokines and up-regulate molecules that promote adhesion of neutrophils and monocytes to the endothelium

(114). Besides, it has already been demonstrated that platelets can contribute to the inflammatory milieu existing in chronic HF patients via induction of the release of monocyte chemotactic protein-1 (MCP-1:CCL2). MCP-1 was markedly elevated in an in-vitro platelet–endothelial cell coculture model and in the serum of chronic HF patients (115).

Activated platelets also express and secrete the chemokines RANTES and CXCL 4, which are deposited in a P-selectin–dependent manner on microvasculature, aortic endothelium, and monocytes (116). Deposition of these proinflammatory cytokines results in activation of monocyte integrins and in increased monocyte recruitment to lesion (117).

The CD40-CD40 ligand system has proved to be critical for the activation of tissue structural cells that include fibroblasts, epithelial cells, and endothelial cells (118). Engagement of CD40 on the surface of these cells induces production of several proinflammatory cytokines, including IL-1, IL-6, and IL-8.

Therefore the inflammatory response to any kind of injury is mediated, in part, by cytokines that have potent biological consequences (108). Following section will focus on these cytokines and chemokines that have role in inflammation especially seen in cardiovascular disorders.

2.2.4. Cytokines

Cytokines form a vast array of relatively low molecular weight (generally 15-30 kDa), pharmacologically active proteins. They differ from the classic polypeptide hormones in that polypeptide hormones are generally produced by a specific cell type in specific organ and tend to act at a distance in an endocrine fashion, whereas cytokines are produced by a broad variety of cell types and tend to act at short distances in a juxtacrine, paracrine, and autocrine fashion. However, when cytokines are expressed at sufficiently high concentrations, these molecules may spill over into the circulation, where they may exert endocrine effects. Once they were synthesized they may have direct membrane effects but in general, they are thought to exert their effects by binding to specific receptors on the surface of the cell (119). They play a

very important role in the cellular interactions (120). They participate in a wide range of biological processes.

The group of cytokines that are responsible both for initiating the primary host response to a bacterial infection, as well as initiating the repair of tissue after tissue injury have been termed “proinflammatory cytokines.”

There is abundant evidence that proinflammatory cytokines are important for the activation of the acquired immune response, which, although usually beneficial, can also lead to pathology such as detrimental autoimmune responses (121). Since there is a mixture of other signals and cytokines in the inflammatory site, it is difficult to determine the net effect of cytokines at any indicated time (122).

Proinflammatory cytokines were originally thought to be secreted only by classic immune cells. It is now known that virtually every nucleated cell type in the myocardium, including the cardiac myocyte, is able to secrete proinflammatory cytokines in response to various forms of stress and injury (123, 124).

Direct myocardial stress, such as mechanical stretch, oxidative stress, and hypoxia in the setting of ischemia, will rapidly induce cytokines such as tumor necrosis factor-α (TNF-α) or IL-6. This can enhance survival or accelerate myocyte necrosis and apoptosis and decrease contractility. This is also followed by cytokine amplification through transmigration of macrophages and neutrophils. During the chronic phase postinfarction, the activation of MMPs and TIMPs contribute to the laying down of collagen and wound repair. The elaboration of angiogenic and progenitor cell mobilization factors further contributes to the healing of the wound (figure 2.10) (125).

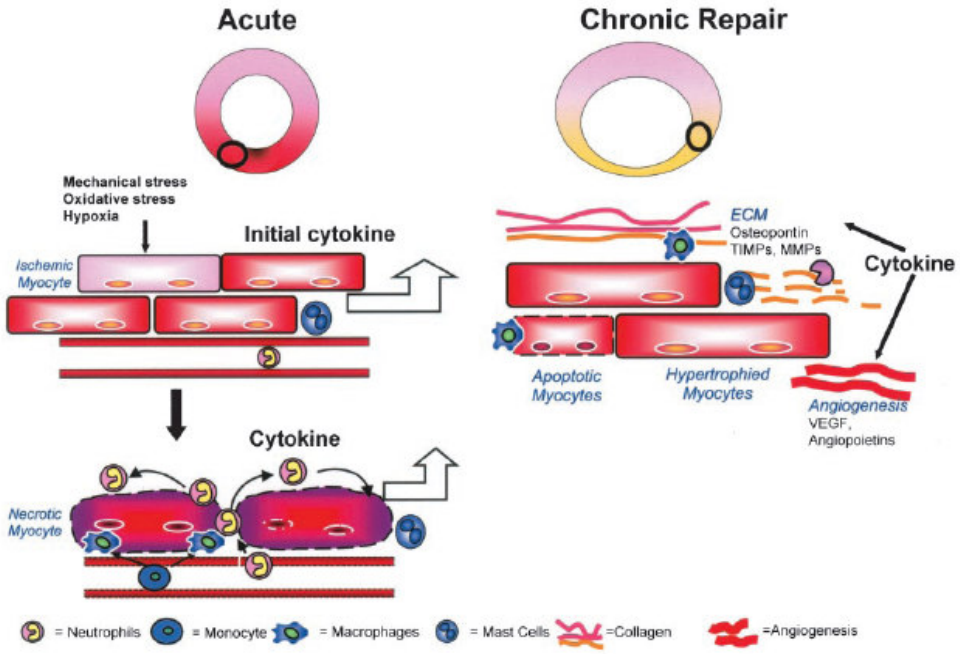


Figure 2.10. The induction of inflammatory cytokines after myocardial ischemia and their effect on acute and chronic cardiac remodeling postinfarction. VEGF, vascular endothelial growth factor (125).

Effects of cytokines on the CVS include promotion of inflammation, intravascular coagulation, free-radical generation, endothelial injury and cardiomyocyte or endothelial cell apoptosis (126, 127). Therefore, a wide range of cardiac diseases have been associated with inflammation and cytokine modulation. These include cardiac reperfusion injury, myocarditis, allograft rejection, sepsis-associated cardiac dysfunction, and chronic HF (128). In these conditions, cytokines are produced by both immunological and CVS structural cells (129). These are known to undergo up-regulation in patients with chronic HF and have been implicated in the pathophysiology of the disease. Cytokines can affect myocardial function through their effects on both myocyte contractility and the ECM (130). Cytokines have been shown to have direct and indirect effects on myocardial function. These effects of cytokines are time, concentration, and subtype specific (131, 132).

Most aspects of the HF syndrome can be mediated by known effects of proinflammatory cytokines (133). Cytokines, when expressed at sufficiently high

concentrations, are sufficient to mimic some aspects of the HF phenotype, including progressive LV dysfunction, pulmonary edema, LV remodeling, fetal gene expression, and cardiomyopathy (134-136).

A glossary of major proinflammatory cytokines and other cytokine-related inflammatory factors associated with the pathophysiology of chronic HF (such as chemokines) and their biologic effects on the CVS in the setting of chronic HF are described briefly in the following section.

2.2.4.1. TNF-α in HF

This cytokine was originally identified for its potent toxicity against tumor cells, hence its name. The TNF-α molecule (a 157-amino acid polypeptide) exists as a membrane bound and a secreted molecule, both bioactive (137). The activated macrophage is the main source of TNF-α containing both cell associated and membrane bound TNF-α (138). Other cells releasing TNF-α include lymphocytes, fibroblasts, neutrophils, smooth muscle and mast cells (138). Furthermore, adult mammalian myocardial cells are able to produce TNF-α after extracellular stimuli such as endotoxin, hypoxia or increased mechanical stress (139).

TNF-α acts at the cellular level via both type I and type II receptors (TNFRI and TN-FRII respectively) and recently, it has been suggested that TNFRI and TNFRII are present in the human myocardium (140). Myocardium physiologically does not contain TNF-α, however, in failing myocardium, TNF-α expression is increased and the receptors for TNF-α are down-regulated (140).

TNF-α activates multiple transduction pathways, inducing or suppressing a wide variety of genes, including those encoding the production of other cytokines, adhesion molecules and inducible nitric oxide synthase (iNOS) (141). Additionally, it orchestrates the inflammatory response through activation of proinflammatory cytokine (such as IL-1β and IL-6) genes, as well as its own production (141).

TNF-α effects on cardiac function are dependent from the amount and duration of cytokine expression. Short term expression within the heart may be an adaptive response to different forms of ‘stress’, whereas long term expression may be maladaptive by producing cardiac decompensation (142). Excessive TNF-α levels

can produce LV dysfunction and cardiomyopathy and may be related with the clinical manifestations and the progression of chronic HF (143, 144).

This proinflammatory cytokine participates in the pathophysiology of HF progress, at least partially, by stimulating myocyte hypertrophy through the generation of reactive oxygen intermediates in cardiac myocytes, by inducing ventricular remodeling through stimulating ECM protein production and increased turnover of matrix, by causing cardiomyocyte loss through necrosis and apoptosis, and by depressing myocardial function through the nitric oxide-dependent and sphingomyelinase pathways (145, 146).

2.2.4.2. IL-6

IL-6 is another multifunctional proinflammatory cytokine that mediates both immune and inflammatory responses.

The recent interest in studying IL-6 in HF has been prompted by the observation that IL-6, like TNF-α, can produce not only myocardial dysfunction (147), but also muscle wasting (148). Recently, elevated levels of IL-6 have been identified in patients with HF (149, 150).

IL-6 is produced by a variety of different cell types including mononuclear phagocytes, some activated T-cells, vascular endothelial cells and fibroblasts (151). Although, IL-6 may be locally produced in the myocardium, data suggest that IL-6 is peripherally released in chronic HF patients (152). Although the underlying mechanism of increased elaboration of IL-6 in chronic HF is not well known, TNF-α seems to be sufficient to induce IL-6 gene and protein expression in a variety of cell types, suggesting that there may be a 'cytokine cascade' in the setting of chronic HF (145, 153).

Some reports indicated a significant correlation between the elevated levels of TNF-α and IL-6 in severe chronic HF (154) as well as the others reported a statistically significant correlation between elevated levels of IL-6 and elevated right heart pressure on chronic HF patients (155). Furthermore it has been shown that; 5-HT markedly increased the production of IL-6 and TNF-α in adult mice CFs, which was mimicked partially by the 5-HT_{2B}R preferential agonist BW (156).

It was also reported that serum IL-6 level was identified during follow-up of severe chronic HF patients as the most powerful independent predictor of new HF episodes, death or need for heart transplantation, a better predictor than TNF-α, plasma neurohormones or LV function (157).

2.2.4.3. Granulocyte macrophage colony stimulating factor (GM-CSF)

GM-CSF is a member of the large family of hemopoietic cell colony-stimulating factors and regulates the proliferation and differentiation of myeloid progenitor cells (158). This inflammatory factor in addition to its growth-promoting effects it stimulates various functional activities of mature neutrophils, monocytes and eosinophils, including regulation of leukocyte adhesion, augmentation of surface antigen expression, superoxide anion generation and enhancement or induction of cytokine production (159). Additionaly it may contribute to the pathophysiologic events involved in atherosclerosis and inflammation (160, 161). The biologic effects of GM-CSF on the immune system are summarized in fig. 2.11. It was also demonstrated that GM-CSF levels were found to be elevated in chronic HF patients which might be associated with the hemodynamic deterioration and neurohormonal activation characterizing this syndrome (162). GM-CSF may represent the common denominator of the pathophysiologic sequelae leading to abnormal nitric oxide-synthase expression in human myocardium and vascular endothelium of patients with chronic HF (162).

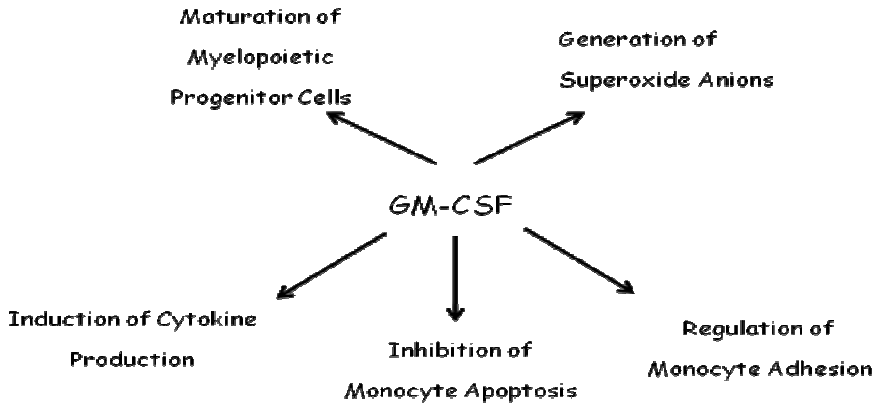


Figure 2.11. Biologic effects of GM-CSF on immune system.

2.2.5. Chemokines

Chemokines are low molecular-weight peptides that are potent chemoattractants for different leucocyte subpopulations and also have biological effects on endothelial cells, fibroblasts and vascular smooth muscle cells (163). The chemokine superfamily is divided into four groups (CXC, CX3C, CC and C) according to the relative positioning of the first two closely paired and highly conserved cysteines of their amino acid sequences (163) (figure 2.12). Their actions are mediated by a family of G-protein-coupled receptors having 18 members (164).

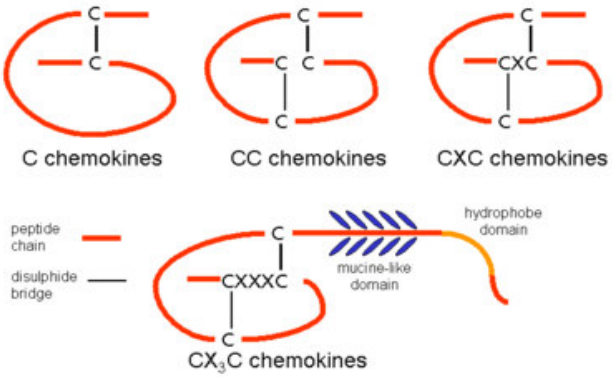


Figure 2.12. Structure of chemokine classes (163).

The major biological functions of chemokines are as follows.

(a) Chemoattractants provide directional cues for leukocyte motility through the formation of gradients that migrating cells can sense. Migrating cells undergo a profound transformation that results in a redistribution of chemoattractant receptors, integrins, cytoskeletal proteins and intracellular regulatory molecules (figure 2.13-a).

(b) The signaling of integrin activation during the multistep process of leukocyte–endothelial cell interactions (figure 2.13-b).

(c) Chemokines stimulate leukocyte degranulation or release of inflammatory mediators. For example, MCP-1 is a potent stimulator of histamine release by basophils and CXCL8 stimulates neutrophil granule exocytosis. Chemokines also stimulate the respiratory burst, which results in production of reactive oxygen intermediates (figure 2.13-c).

(d) Some chemokines also stimulate angiogenesis or angiostasis. The "ELR" CXC chemokines and MCP-1 possess angiogenic properties, whereas CXCR3 ligands, such as CXCL10 and CCL21, possess angiostatic properties. Chemokines have been reported to participate in tumor suppression or in inflammatory responses where angiogenesis is an important requirement (figure 2.13-d).

These functions of chemokines have been observed in a combination in various biological responses. For example, tumor rejection involves the recruitment of leukocytes from blood, their chemotaxis to the tumor and also angiostasis; allergic inflammation involves leukocyte recruitment and release of inflammatory mediators.

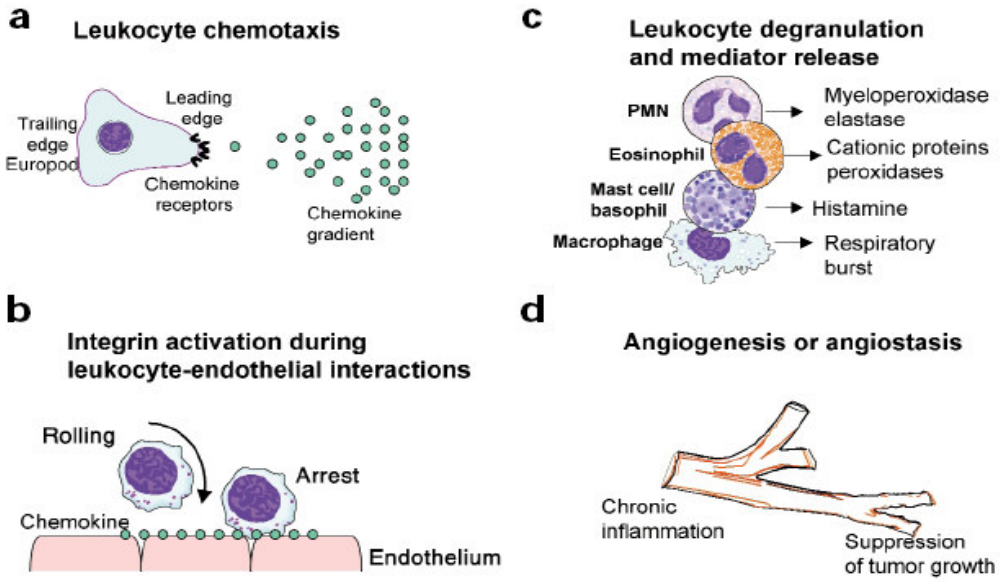


Figure 2.13. Major biological functions of chemokines (165).

Chemokine-dependent functions are essential for the control of infection, wound healing and haematopoiesis while excessive chemokine activation may result in inappropriate inflammation leading to cell and tissue damage (165). Thus, chemokines have been suggested to play a pathogenic role in several immune-mediated and inflammatory disorders (165). Recently, chemokines have also been implicated in the pathogenesis of several cardiovascular disorders. Patients with HF have been shown to have significantly elevated levels of all chemokines, including MCP-1, macrophage inflammatory protein-1α, RANTES and IL-8 (166).

2.2.5.1. MCP-1

MCP-1, is a potent chemoattractant for monocytes, T cells, and NK cells and has been implicated in diseases characterized by monocyte-rich infiltrates (167) and thought to play an important role in controlling inflammation and immune responses (168). It is a member of the largest chemotactic cytokine subfamily, C-C chemokines; this class of chemokines does not have an amino acid separating the first two N-terminal cysteine residues (169).

Its expression and functional significance have been documented in a wide variety of disease processes, such as atherosclerosis (170), multiple sclerosis, rheumatoid arthritis, stroke, and nephritis (167) and MCP-1 has also been implicated in the pathogenesis of myocarditis, acute myocardial infarction and ischemia-induced myocardial damage (171–173).

The involvement of chemokines in the pathogenesis of atherosclerosis has been widely investigated, and its central role in this process has been clearly clarified (174-176). Cellular interactions in the initiation of atherosclerosis and the role of MCP-1 are shown in figure 2.14.

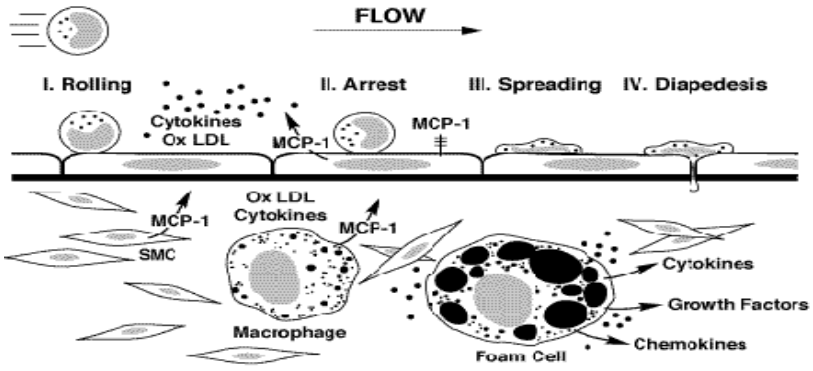


Figure 2.14. Cellular interactions in the initiation of atherosclerosis and the role of MCP-1 (174).

Furthermore a recent report showed that blocking of MCP-1 reduces severity of experimental autoimmune myocarditis in mice (177). Besides detrimental effects brought about by the inflammatory capacity of monocytes and macrophages, MCP-1 seems to be also involved in cardiovascular repair and protection. Infiltrating

mononuclear cells appear to orchestrate cardiac repair processes through a complex cascade involving cytokines and growth factors (178). A recent report showed that macrophage inflammatory protein 2 dependent MCP-1 expression protects cardiac myocytes from cell death (179) and can also counteract directly hypoxia induced cardiac myocyte apoptosis (180). By drilling tunnels through myocardial tissue, monocytes and macrophages were also reported to increase angiogenesis in the heart (181).

MCP-1 is not only chemotactic for monocytes but also for endothelial cells and thus can induce endothelial cell sprouting and the chemokine seems to play a major role in the growth of collateral vessels in ischemic tissue through the recruitment of monocytes (182, 183). In MCP-1 null mice suppression of macrophage recruitment as well as a decreased infiltration with myofibroblasts leads to a decreased number of mature vessels with a muscular coat in the infarct zone of the heart (184). A recent report shows that MCP-1 can directly act on endothelial cells and induce angiogenesis through induction of VEGF (185). Given the aforementioned evidence, MCP-1 expression might contribute to the development of HF but also to the repair process.

Recently, Aukrust et al. (166) have reported that circulating levels of MCP-1 were increased in patients with chronic HF and have suggested that it was involved not only in the pathogenesis of atherosclerosis and ischemia-induced myocardial injury, but also in the development of chronic HF. In this study (166), the elevated levels of MCP-1 in chronic HF were correlated with the degree of LV dysfunction, as well as with enhanced circulating monocyte activity and increased oxidative stress. On the other hand, experimentally, it has been suggested (186) that mechanical overload induces the myocardial expression of MCP-1, which attracts and activates monocytes and macrophages, and that these recruited cells produce proinflammatory cytokines which contribute to the pathogenesis of chronic HF (187).

MCP-1 may be produced in various cell types. However at present data on MCP-1 production in the myocardium are limited (188). Recently it has been demonstrated that when TNF-α is used in order to mimic inflammatory conditions characteristic for cardiac injury; human cardiac myocytes and fibroblasts express MCP-1 (189).

3. MATERIALS and METHODS

3.1. Materials

The mouse anti alpha smooth muscle actine (α-SMA) antibody was obtained from Sigma-Aldrich, the anti–α-tubulin antibody from Millipore and the anti-5-HT receptor antibodies from BD Pharmingen. Cell culture media, foetal bovine serum (FBS), and supplements were purchased from Invitrogen. All other chemicals were of analytic grade and were obtained from Sigma-Aldrich unless otherwise indicated.

3.2. Methods

3.2.1. Cells and culture conditions

Due to some technical limitations and especially to study inflammation in adult cells instead of neonatal rat CFs, adult mouse CFs were used in some experiments.

3.2.1.1. Neonatal Rat CFs Culture

Wistar rats were used for neonatal rat CF primary cell culture. The rats were treated in accordance with the protocol approved by the institutional animal care and use committee.

CFs were prepared by enzymatic digestion and the selective plating technique as previously described (190). Briefly, 3-4 days old rats were killed by decapitation, the heart was removed, and the ventricles were cut from the atria, minced and washed twice with Earle's Balanced Salt Solutions (EBSS). The minced tissue was suspended in 5 mL of EBSS containing 0,05% (w/v) collagenase and incubated at 37°C for 15 min. The dissociated cells were collected by centrifugation, suspended in DMEM/F12 (GIBCO) supplemented with antibiotics (100 U/mL penicillin, 100 U/mL streptomycin) and 10% (v/v) foetal bovine serum, plated onto the culture plates according to the experimental design, and incubated at 37°C in a humidified

atmosphere containing 5% CO_2 for 2 h. Following incubation, the non-adherent cells were removed, the plates were washed, and the attached CFs were grown in DMEM/F12 supplemented with antibiotics and 10% foetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. The cultured CFs were usually confluent in 3–4 days.

3.2.1.2. Adult Mice CFs Culture

C57BL/6J mice are used in a wide variety of research areas including cardiovascular biology besides this mouse strain is a useful animal model to study fibrosis and inflammation. Male C57BL/6J mice were used for adult mice CF primary cell culture. Mice were treated in accordance with the protocol approved by the institutional animal care and use committee. Mice were anesthetized with an intraperitoneal injection of 100 lU (30 mg/kg) phenobarbital. Primary cell culture protocol for rat new born CF was used with some modifications. Whenever EBSS is needed instead, Hank's Buffered Salt Solution (HBSS) buffer was used.

3.2.1.3. Macrophage Monocyte Cell Line Culture

The mouse macrophage monocyte cell line J774 was a kind gift from Dr. Bernard Pipy and was maintained as an adherent culture. It was grown in DMEM (Gibco, France) supplemented with 2mM L-glutamine, 10% foetal calf serum (Gibco) and penicillin–streptomycin (100 U/mL) (Eurobio, France). Cells were starved for 24 h with DMEM/F12 supplemented with insulin/transferrin/selenium (I/T/S) prior to chemotaxis assay.

3.2.1.4. The Isolation of Platelets from Human Blood

Peripheral blood from healthy volunteers was collected in EDTA tubes and centrifuged at 150 *g* for 10 minutes at room temperature. The pool platelet-rich plasma was carefully aspirated and centrifuged at 1500 *g* for 15 minutes to obtain pellet platelets. Then the cell pellet was washed with excess volume of phosphate-

buffered saline to eliminate plasma. The pellet was resuspended in phosphate buffered saline (PBS) and recentrifuged at 200 *g* for 5 minutes to remove any remaining red and white blood cells. Platelets were counted and checked for contamination on the MICROS-60 CS/18 (ABX-Diagnostics). The platelet suspensions were adjusted to the concentration of $4x10^5$/µl in PBS, then aliquoted and immediately frozen at -80°C. Platelet lysis and consequent release of chemotactic and growth factors, was obtained by freezing and thawing the cells in a single cycle. This last preparation is defined as PL.

3.2.2. Cell treatment

Experiments were performed on cells at passage one or two. After 2 passages, most of cells acquired a myofibroblast-like phenotype. At confluence, CFs were subcultured (1:3 dilution) to passage 1 or split in 12 well plate to perform ELISA. Confluent primary CF culture were washed three times with PBS and unless indicated switched to a serum-free DMEM/F12 medium for 24 h before treatment. Depending on the experimental procedure stimulants were used for different time intervals in indicated concentrations (PL (1 to $4x10^6$/mL) or 5-HT (1 to 5 µM) or DOI (1 µM) and/or ketanserin (0.1 µM) and/or SB206553 (0.1 µM; 5-HT_{2B} receptor antagonist)).

3.2.3. FACS Analysis

The purity of the neonatal rat CFs was assessed based on morphology and immunostaining against vimentin, α-SMA, CD 31, and CD 45.

Neonatal rat CFs are adherent cells which require trypsin treatment in order to be detached from cell culture dishes. CFs were detached with 0.5% (w/v) trypsin-EDTA (Gibco, Invitrogen) and then cells were taken up in ice-cold PBS containing 10% (v/v) FBS. Cell samples were washed twice with PBS, and were resuspended in PBS. After incubating cells with anti-α-SMA, anti-vimentin, anti-CD45 and anti-CD31 at room temperature for 1h, cells were washed 2 times with PBS. After incubating neonatal rat CFs with corresponding secondary antibodies at room

temperature for 30 min., cells were washed with PBS. Cell preparations were resuspended in PBS at a density of ~6x10^5 cells/mL and were immediately analyzed by flow cytometry (FACS Calibur, Becton-Dickinson, San Jose, CA, USA). As α-SMA and vimentin are not cell surface markers, CFs were permeabilised with 0,05% (v/v) Triton-X-100 for 10 min. and were blocked with PBS containing 1% (v/v) FBS prior to first washing step with PBS.

3.2.4. Immunohistochemistry

For immunohistochemical analyse, cells seeded on coverslips were fixed with 4% (v/v) paraformaldehyde for 20 minutes and permeabilized with 0.1% (v/v) Triton-X-100 for 5 minutes. Fixed cells were incubated with primary antibody (monoclonal rat anti-vimentin directly conjugated with FITC; 1:1000) at room temperature for one hour, followed by several washes with PBS and incubation with appropriate secondary antibody for 30 minutes. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to allow identification of the nucleus. The coverslips were then washed with PBS and mounted onto slides with Aquamount (Lerner Laboratories) for immunofluorescent microscopy. Stained cells were viewed with an Olympus B-Max 60 microscope equipped with epiflourescence (Olympus, Center Valley, PA, USA) and pictures acquired digitally with a Retiga cooled CCD camera (Q Imaging, Burnaby, BC, Canada).

3.2.5. Western Blot Analyses

For western blot sample preparation, CFs cell pellets treated with either 5-HT (1 to 5 µM) or PL (1 to 4x10^6/mL) or DOI (1 µM) for 48 h were prepared using lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton-X-100, 10 µg/mL PMSF, supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After sonication protein contents of the samples were determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) in order to normalize the protein contents of samples for western blot analyses.

Equal amounts of protein samples (3 μg) were solubilised in loading buffer (60 mM Tris–HCl, pH 6.8 containing 2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.05% bromophenol blue) at 100°C for 5 min.

Protein extracts (from CFs or brain tissue) were separated on a 10% SDS–PAGE and were transferred to PVDF membranes using electroblotter (Trans-Blot, Bio-Rad Laboratories, CA, USA) for 45 min. at 450 mA. The blots were blocked with 5% non-fat dried milk in washing buffer (Tris-Buffered Saline (TBS), pH 7.5 and 0.1% (v/v) Tween 20) overnight at 4°C, washed twice, and incubated for 1 hr at room temperature with antibody against α-SMA (1:7,000) (Chemicon, CA, USA) or anti-5-HT_{2A} (1:1000). Membranes were then incubated with secondary HRP-conjugated antibody (1:10,000; Molecular Probes/Invitrogen) for 30 min. at room temperature and proteins were detected using enhanced chemiluminescence reagents (ECL, Millipore, France).

Each membrane was stripped and re-probed with a polyclonal anti mouse α-tubulin (1:1000; Santa Cruz Biotechnology, CA, USA) antibody that served as a loading control. Bands were quantified using an imaging densitometer. The densitometric plots were normalized to the intensity of the α-tubulin band.

3.2.6. Enzyme-Linked ImmunoSorbent Assays (ELISAs)

The ELISA kit for the rat MCP-1 was purchased from Biosource (Pierce Biotechnology, Inc., IL). The ELISA kits for the rat TGF-β and the mouse IL-6, TNF-α, GM-CSF were purchased from eBioscience (eBioscience, San Diego, CA). The measurement of cytokines or chemokines by ELISA was performed according to the manufacturer's instructions.

3.2.7. RNA isolation, RT-PCR and quantitative real-time RT-PCR

Cells were scraped by lysis buffer (RNeasy™ RLT buffer, Qiagen, CA) at the end of the stimulation time period (for 5HT receptor expressions 48 h; for TGF-β1 expression 2 h; MMPs and TIMPs expressions 2h and 6 h; for IL-6 and GMCSF expressions 2 h and for MCP-1 expression 2 h, 6, 12 h and 24 h). Total RNA was

extracted using RNeasy Mini Kits purchased from Qiagen (Valencia, CA) according to the manufacturer's instructions. Total RNA was quantified in each sample using a ND-1000 spectrophotometer (NanoDrop Technologies).

First-strand cDNA was synthesized from up to1 µg of total RNA by reverse transcription for 60 minutes at 42°C in a final volume of 20 µl of RT buffer with 100 U of Superscript II (Invitrogen) 0.25 µg random primers, 0.5 mM dNTPs, 5 mM DTT, 32 U Rnase inhibitor according to manufacturer instructions. Five µl of first strand cDNA was then used to amplify serotoninergic receptors fragments by PCR. Reaction mixture containing PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 60 nM of primers, 2 U Taq polymerase and reverse transcription reaction was denatured at 93°C for 2 min 30 s and then amplified by 35 cycles with a DNA thermal cycler (TRIO-ThermoblockTM Biometra-Göttingen, Germany). The final extension step was prolonged to 10 min. The absence of contaminants was checked by RT-PCR assays of negative control samples in which the Supersript TM was omitted. PCR products of 5-HT receptors and their negative controls were separated on 1.6% agarose gel. All primer sets used (Table 1) were previously assessed to check for amplification in rat brain tissue.

Real-Time PCR analysis was performed in 96-well plates using an ABI Prism 7000 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mixture (Applied Biosystems, Foster City, CA, USA). Amplification reactions (25 µl) were carried out in triplicate with 5 µl of 1:5 diluted template cDNA according to manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Each assay was normalized by amplifying the housekeeping cDNA 36B4 from the same cDNA sample. The suitable use of 36B4 as internal control for gene expression was previously validated by using the GeNORM algorithm. The parameters included a single cycle of 94°C for 10 min followed by 40 cycles of 95°C for 15 s, annealing, and 60°C for 1 min.

Table 3.1. Primer sets used in RT-PCR experiments.

PRIMER	5' SEQUENCE 3'
5-HT$_{1B}$R	F : TCCGGGTCTCCTGTGTACGT R : GGCGTCTGAGACTCGCACTT
5-HT$_{1D}$R	F : ACTGTCCTCTCCAATGCCTTCGT R : ATGCGGCCAAGGATAATGAGC
5-HT$_{1E}$R	F : CTGCCTCGTGATCACTGCAATCAT R : TGTAGAAGGCTCCAAACGTGGAGT
5-HT$_{1F}$R	F : TACTCGAGTATTCATTTCTTCAACTATA R : TATCTAGACTTTAAAATTAGGTTTCTT
5-HT$_{2A}$R	F : TCCAGAACCAAAGCCTTCCTGA R : TGCAGGATTCTTTGCAGATGAC
5-HT$_{2B}$R	F : GTCTGGAACTGGACTGAGTCAC R : GGGAAATGGCACAGAGATGCATGA
5-HT$_{2C}$R	F : CCAGTAGCAGCTATAGTAACTGAC R : ATGGCCTTAGTCCGCGAATTGAAC
5-HT$_{4}$R	F : GGGACAGGCAGCTCAGGAAAATAA R : GGCATGCTCCTTAGCAGTGACATA
5-HT$_{7}$R	F : GAGCAGATCAACTATGGCAGAG R : GTGGAGTAGATCGTGTAGCCAA
TGF- β1	F : GGCCCTGCCCCTACATTT R : GCACGCAGCACGGTGAT
MMP-3	F : ACTGTCCTCTCCAATGCCTTCGT R : ATGCGGCCAAGGATAATGAGC
MMP-13	F : CTGCCTCGTGATCACTGCAATCAT R : TGTAGAAGGCTCCAAACGTGGAGT
GM-CSF	F : GCC AGC CAC TAC CAG ACG AA R: CCTCAAAGGTGG TGACTTCTATTTC
MCP-1	F: ATQCAQTTAATQCCCCACTCA R: ACACCTQCTQCTQQTQATTCT
IL-6	F: AGTCAACTCCATCTGCCCTTCA R: CAGTGGCTGTCAACAACATCAGT
36B4	F: GAAGCCACACTGCTGAACATGT R: AGCACCTCTGGGCTGTAGATG

F: Sequence of the forward primer, R: Sequence of the reverse prime

3.2.8. Cell migration assay

After 24 h of serum starvation with DMEM/F12 supplemented with Insulin Transferine Selenium (I/T/S) (invitrogen), CFs were harvested in trypsin-EDTA, concentrated by centrifugation, washed with PBS, and resuspended in DMEM/F12 - I/T/S. 40,000 cells in 200µl were loaded onto PET semipermeable membranes (8 µm pore size, 6.5-mm diameter) in modified Boyden chambers (BD Falcon Cell Culture Insert, BD). Same medium containing vehicle, 5-HT or 5-HT_{2A} agonist DOI was loaded to the lower side of the migration chamber. After different time periods, the migration was stopped by fixing the cells in ethanol. Nonmigrated cells remaining on the upper surface of the membrane were mechanically removed with a PBS-soaked swab and migrated cells were stained with 10% crystal violet for 10 min. The membranes were then cut from the chambers, and mounted on glass slides with PBS. Migrated cells were visualized and counted with a microscope. Five to six fields were counted on each membrane and averaged duplicate determinations were performed for each set of experiments.

3.2.9. Cell proliferation

Cells were plated on 12 wells cell culture plate at a density of 20,000 cells per well and cultured for 24 h. After 12 h of serum starvation, cells were incubated for 48 h with different concentration of platelets or 10 µM 5-HT in presence or absence of 1µM ketanserin. Number of cells was determined using a Coulter particles counter.

3.2.10. *In Vitro* Chemotactic Assay

100,000 CFs were seeded in 12-well plate. Confluent primary CFs were starved for 24 h with DMEM/F12 supplemented with I/T/S (invitrogen), CFs were treated with 5-HT and PL and after 14 h conditioned medium was collected for further use. In order to investigate the direct effect of PL or 5-HT on chemotaxis of J774 cell line, PL or 5-HT was incubated in absence of CFs in DMEM/F12 supplemented with I/T/S and supernatant from this incubation was also collected after 14 h.

The experiments were performed by using special wells consisting of two compartments separated by a porous membrane (Transwell Permeable Supports, polyester membrane, pore size 0.5 μm, Costar). The conditioned medium from treated CFs or supernatant from PL and 5-HT incubation were filled into the lower compartment (500μL) and 60,000 J774 cells suspended in 200μL of DMEM/F12 medium supplemented with I/T/S were transferred to the upper part of Transwell™ insert. After the incubation at 37 °C for 4 h in an atmosphere of 95% air and 5% CO_2, cells were fixed with ethanol. Nonmigrated J774 cells remaining on the upper surface of the membrane were mechanically removed with a PBS-soaked swab and migrated cells were stained with 10% crystal violet for 10 min. The membranes were then cut from the chambers, and mounted on glass slides with PBS. Migrated cells were visualized and counted with a microscope. Five to six fields were counted on each membrane and averaged duplicate determinations were performed for each experimental condition.

3.2.11. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 4.03 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance between experimental groups was determined by t-tests and ANOVA for multiple comparisons.

All values are expressed as mean±SEM. $P< 0.05$ was considered significant.

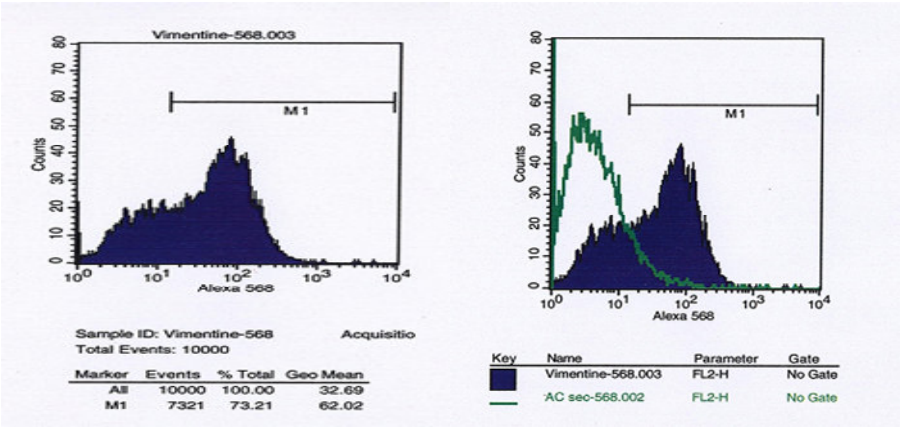
4. RESULTS

4.1. Fibrosis

4.1.1. Neonatal rat CF primary cell culture characterization

CFs were isolated from neonatal rat hearts and propagated in culture. To characterize these cells, fluorescent cell sorting (FACS) was performed using antibodies against fibroblast intracytoplasmic marker, vimentin. Almost all cells (73.2 %) were positive for this marker, showing a typical CF-like immunophenotype (figure 4.1A). On the other hand they were negative for CD45 and CD31, markers associated with hematopoietic cells. Furthermore immunofluorescence analysis was performed against vimentine and approximately all cells were vimentine positive (figure 4.1B).

A



B

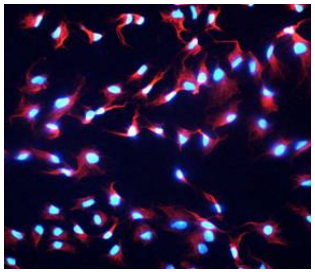


Figure 4.1. Purity of primary neonatal rat CF cell culture. (A) Results of FACS analysis of primary neonatal rat CF cell culture for vimentin. (B) Immunohistochemistry results against Vimentin. Almost all cells were vimentin positive in cell culture. Figures are representatives of three independent experiments.

4.1.2. Expression of 5-HT receptors

Specific signals for the 5-HT_{1B}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_4 receptors were consistently detected by gel electrophoresis in isolated neonatal rat CFs (Figure 4.2A). In all preparations, the intensity of the 5HT2A receptor immunodetected by Western Blotting from cardiac fibroblasts was generally lower than that found in control brain tissue (figure 4.2B). In all PCR experiments, no products were observed if amplifications were performed in cDNA samples devoid of reverse transcriptase (RT) enzyme (-lanes, in Figure 4.2A).

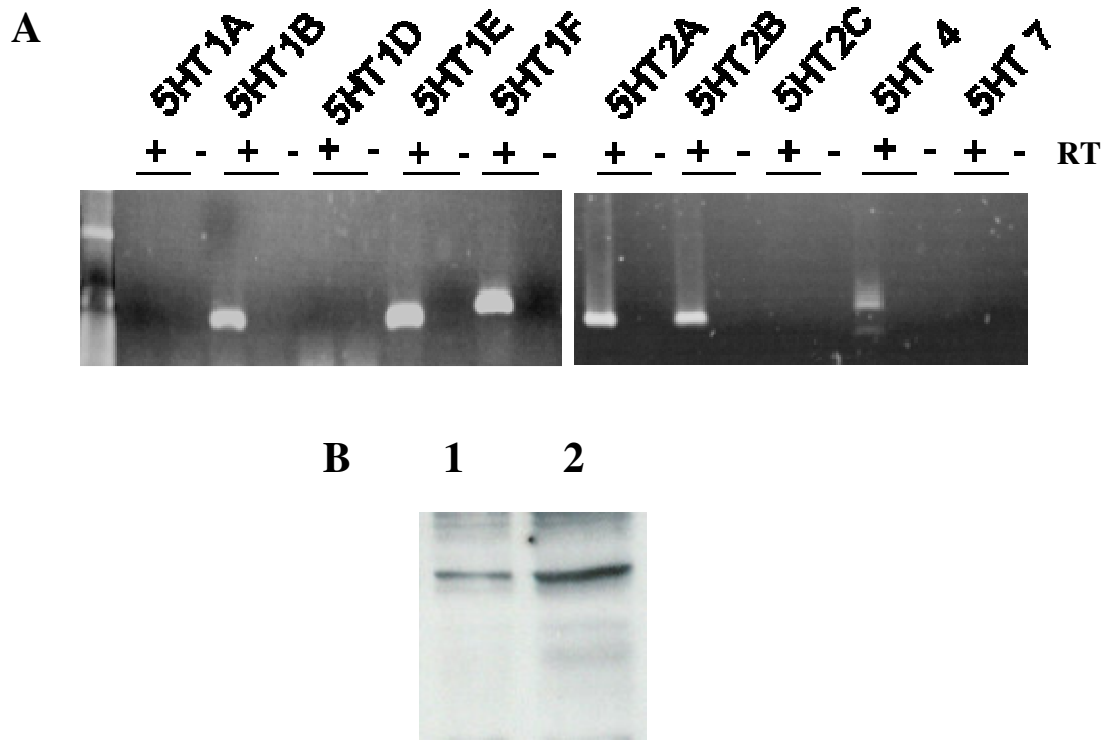


Figure 4.2. Expression of 5-HT receptors in isolated neonatal rat CFs. **(A)** Agarose gel electrophoresis of PCR products for the 5-HT receptors expression from culture of neonatal rat CFs. Messages for 5-HT_{1B}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_4 receptors were detected in these cells. Samples with and without reverse transcriptase (+ or - RT) are shown. **(B)** Immunodetection of 5-HT2A in cardiac fibroblasts (1) with respect to control brain tissue (2).

4.1.3. α–SMA expression in PL and 5-HT-induced rat CFs

A critical event in the initiation of fibrosis is the differentiation of CFs into myofibroblasts. Increase in the expression of α-SMA has been predominantly used as a marker of this differentiation. To determine whether protein level of α-SMA was modulated by PL and 5-HT, ray CFs were treated with various concentrations of PL (1 to 4x10 ^{6}/mL) or 5-HT (1 to 5 µM), and protein samples were collected after 48 hours. Protein expression of α-SMA (relative to α-tubulin) was determined by Western blotting. Figure 4.3 and figure 4.4 show a clear induction of α-SMA protein expression with PL and 5-HT respectively.

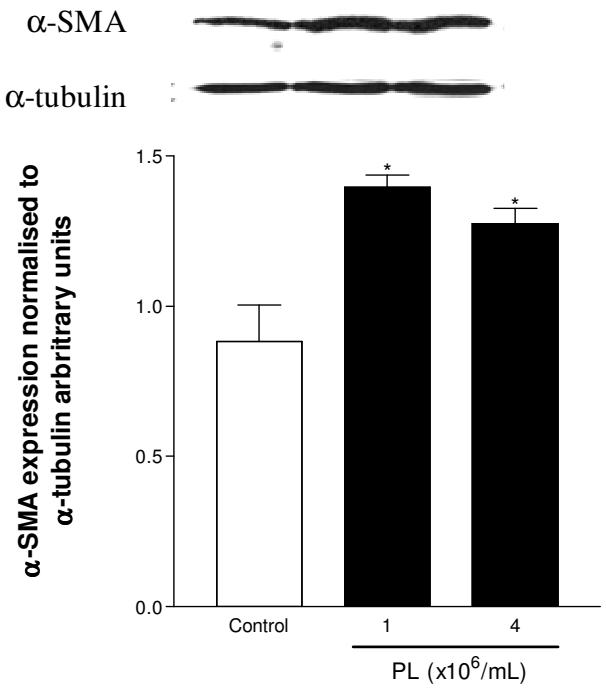


Figure 4.3. Effect of PL on α-SMA protein expression in CFs. Cells were treated with PL from 1 to 4x10^6/mL. Cell extracts were analyzed by Western blot. Changes in α-SMA expression were assessed using monoclonal anti α-SMA antibody. α-tubulin was used as reference protein. A representative blot is shown in the upper panel of the figure and the densitometric analysis is reported in the lower graph. Results in the graphs are the mean ± SEM of the ratio α-SMA/α-tubulin from three independent experiments. *P < 0.05.

As shown in figure 4.2, CFs express the 5-HT_{2A} receptor subtype as determined by RT-PCR and Western blotting. The fact that 5-HT effect was mimicked by the 5-HT_{2A} receptor agonist DOI suggests the implication of this receptor subtype in 5-HT mediated differentiation of CFs into myofibroblasts.

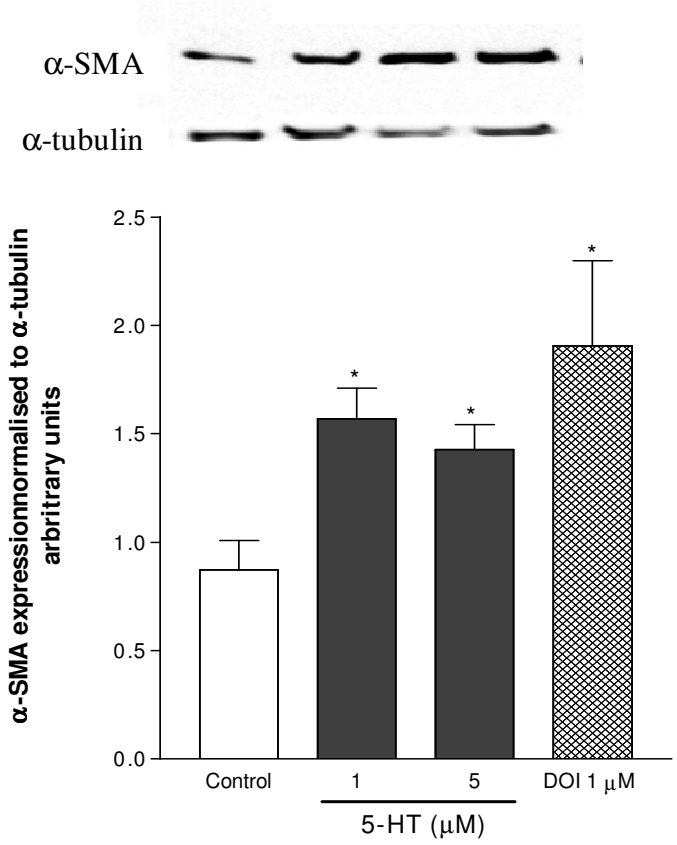


Figure 4.4. Effect of 5-HT on α-SMA protein expression in CFs. Cells were serum-deprived for 24 h and treated with 5-HT (1 or 5 μM) or the 5-HT_{2A} receptor agonist DOI (1 μM) for 48 h. Changes in α-SMA expression were assessed using monoclonal anti α-SMA antibody. α-tubulin was used as reference protein. A representative blot is shown in the upper panel of the figure and the densitometric analyses are reported in the lower graphs. Results in the graphs are the mean ± SEM of the ratio α-SMA/α-tubulin from three independent experiments. *$P < 0.05$.

4.1.4. Migration Assay

PL and 5-HT effects on rat CF chemotaxis were investigated at 6 h, 14 h, 22 h time points by using a modified Boyden chamber assay. Photomicrographs were taken under microscopy for PL ($8x10^6$/mL) and 5-HT (1µM) induced migration after 22 h (figure 4.5A). Migration velocity of rat CFs was time-dependent (figure 4.5B) and both PL ($8x10^6$/mL) and 5-HT (1µM) significantly increased the number of migrated cells at 22 h (figure 4.5B). Moreover, PL increased cell migration up to approximately 6-fold over baseline in a dose-dependent manner at 22 h (figure 4.5C). To determine whether the change in cell migration was due to an increase in cell proliferation, we measured cell growth in response to PL and 5-HT. Concentrations of PL that induced migration increased cell proliferation up to 1.75-fold over baseline after 48h (figure 4.6 A). Although PL is mitogenic for rat CFs, the potent chemotatic effect of PL measured after 24 h can not be totally attributed to cell proliferation.

Moreover PL effect on rat CFs migration was abolished by 5-HT_{2A} antagonist, ketanserin (figure 4.5D) whereas cell proliferation remained unchanged (figure 4.6A). In contrast, 5-HT exhibited a modest and non significant effect on rat CF growth after 48h (figure 4.6B). 5-HT effect on migration was abolished by ketanserin and mimicked by the 5-HT_{2A} agonist DOI (figure 4.5D) suggesting that 5-HT effect was mediated by 5-HT_{2A} receptor subtype. These results showed that 5-HT released by platelets is partially responsible for the chemotactive effect of PL and appears not involved in its proliferative activity. PL and 5-HT behaved as potent chemotactic factors for CFs through the activation of 5-HT_{2A} receptors in modified Boyden chamber assay.

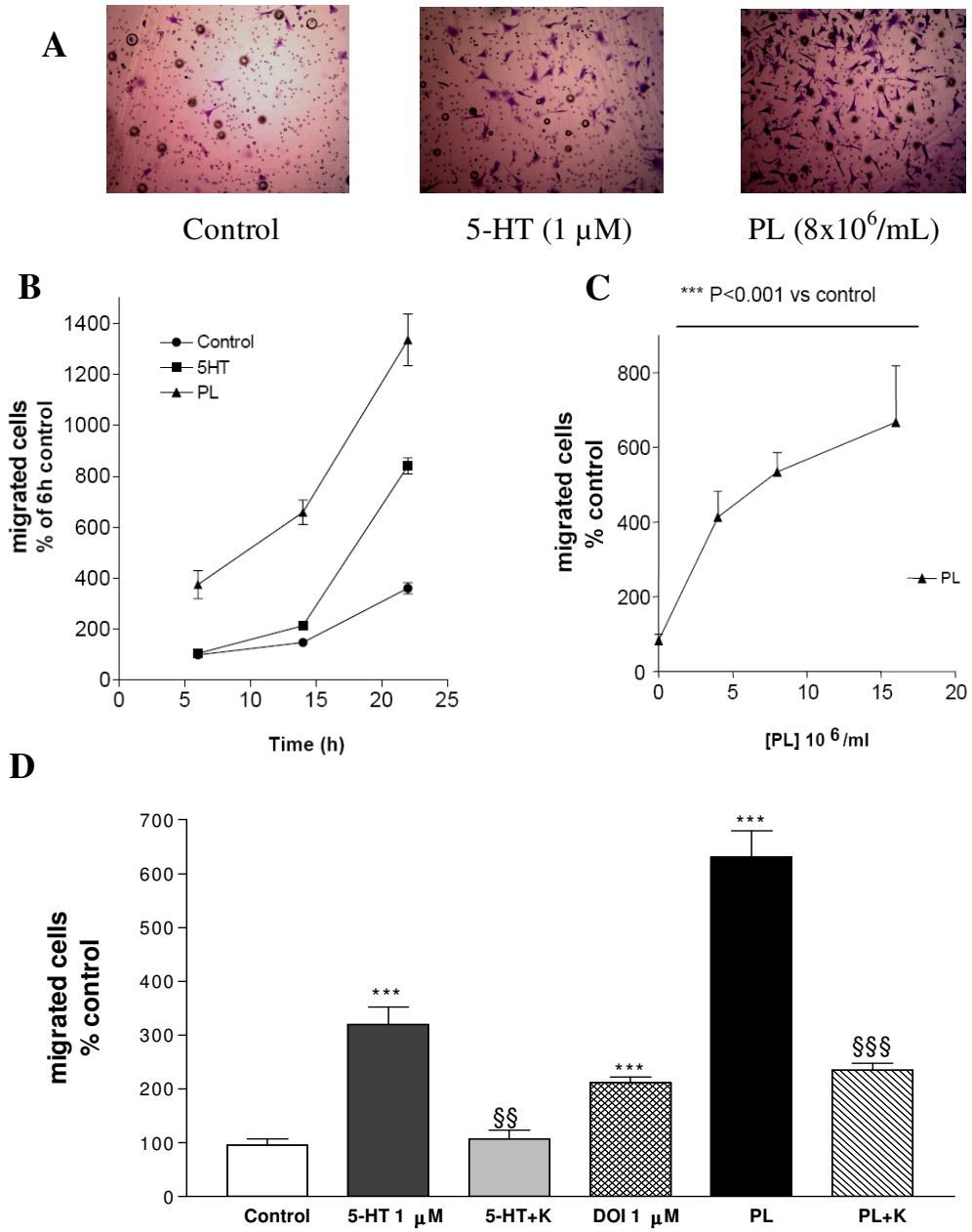


Figure 4.5. PL and 5-HT induced migration assay in rat CFs. (A) The photomicrograph of control and 5-HT or platelet induced migration after 22 h. (B) Time course of PL ($8x10^6$/mL) and 5-HT (1 µM) induced rat CF migration. (C) Dose response curve of PL measured after 22 h treatment. (D) Bar chart presenting the percentage of migrated cells after 22 h following stimulation with PL ($8x10^6$/mL), 5-HT (1 µM) or DOI (1 µM). Ketanserin (K) (0.1 µM) was added to CFs prior to stimulation with 5-HT or PL. (***P<0.001 5-HT, DOI or PL vs control; §§P<0.01 5-HT vs 5-HT+K and §§§P<0.001 PL vs PL+K; all results are average of at least three independent experiments).

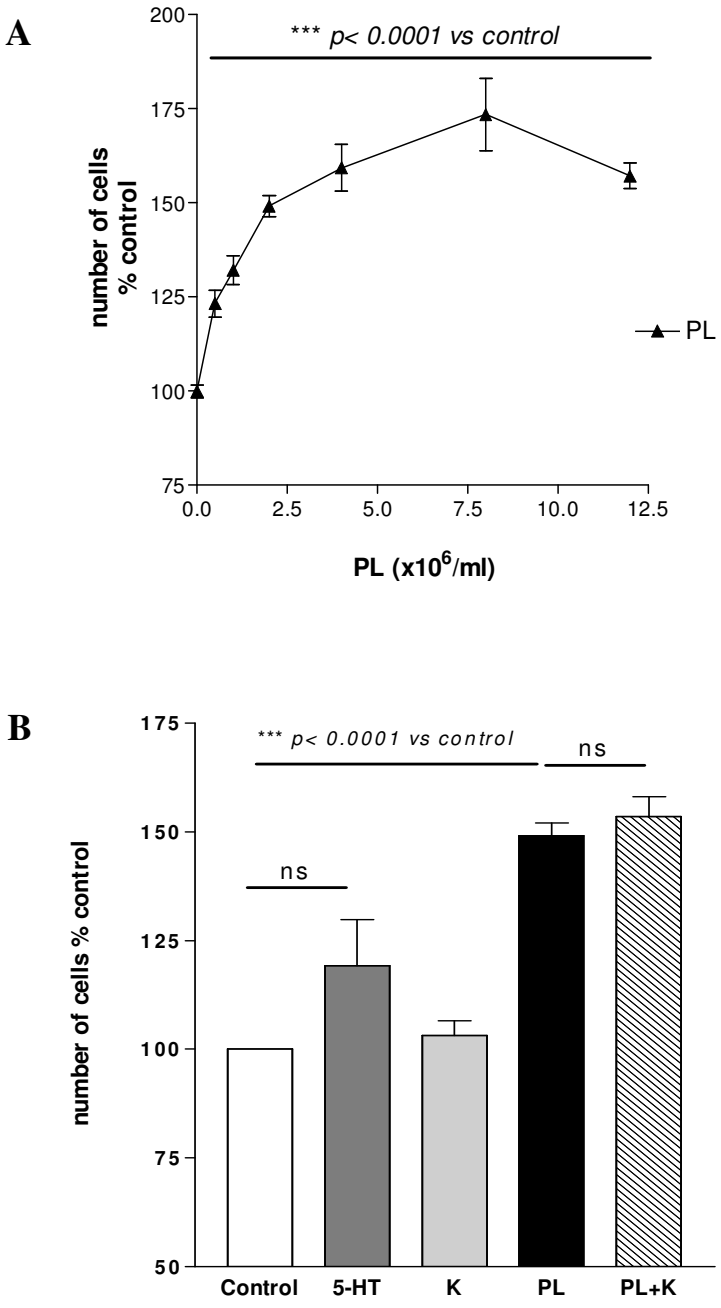


Figure 4.6. Proliferation assay in PL and 5-HT treated rat CFs. (A) Dose dependency of PL induced rat CF proliferation after 48 h treatment. (B) Effect of ketanserin on proliferation of rat CFs induced by 5-HT (1 μM) and PL ($8x10^6$/mL) after 48 h treatment. Results are the average of at least three independent experiments. ***P<0.001 PL vs control.

4.1.5. TGF-β1 expression in PL and 5-HT induced rat CFs

In different tissues, TGF-β1 has been identified as one of the major mediators of the profibrotic activation and the phenotypic conversion of fibroblasts into myofibroblasts. To determine the potential profibrotic effect of PL and 5-HT on CFs, we followed the kinetic of TGF-β1 release in CF culture medium after stimulation. As shown in figure 4.7A, both PL and 5-HT stimulated TGF-β1 secretion by CFs in time-dependent manner. This effect was observed after 4 h stimulation, peaked at 6h and decreased after 12 h. Ketanserin completely prevented the stimulatory effect of PL and 5-HT on TGF-β1 secretion by CFs (Figure 4.7B).

Based on the time course of TGF-β1 secretion, we hypothesized that the effect of PL and 5-HT may involve regulation of TGF-β1 messenger expression. Thus, we next evaluated the effect of PL and 5-HT on TGF-β1 mRNA expression by real time RT-PCR. 5-HT (1 µM) significantly increased TGF-β1 mRNA expression in CFs (figure 4.8A). Ketanserin but not SB206553, a 5-HT_{2B} antagonist, prevented TGF-β1 mRNA up regulation (figure 4.8A). Interestingly, ketanserin also fully blocked TGF-β1 mRNA expression induced by PL (figure 4.8B). These data showed that the effect of 5-HT on TGF-β1 secretion by CFs revealed via 5-HT_{2A} receptors. Furthermore, platelet derived 5-HT appeared to be the major contributor of PL effect on TGF-β1 expression and secretion.

As a result, it was suggested that PL and 5-HT increase TGF-β1 expression and secretion by CFs via 5-HT_{2A} receptors activation.

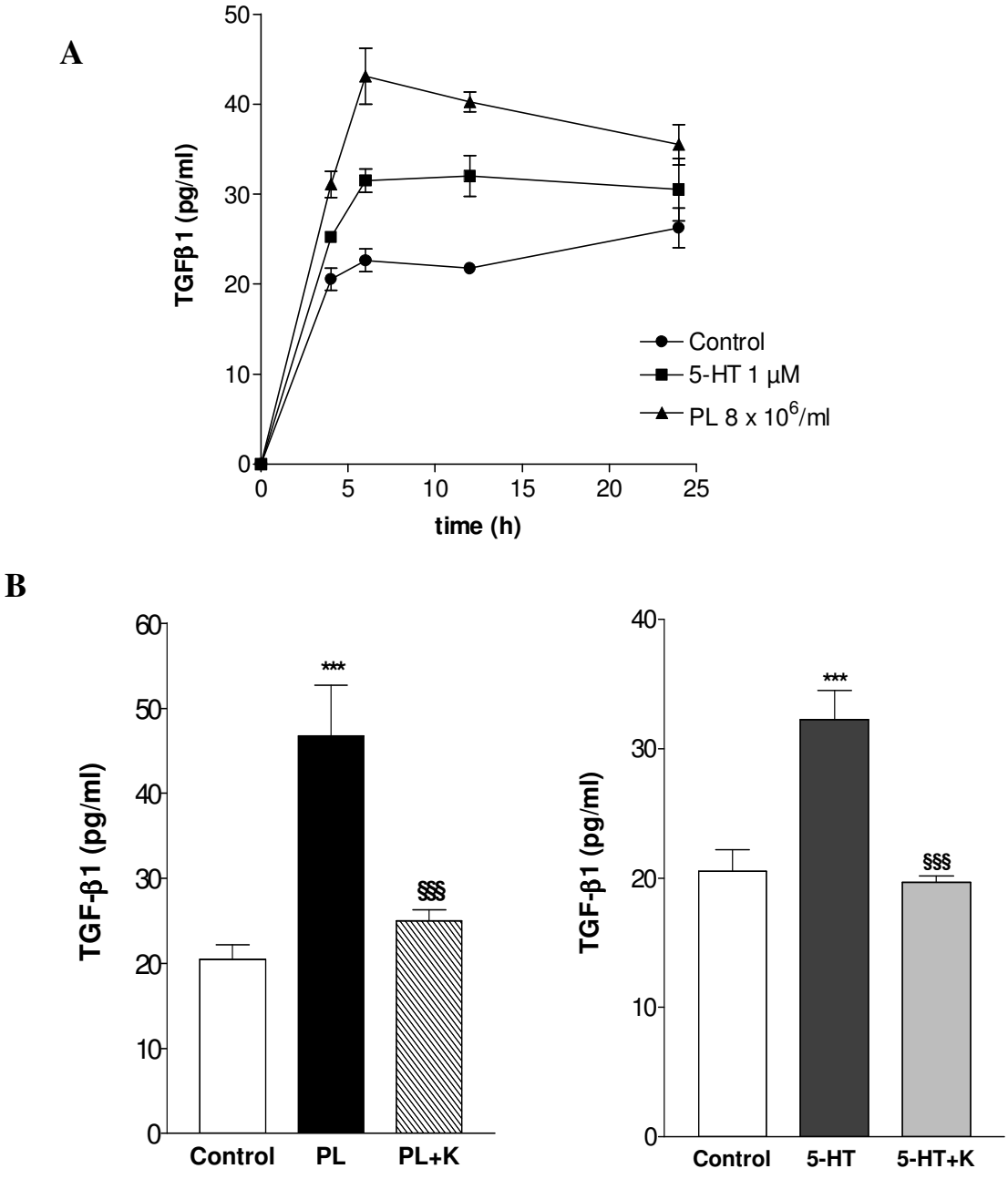


Figure 4.7. Effects of 1 µM 5-HT and $8x10^6$/mL PL on TGF-β protein level. (A) Time-dependent effects of PL and 5-HT on TGF-β protein level in supernatants of CFs. (B) Effects of PL (left panel) and 5-HT (right panel) on TGF-β secretion in the absence and in the presence of ketanserin (0.1 µM) in cultured rat CFs 6 h after treatment. The data represent means ± SEM of three independent experiments. ***P<0.001, vs control; §§§ P<0.001 PL vs PL+K and 5-HT vs 5-HT+K.

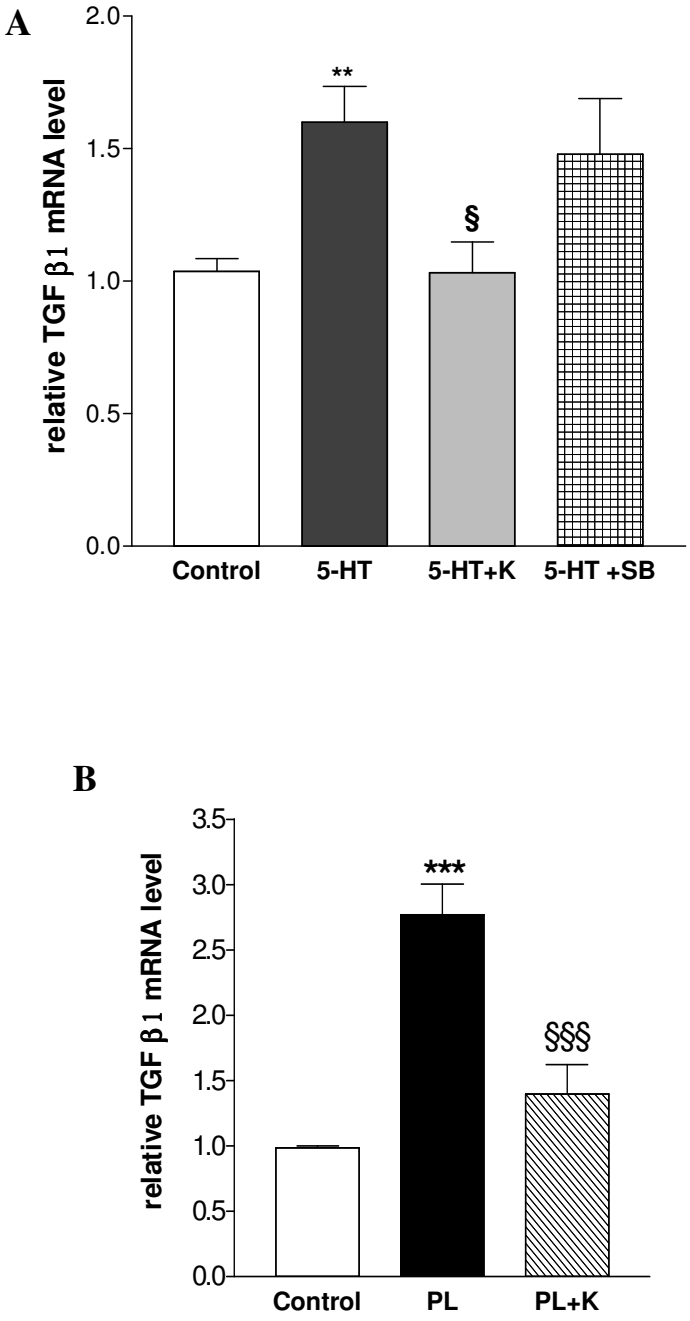


Figure 4.8. Effects of 1 μM 5-HT (A) and $8x10^6$/mL PL (B) on TGF-β mRNA. Ketanserin (0.1 μM) or SB 206553 (1 μM) were added to CFs 30 minutes prior addition of 5-HT or PL. TGF-β transcripts was determined after 2 h by the real-time RT-PCR and normalized to 36B4. **P<0.01, 5-HT vs control;***P<0.001, PL vs control; §P<0.05 5-HT vs 5-HT+K and §§§ P<0.001 PL vs PL+K.

4.1.6. PL and 5-HT regulated MMPs expression in rat CFs

ECM homeostasis is an orchestrated process between synthesis and degradation. A family of proteolytic enzymes, MMPs, has a predominant role in this homeostasis by cleaving ECM components. The activity of MMPs is regulated by proteolysis via other MMPs and by the endogenous TIMPs synthesized by many cell types including CFs. We followed MMPs and TIMPs expression by real time PCR after treatment with PL and 5-HT. We observed no significant modification for the expression of TIMP-1, TIMP-2, TIMP-3 after 2h or 6h stimulation. In contrast, 5-HT treatment induced a sustained increase in MMP-3, MMP-13 mRNA up to 6 hours (figure 4.9A and figure 4.9B respectively) whereas MMP-2 and MMP-9 expression were slightly decreased or unchanged respectively.

Moreover, PL showed the similar effect of 5-HT and induced an even higher increase in MMP-3 and MMP-13 mRNA (figure 4.9A and figure 4.9B, respectively). Pretreatment with ketanserin abolished both 5-HT and PL effect on MMPs expression indicating the overexpression of MMP-3 and MMP-13 was mediated by 5-HT_{2A} receptors (table 4.1).

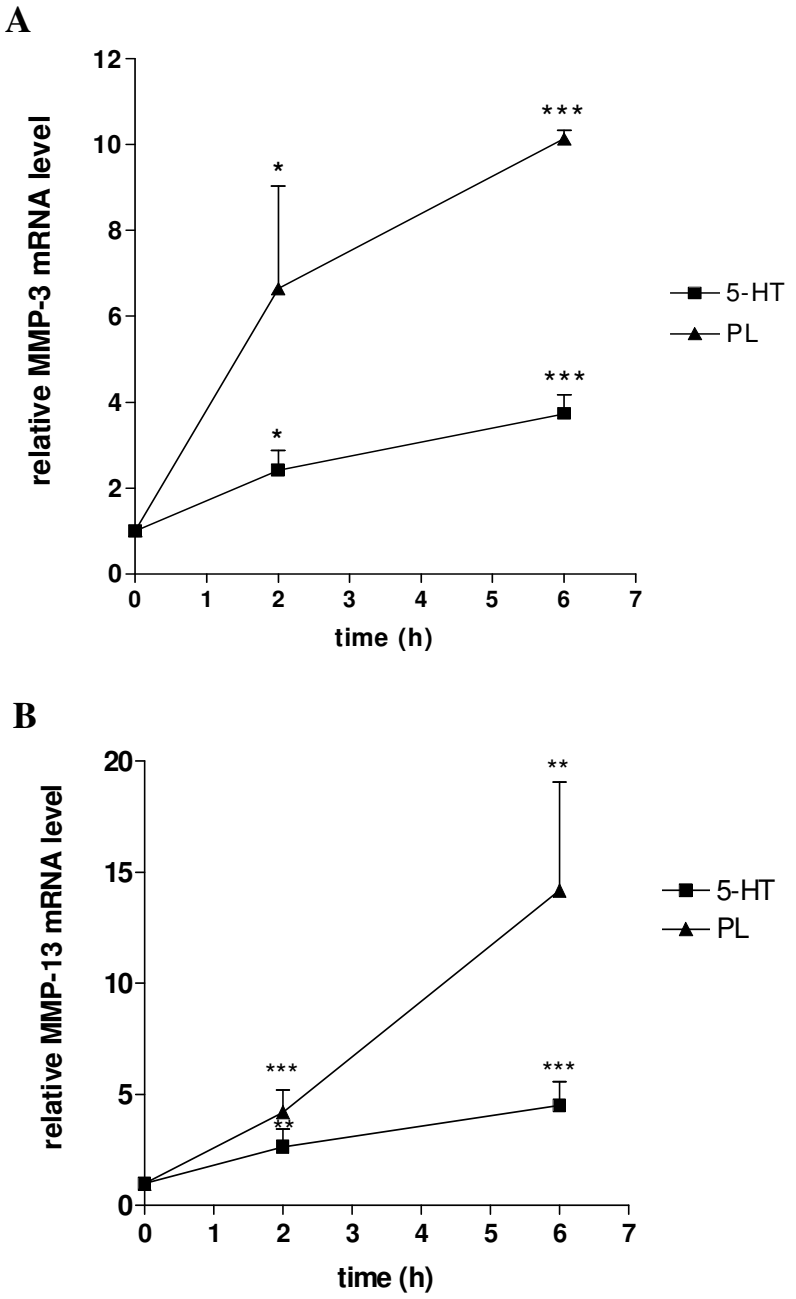


Figure 4.9. Effects of 5-HT and PL on (A) MMP-3 and (B) MMP-13 mRNA expressions. MMP-3 and MMP-13 mRNA were identified by Real Time RT-PCR and normalized to 36B4 used as a reference transcript. CFs were treated with 1 µM 5-HT or PL from $8x10^6$ platelets/mL after 24 h serum starvation. Data are expressed as fold-increase vs. control for each time point. *P<0.05, vs control **P<0.01, vs control; ***P<0.001 vs control.

Table 4.1: Effect of ketanserin (0.1 µM) on PL ($8x10^6$ platelets/mL) and 5-HT (1 µM) induced MMPs mRNA expression for 2 h. Ketanserin was added to CFs 30 minutes prior addition of 5-HT or PL. MMP-3 and MMP-13 mRNA were identified by Real Time RT-PCR and normalized to 36B4 used as a reference transcript.

	5-HT	**5HT+K**	**PL**	**PLT+K**
MMP-3	2.46±0.30 **(**P<0.01 vs Control)**	0.81±0.09 **(***P<0.001 vs 5-HT)**	5.35±1.56 **(*P<0.05 vs Control)**	1.25±0.06 **(**P<0.01 vs PL)**
MMP-13	2.64±0.78 **(**P<0.01 vs Control)**	0.82±0.16 **(**P<0.01 vs 5-HT)**	4.27±0.97 **(***P<0.001 vs Control)**	1.04±0.04 **(**P<0.01 vs PL)**

Values are expressed as fold change with respect to the control normalized to 1. The data represent means ± SEM of four to six independent experiments.

4.2. Inflammation

4.2.1. IL-6 expression in PL and 5-HT induced CFs

In response to myocardial injury or infarction, activated CFs migrate into the infarct zone and release MMPs, counterbalanced by TIMPs, leading to net degradation of damaged ECM (191). These fibroblast responses are regulated by pro-inflammatory cytokines including TGF-β, IL-6 and TNFα produced by resident cells at sites of injury, including fibroblasts themselves (178). To determine the potential proinflammatory effects of PL and 5-HT on CFs, we followed the release of IL-6 in CF culture medium after stimulation.

As shown in fig. 4.10 and fig. 4.11 respectively, both PL and 5-HT stimulated IL-6 secretion by mouse CFs in a time- and concentration-dependent manner. PL stimulated IL-6 secretion by mouse CFs was investigated at the concentration range of 0.5-10x10^6/mL. The highest effect was observed at the concentration of 8x10^6/mL (figure 4.10A). IL-6 secretion was increased upto 14 h stimulation and remained constant up to 24 h at the concentration of 8x10^6/mL for PL stimulation (figure 4.10B). Ketanserin partially prevented the stimulatory effect of PL on IL-6 secretion by mouse CFs (figure 4.10C). 5-HT also stimulated IL-6 secretion by mouse CFs was determined at the concentration range of 0.01-1 μM (figure 4.11A). IL-6 secretion was increased upto 14 h stimulation and remained constant up to 24 h at the concentration of 1 μM for 5-HT stimulation (figure 4.11B). Ketanserin prevented the stimulatory effect of 5-HT on IL-6 secretion by mouse CFs (figure 4.11C). We next evaluated the effect of PL and 5-HT on IL-6 mRNA expression by real time RT-PCR. PL (8x10^6/mL) and 5-HT (1 μM) significantly increased IL-6 mRNA expression in rat CFs. Ketanserin prevented IL-6 mRNA up regulation observed by 5-HT stimulation (figure 4.12B). Interestingly, ketanserin partially blocked IL-6 mRNA expression induced by PL (figure 4.12A). These data showed that the effect of 5-HT on IL-6 secretion by CFs revealed via 5-HT_{2A} receptors. Furthermore, platelet derived 5-HT appeared as the major contributor of PL effect on IL-6 expression and secretion.

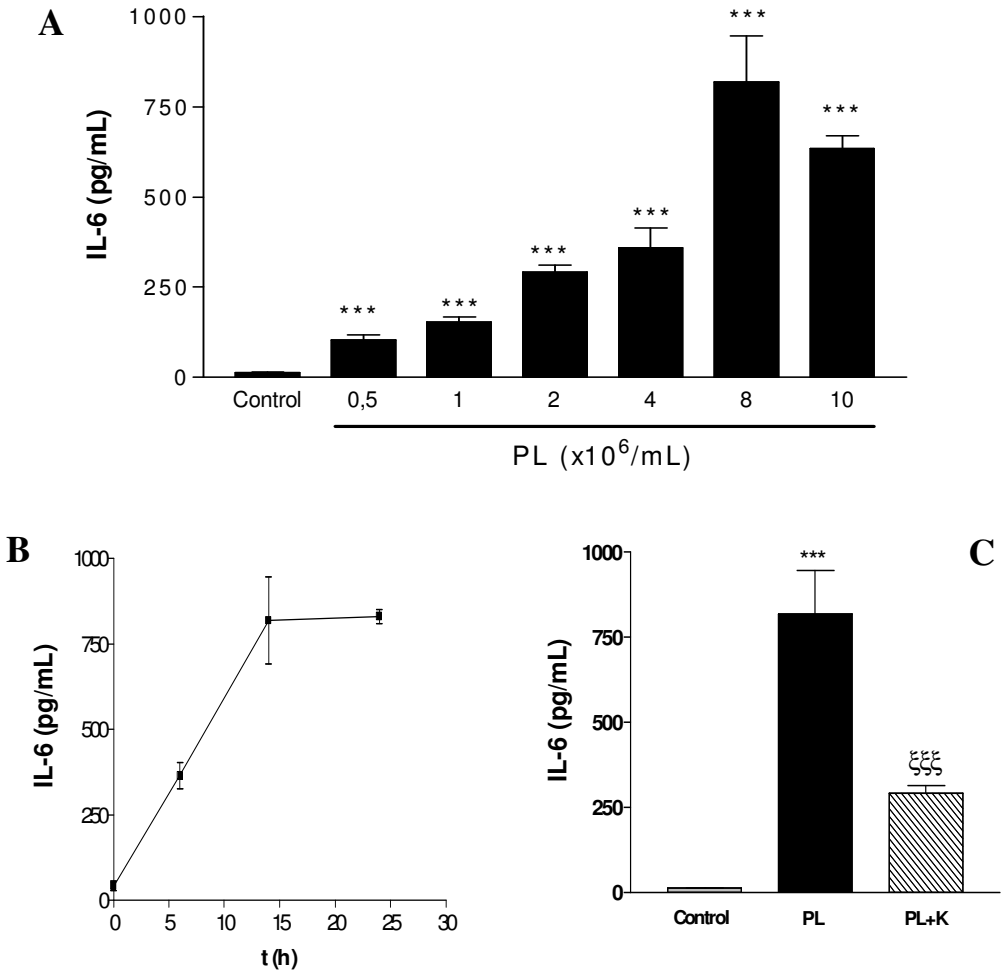


Figure 4.10. Effect of PL on IL-6 protein level. (A) Dose-dependent effect of PL on IL-6 protein level in supernatants of mouse CFs after 14 h. (B) Time-dependent effect of 5-HT on IL-6 protein level in supernatants of mouse CFs. (C) Effect of PL on IL-6 secretion in the absence and in the presence of ketanserin (0.1 μM) in cultured mouse CFs after 14 h. The data represent means ± SEM of at least three independent experiments. ***P<0.001 PL vs control; ξξξ P<0.001 PL vs PL+K.

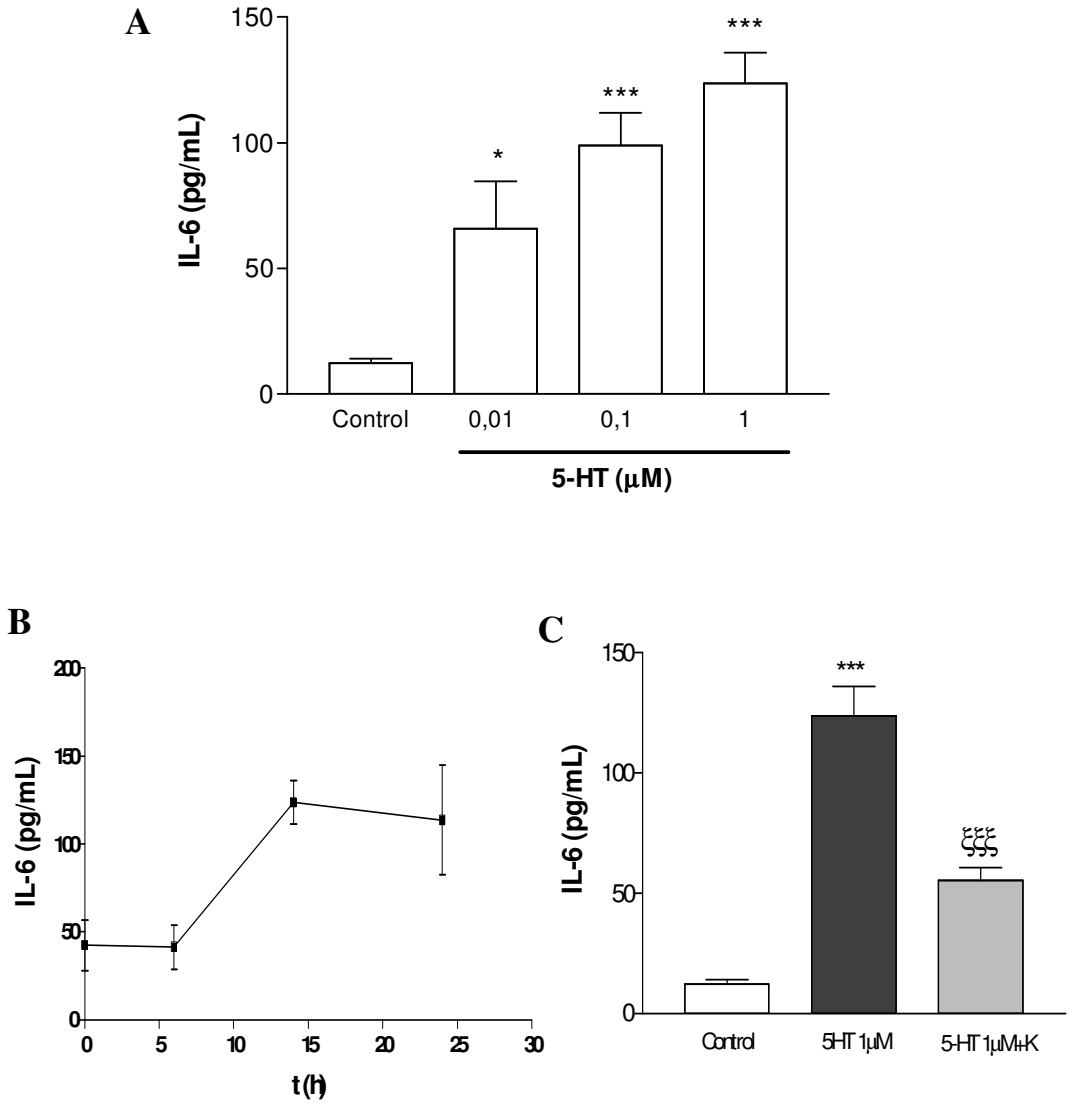


Figure 4.11. Effect of 1 µM 5-HT on IL-6 protein level. (A) Dose-dependent effect of 5-HT on IL-6 protein level in supernatants of mouse CFs after 14 h. (B) Time-dependent effect of 5-HT on IL-6 protein level in supernatants of mouse CFs. (C) Effect of 5-HT on IL-6 secretion in the absence and in the presence of ketanserin (0.1 µM) in cultured mouse CFs after 14 h. The data represent means ± SEM of at least three independent experiments. ***P<0.001 5-HT vs control, *P<0.05 5-HT vs control; ξ ξ ξ P<0.001 5-HT vs 5-HT+K.

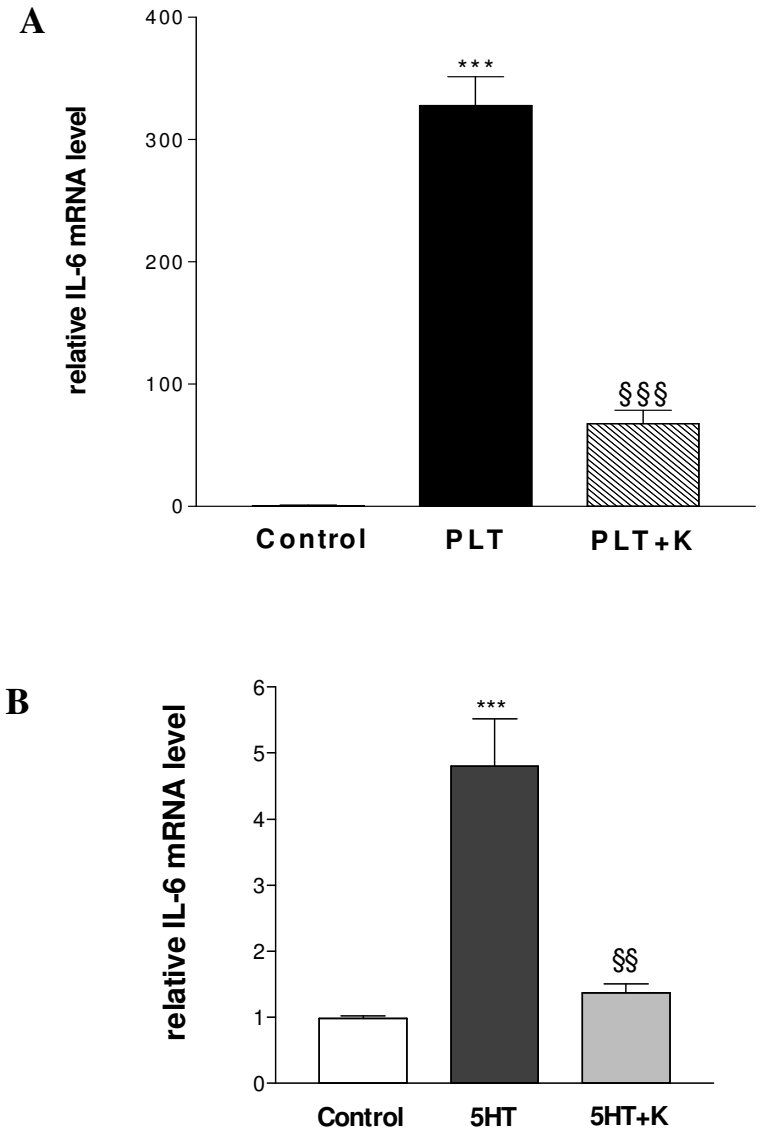


Figure 4.12. Effects of 1 µM 5-HT (A) and 8x10 ^{6}/mL PL (B) on IL-6 mRNA. Ketanserin (0.1 µM) was added to mouse CFs 30 minutes prior to addition of 5-HT or PL. IL-6 transcripts was determined by the real-time RT-PCR and normalized to 36B4. ***P<0.001, PL vs control and 5-HT vs control; §§§ P<0.001 PL vs PL+K; §§ P<0.01 5-HT vs 5-HT+K.

4.2.2. TNF-α expression in PL and 5-HT induced CFs

To determine whether PL and 5-HT stimulation in CFs is capable of inducing TNF-α, CFs were cultured in presence of 1 μM 5-HT or $8x10^6$/mL PL. Figure 4.13A shows the time course of TNF-α production after PL and 5-HT stimulation of CFs. During the incubation period, a sharp rise in TNF-α levels was observed from 2 h to 6 h and peaked at 4 h. The secreted TNF-α concentrations obtained from PL and 5-HT stimulated CFs at 2 h, 4 h and 6 h were significantly differed from those obtained from unstimulated CFs (figure 4.13A).

Since TNF-α expression was hypothesized to be induced in 5-HT_{2A} receptor-dependent manner in CFs stimulated by PL and 5-HT, attempts were made to determine whether TNF-α production could be inhibited by ketanserin. Ketanserin was added to CFs cultures 30 min prior to addition of PL or 5-HT. The amount of TNF-α in the supernatants was assayed by ELISA at 4 h following treatment. As shown in Figure 4.13B, TNF-α production induced by PL or 5-HT was completely blocked by the addition of ketanserin. This TNF-α induction in CFs as a response to PL or 5-HT stimulation was appeared through 5-HT_{2A} receptor.

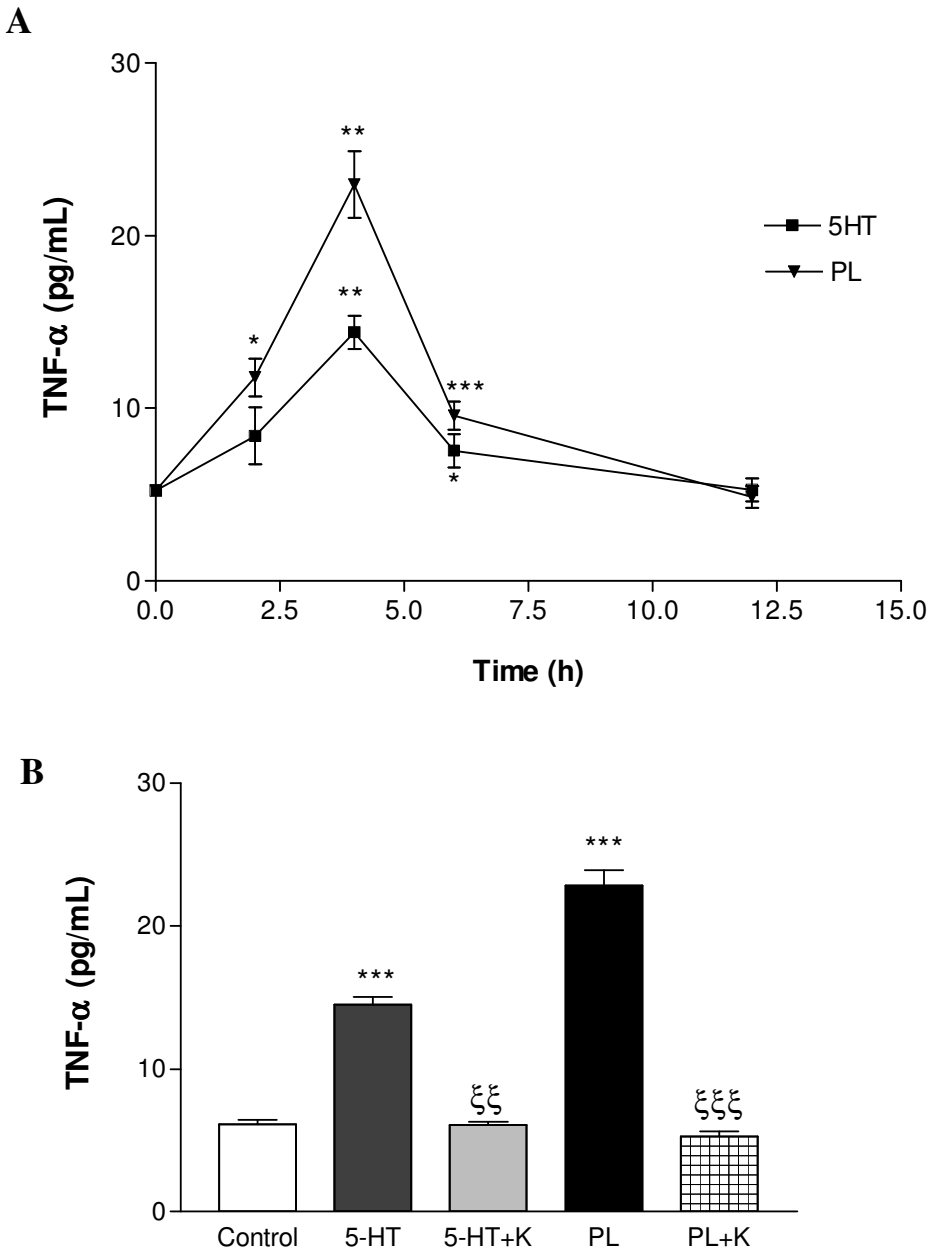


Figure 4.13. Secreted TNF-α levels in mouse CFs after treatment with PL and 5-HT in presence or absence of ketanserin. (A) Time-dependent effect of 5-HT on TNF-α protein level in supernatants of mouse CFs. (B) Effect of 5-HT and PL on TNF-α secretion in the absence and in the presence of ketanserin (0.1 μM) in cultured mouse CFs for 4 h. The data represent means ± SEM of at least three independent experiments. ***P<0.001, **P<0.01, *P<0.05 vs control; ξ ξ ξ P<0.001 PL vs PL+K; ξ ξ P<0.01 5-HT vs 5-HT+K.

4.2.3. GM-CSF expression in PL and 5-HT induced CFs

As presented in the figure 4.14, PL and 5-HT increased the level of GM-CSF mRNA expression as early as 2 h after stimulation of rat CFs. The observed increase in stimulation with PL was approximately 500-fold of untreated cells whereas the increase with 5-HT stimulation was only 1.5-fold of unstimulated rat CFs. It was suggested that the upregulated expression of GM-CSF in 5-HT or PL treated rat CFs was significantly suppressed by ketanserin (figure 4.14).

To investigate whether the increase in mRNA expression also observed in protein level, we measured the levels of secreted GM-CSF in PL and 5-HT treated mouse CFs supernatants. In agreement with the gene expression data; significant increase was observed in protein level with ELISA in response to PL and 5-HT treatment in mouse CFs. GM-CSF protein level was significantly elevated following treatment starting at 2 h, this was observed up to 4 h (Figure 4.15) and returned to basal level after 6 h. Maximum induction was observed for 4 h stimulation therefore inhibition studies with ketanserin were carried in 4h stimulation conditions. As expected ketanserin abrogated the induction of GM-CSF secretion in PL and 5-HT treated mouse CFs (figure 4.15).

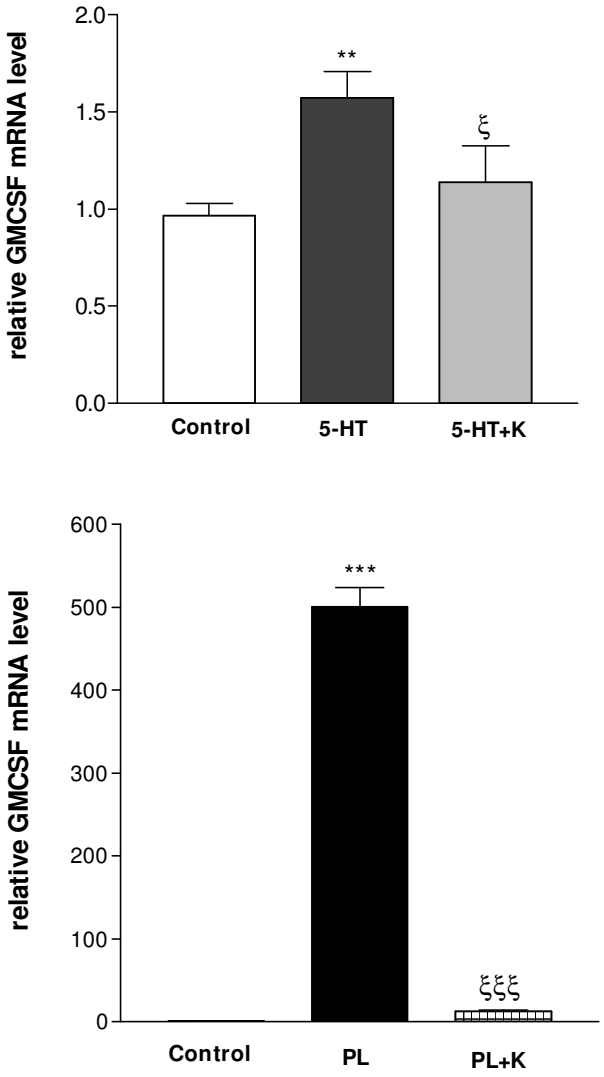


Figure 4.14. Effects of 1 μM 5-HT (upper panel) and 8x10 ^{6}/mL PL (lower panel) on GM-CSF mRNA. Ketanserin (0.1 μM) was added to mouse CFs 30 minutes prior to addition of 5-HT or PL. GM-CSF transcripts was determined by the real-time RT-PCR and normalized to 36B4. **P<0.01, 5-HT vs control;***P<0.001, PL vs control; §P<0.05 5-HT vs 5-HT+K and §§§ P<0.001 PL vs PL+K.

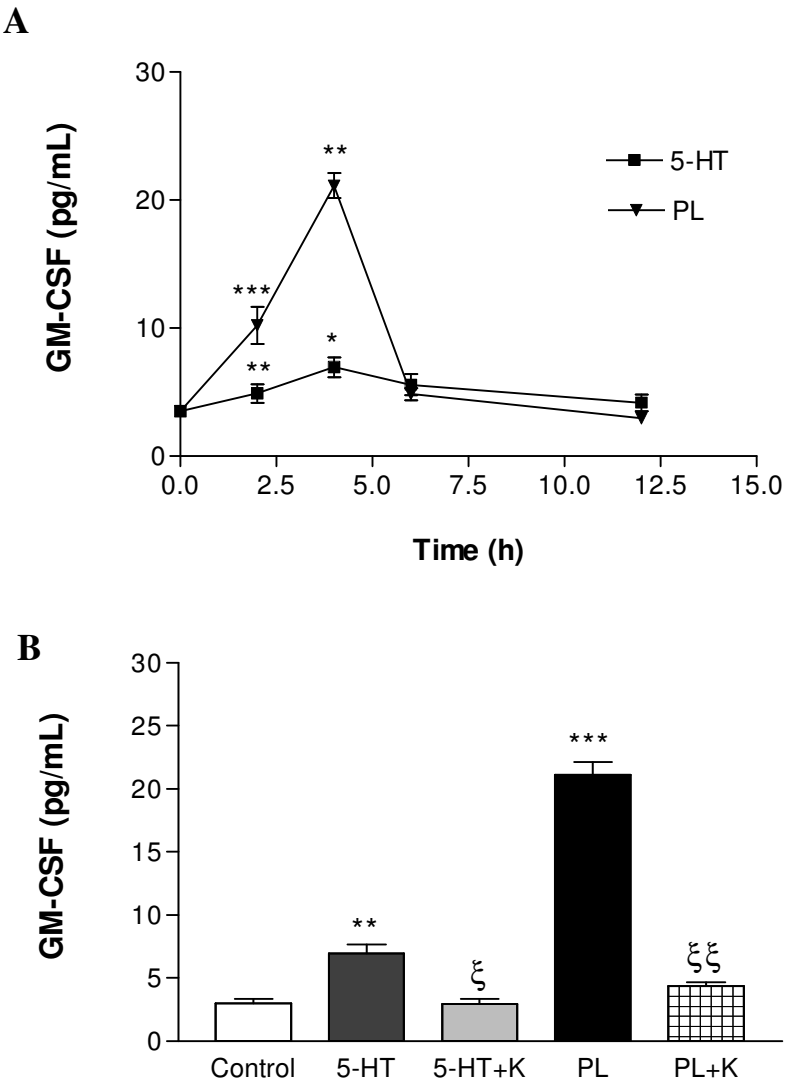


Figure 4.15. Effects of 8x10 ^{6}/mL PL and 1 μM 5-HT on GM-CSF protein level. (A) Time-dependent effect of PL and 5-HT on GM-CSF protein level in supernatants of mouse CFs. (B) Effect of PL and 5-HT on GM-CSF secretion in the absence and in the presence of ketanserin (0.1 μM) in cultured mouse CFs after 4 h treatment. The data represent means ± SEM of at least three independent experiments. ***P<0.001, ** P<0.01 * P<0.05 vs control; ξ P<0.05 5-HT vs 5-HT+K, ξ ξ P<0.01 PL vs PL+K.

4.2.4. MCP-1 expression in PL and 5-HT induced CFs

The primary biological function of MCP-1 appears to be the recruitment of monocytes and other mononuclear cells to sites of inflammation (192). Indeed, MCP-1 expression appears to be an important component of a hierarchical, differential pattern of chemokine expression and immobilization (193). Moreover, MCP-1 has been shown to be upregulated in heart injury (194). Thus, to investigate whether this inflammatory chemokine is secreted in response to PL and 5-HT stimulation in CFs we determined the level of MCP-1.

Total RNA was isolated 0 h, 4 h, 6 h, 12 h and 24 h after PL and 5-HT stimulation of rat CFs and subjected to quantitative real-time PCR. MCP-1 mRNA expression was significantly increased as compared with that of the untreated controls in a time-dependent manner in response to the treatment with $8x10^6$/mL PL and 1 μM 5-HT (figure 4.16 A and B, respectively). A significant increase occurred at 4 h after stimulation and this increase was peaked at 6 h. At 12 h, increase was still significant for PL stimulated cells whereas in 5-HT stimulated cells no increase was observed compared to that of unstimulated cells. At 24 h, MCP-1 mRNA level was returned to that of the untreated controls in PL stimulated cells.

The levels of secreted MCP-1 in media of CFs exposed to PL and 5-HT were significantly higher than those measured in unstimulated CFs (figure 4.17). The increased release of MCP-1 from CFs exposed to PL or 5-HT was apparent in 12 hours after stimulation. The levels of MCP-1 released by CFs exposed to PL or 5-HT were 1.5- and 10-fold higher than that of control respectively at 12 hours (figure 4.17) and this increase remained elevated upto 24 hours (figure 4.17).

To study the inhibition capability of ketanserin, we determined MCP-1 production in supernatants collected from 5-HT+K and PL+K treated CFs. Addition of ketanserin (0.1 μM) 30 min. prior to stimulation with 5-HT or PL led to a significant inhibition of MCP-1 increase 12 h and 24 h after treatment in CFs (figure 4.17). Thus, the induction of MCP-1 by PL and 5-HT could be inhibited by the addition of ketanserin prior to treatment.

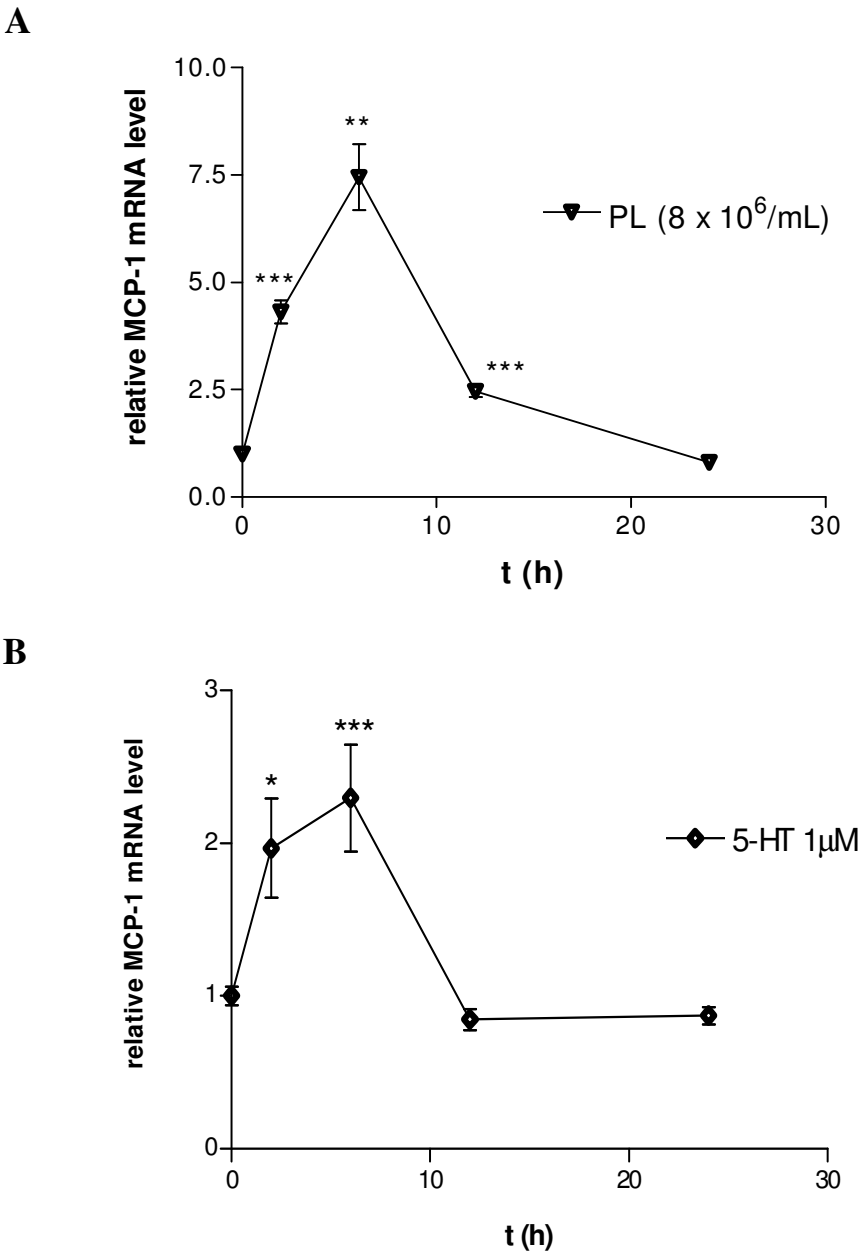


Figure 4.16. Time-course of quantitative real-time RT-PCR analysis of MCP-1 expression in neonatal rat CFs after PL (A) and 5-HT (B) stimulation. Results were normalized to 36B4 expression (***P<0.001, **P<0.01, *P<0.05 vs t=0).

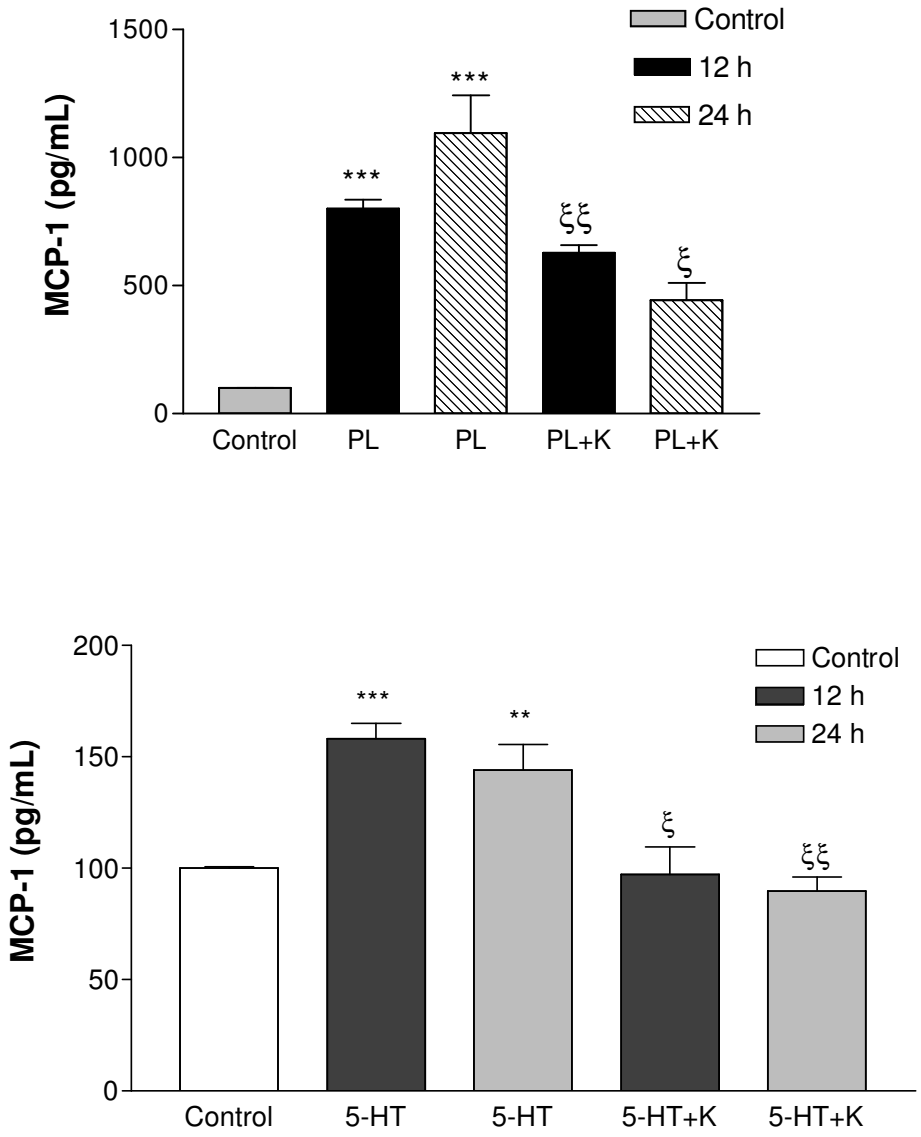


Figure 4.17. Effects of $8x10^6$/mL PL (upper panel) and 1 µM 5-HT (lower panel) on MCP-1 mRNA for 12 h and 24 h. Ketanserin (0.1 µM) was added to CFs 30 minutes prior to addition of 5-HT or PL. MCP-1 transcripts was determined by the real-time RT-PCR and normalized to 36B4. ***P<0.001, PL vs control; **P<0.01, 5-HT vs control; §P<0.05 PL (24 h) vs PL+K (24 h) and 5-HT (12 h) vs 5-HT+K (12 h) §§ P<0.01 PL (12 h) vs PL+K (12 h) and 5-HT (24 h) vs 5-HT+K (24 h)

4.2.5. Chemotaxis Assay

In order to evaluate the effect of cytokines and chemokines secreted from mouse CFs in response to PL or 5-HT treatment, it was decided to perform *in vitro* chemotaxis assay by J774 mouse macrophage cell line. As expected, PL stimulated mouse CFs conditioned media recruited J774 cells about ten fold over basal control (figure 4.18). When the chemotactic response to 5-HT treated mouse CFs conditioned media was tested, it was evaluated that 5-HT treated mouse CFs conditioned media also stimulated the monocyte transmigration (figure 4.18).

In contrast, loading the supernatant of PL or 5-HT incubation without conditioning into lower compartment of the transwell insert did not effect transmigration compared to control. Therefore, observed transmigratory effects of conditioned media can be attributed to the secreted cytokines from mouse CFs in response to PL or 5-HT stimulation. As a result, PL and 5-HT exhibited a potent chemoattractive effect on the J774 macrophage cell line *in vitro* via secreted cytokines from mouse CFs.

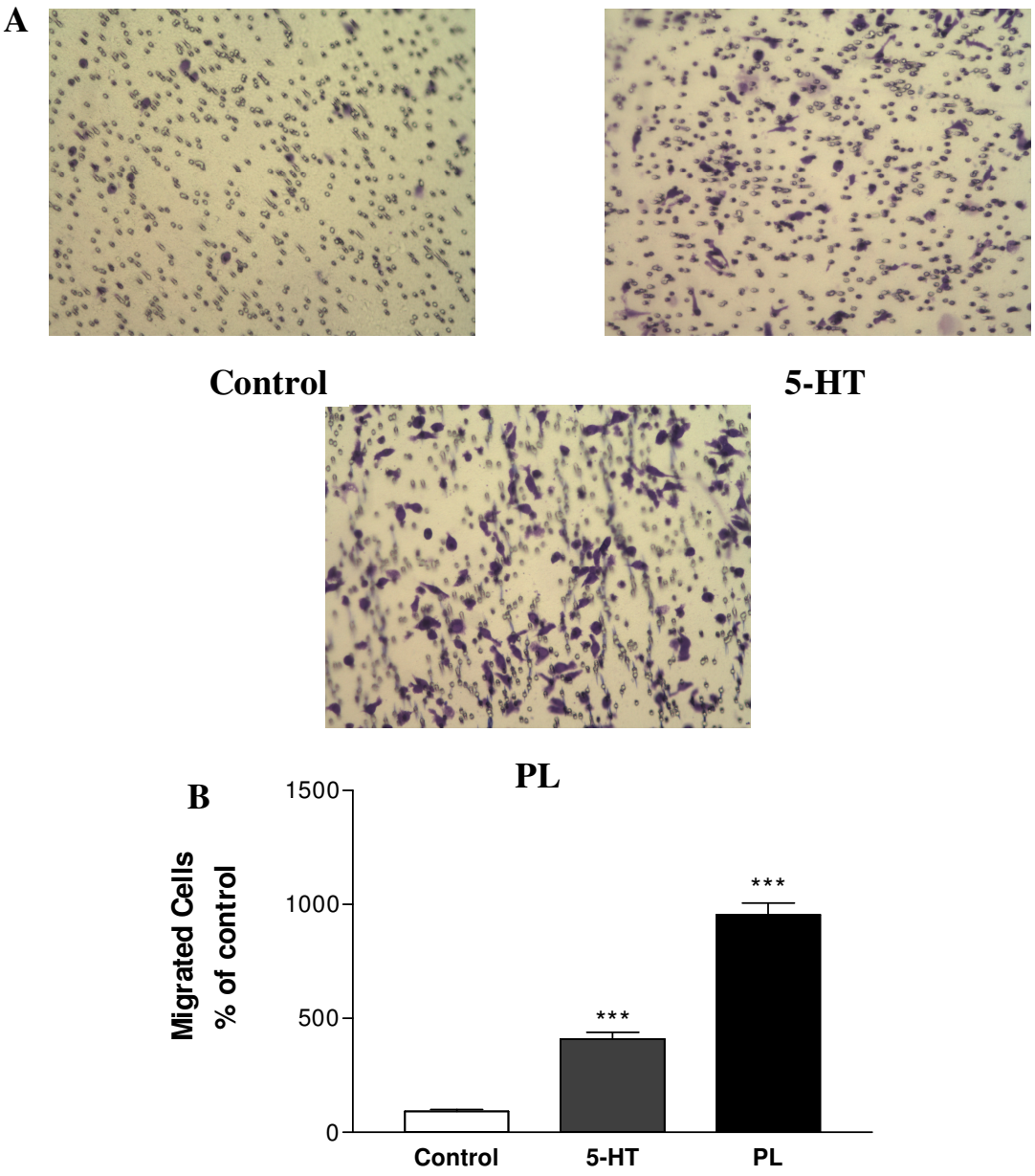


Figure 4.18. Chemotactic response of J774 macrophage cell line to conditioned medium (PL or 5-HT induced) from mouse CFs. (A) The photomicrograph of control and conditioned medium (PL or 5-HT) induced chemotaxis. (B) Bar chart presenting the percentage of transmigrated cells after 4 h following incubation with medium alone or conditioned medium (PL or 5-HT induced). ***P<0.001 conditioned medium from 5-HT or PL treated mouse CFs vs medium from untreated mouse CFs; all results are average of three independent experiment.

5. DISCUSSION

In the present study, it has been shown that platelets and 5-HT play an important role in the functional regulation of CFs. All experiments were carried with either pure neonatal rat CFs or pure adult mice CFs. Hypothesis were based on two different aspects of injured heart; fibrosis and inflammation. In the following section, results related with fibrosis and inflammation will be discussed subsequently.

Before proceeding on effects of PL or 5-HT on CFs, expression of six 5-HT receptor subtypes was demonstrated in the neonatal rat CFs; 5-HT_{1B}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_4. Accordingly appropriate inhibitors were used throughout the study.

Phenotypic conversion into myofibroblasts and recruitment of fibroblasts at sites of heart injury is necessary for normal healing and the development of fibrosis (87, 195). These events mainly result from the migration and differentiation of resident fibroblasts in response to secreted profibrotic cytokines and growth factors. Previous studies supplied indirect evidence suggesting that some cytokines expressed in platelets regulate transdifferentiation and migration of fibroblasts (196). In this study it was shown for the first time that PL stimulated both CF transdifferentiation into myofibroblasts and their migration. Indeed, it was also found that α-SMA expression, used as a marker of myofibroblast differentiation, was stimulated by PL. This phenomenon was associated with an increase in CF migration. Additional experiments allowed identifying 5-HT as one of the major factors regulating the effects of platelets on CFs. This conclusion was supported by the demonstration that stimulation of α-SMA expression and migration of CFs was mimicked by 5-HT. In addition, it was shown that these effects were mediated by the 5-HT_{2A} receptor subtype. Indeed, the 5-HT_{2A} receptor agonist DOI induced an over expression of α-SMA and the 5-HT_{2A} receptor antagonist, ketanserin prevented PL and 5-HT migration of CFs. Interestingly, it was observed that ketanserin totally reversed the effect of 5-HT but partially prevented PL induced effect on fibroblast migration, indicating that other platelet derived factors were chemotractive for CFs. The mechanism by which 5-HT and platelet derived 5-HT regulated fibroblast migration was not further investigated and may involve cytokines rapidly released by the cells

after 5-HT stimulation. Indeed, recent reports by Mitchell et al. showed that two cytokines rapidly released after 5-HT stimulation by mouse CFs (197), IL-1β and TNF-α stimulated the migration of CFs (198).

TGF-β1 is a key mediator of transdifferentiation and migration of fibroblasts. In this study it was shown that both 5-HT and PL up-regulated TGF-β1 mRNA expression and secretion measured by real time RT-PCR and Elisa respectively. Interestingly, ketanserin almost completely prevented PL induction of TGF-β1 mRNA expression and secretion by CFs. These data indicated that, in this experimental conditions, platelet derived 5-HT is the major contributor of platelet increase in TGF-β1 expression and this effect is mediated by the stimulation of 5-HT_{2A} receptors. It is conceivable that the up-regulation of TGF-β1 may participate in migration and differentiation of CFs induced by PL and 5-HT.

The ultimate structural hallmark of reparative and reactive fibrosis is the significant increase in ECM deposit in scar tissue and between myocytes respectively. However, before the final phase of ECM deposit and synthesis inside the infarcted areas, the early phase of remodeling is characterized by an activation of MMPs and a net decrease in ECM (199). Besides, in different chronic cardiopathies, fibrosis is not only associated with ECM accumulation but also with an alteration in the ECM organization. Indeed, in such pathological conditions, regular ECM is replaced by a thickened, and poorly organized one (100). The observation that maximal platelet aggregation and 5-HT release occur initially (2-30 min) after reperfusion of the ischemic heart, suggests a potential role of platelets and 5-HT in the early ventricular remodeling (200, 201). It was observed that PL and 5-HT induced an up-regulation of MMP-3 and MMP-13 expression, suggesting that platelets may participate in matrix degradation. Interestingly, both PL and 5-HT effects were prevented by ketanserin indicating that 5-HT is necessary for PL effect. Moreover, increase in MMPs mRNA by PL was higher than that observed with 5-HT, suggesting that other factors released by platelets may amplify 5-HT effect. Taken together, these data indicate that platelets and platelet derived 5-HT may participate into fibrosis process by enhancing fibroblast activation in terms of migration, proliferation and differentiation. By increasing at the same time MMPs

and TGF-β1 expression, platelets and 5-HT may play an important role in matrix turnover.

Another aspect that this thesis focused on was the potential proinflammatory effect of PL and 5-HT. Platelet activation and inflammation are two features observed in heart failure. Inflammatory response is the first phase in the process of cardiac repair following myocardial infarction can be divided into three overlapping phases: the inflammatory phase, the proliferative phase and the maturation phase (202).

During the inflammatory phase, vascular permeability agents such as vascular endothelial growth factor (VEGF), thrombin, or histamine are generated and released from platelets, mast cells, monocytes, or macrophages in the microcirculation when they are activated in response to an injury. They are also produced and released by activated vascular and stroma cells (203). Consequently, vascular permeability increases.

During injury the disruption of the vascular barrier often results in the recruitment of platelets and leukocytes to the injured site of heart (203). As the blood components spill into the site of injury, the platelets come into contact with exposed collagen and other elements of the ECM or even with cardiac cells in the site of injury. This contact triggers the platelets to release the contents of its specific secretory granules (dense and α-granules). Naturally, like other contents of dense granules; 5-HT is also released into the local microenvironment when platelets are activated during injury (204). The main concern of this part of the thesis is to investigate the effect of this platelet released 5-HT on CFs in the aspect of inflammatory phase.

To determine the potential proinflammatory effect of PL and 5-HT on CFs, initially mRNA and protein levels of one of the proinflammatory cytokines; IL-6 were evaluated. According to IL-6 ELISA results; IL-6 secretion from CFs in response to both PL and 5-HT stimulations were found to be dose-dependent. In order to further determine the roles of PL and 5-HT on IL-6 production, their potential effects on the regulation of IL-6 mRNA expression in neonatal rat CFs following PL and 5-HT stimulations were analysed. It was observed that the level of IL-6 mRNA expression was significantly changed by PL and 5-HT at the

concentrations used in these experiments. This suggests that the regulatory effects of PL and 5-HT on the production of IL-6 occur at the transcriptional level since the IL-6 mRNA content in CFs was significantly altered.

Previous studies have documented the involvement of 5-HT in diverse inflammatory responses despite its controversial effects on cytokine release. A clear inverse relationship between 5-HT levels and inflammation has been reported in depressed patients (205). However, paradoxical effects of 5-HT in inflammation with biphasic responses have been reported at the central and peripheral systems (206) and in blood cells (207). The presence of multiple subtypes of 5-HT receptors, which mediate the inhibitory and stimulatory effects, may be responsible for its complex activities in inflammation. Therefore, determination of the type of involved 5-HT receptor is also important.

Results suggest that the modulatory effect of 5-HT on IL-6 production occurs through the activation of the 5-HT_{2A} receptor subtype. Indeed, ketanserin, a 5-HT_{2A} antagonist, inhibited the stimulatory effect of 5-HT on IL-6 production in CFs. The inhibitory effect of ketanserin observed in PL induced IL-6 production is partial; consequently it was suggested that there are some other factors released by platelets possibly involved in this IL-6 production by CFs. Moreover, although the effect of ketanserin on 5-HT induced IL-6 secretion is significant both in protein level and mRNA level, effect of inhibition in protein level is approximately fifty percent. This result suggests possible involvement of other receptor subtypes at the posttranscriptional level.

A significant correlation between elevated levels of TNF-α and elevated levels of IL-6 has been previously identified in severe chronic HF (154). It has been already known that mammalian myocardial cells are able to produce another proinflammatory cytokine; TNF-α after stimuli such as endotoxin, hypoxia or increased mechanical stress (139). It was demonstrated that TNF-α is released early after myocardial ischemia (208). Furthermore, as already mentioned in the previous part of this thesis discussion maximal platelet aggregation and 5-HT release occur initially (2-30 min) after reperfusion of the ischemic heart. Based on these reports, effects of PL and 5-HT on TNF-α secretion from CFs were investigated.

Time course analysis data on TNF-α protein secretion in response to PL and 5-HT induction indicated that TNF-α protein level in conditioned medium was evident as early as 2 h and peaked at 4 h after stimulation. As expected ketanserin totally inhibited 5-HT or PL induced TNF-α secretion by CFs. Based on these results, it could be assumed that the secretion of TNF-α is an early step response of CFs to PL or 5-HT stimulation via 5-HT_{2A} receptors. Similarly, Yamada et al. (209) reported that TNF-α may play an important role in very early stages of the immune system in murine model of viral myocarditis.

Clinical studies have also shown that circulating M-CSF and GM-CSF, were elevated in patients with acute myocardial infarction according to the severity of the disease, suggesting that these molecules are important mediators in ischemia-induced myocardial injury and reflect important pathogenic mechanisms in post-infarction repair process (210, 211). Up-regulation of these factors into infracted myocardium early during the reperfusion regulates the local infiltration of mononuclear cells and their differentiation into macrophages (178, 212). Furthermore, there is increasing evidence that GM-CSF may play an important pathogenic role in post-infarction LV remodeling and chronic HF (162, 210, 213). Parissis et al. (210) reported that one month after the onset of acute myocardial infarction, enhanced GM-CSF activation is present in patients with severe LV dysfunction, and is significantly correlated with increased LV dimensions. On the basis of this observation, it is tempting to hypothesize that enhanced GM-CSF may be an important factor in mediating the differentiation of mononuclear leukocytes to activated macrophages and their infiltration into the injured and ischemic myocardium during the post-infarction period; GM-CSF may regulate the production of other pro-inflammatory cytokines (214).

Our data indicated that PL and 5-HT stimulations induced GM-CSF expression in CFs. Increased GM-CSF mRNA expression and protein level in conditioned medium were evident 2h and 4h respectively after stimulation. These *in vitro* studies revealed that CFs are also an important source of GM-CSF. Especially strong and significant increase both in mRNA expression and protein level observed upon PL stimulation is considerable and total inhibition of this increase with ketanserin is challenging. This suggests PL effect is mediated by 5-HT and 5-HT_{2A}

receptors. This result is confirmed by observing total inhibition in 5-HT effect by using ketanserin.

MCP-1 has been found to induce monocyte infiltration in murine cardiac muscle with development of a number of pathological changes characteristic of chronic HF, e.g. cardiac hypertrophy, ventricular dilatation and depressed contractile function (171). MCP-1 has also been implicated in the pathogenesis of myocarditis, acute myocardial infarction and ischemia-induced myocardial damage (171, 173). One of the earlier reports suggests that the cardiomyocytes themselves may directly contribute to such an infiltration of inflammatory cells within the myocardium by secreting MCP-1 (188). According to the findings above it was hypothesized that CFs may also have a role in MCP-1 secretion. The present data showed that CFs are capable of secreting MCP-1 in this experimental condition where PL and 5-HT is used as stimulators. Since the MCP-1 protein secretion was observed 12 h after the stimulation, this chemokine secretion can be considered as late response of CFs compared to release of other cytokines. The 5-HT_{2A} receptor involvement was also verified by using ketanserin.

The attraction of leukocytes to tissue is essential for inflammation and the host response to infection. This process is partly controlled by chemokines, which are chemotactic cytokines (163, 215). Recently, chemokines have been implicated in the pathogenesis of several inflammatory disorders such as asthma and rheumatoid arthritis (216, 217), and there is also growing evidence suggesting that these cytokines may play a pathogenic role in several cardiovascular disorders (187). The involvement of chemokines in the pathogenesis of atherosclerosis has been widely investigated, and it seems that monocyte recruitment to the site of injury is mainly regulated by chemokines, particularly MCP-1 (218). On the basis of these reports, in order to test apparent, cumulative effect of released cytokines and especially the chemokine; MCP-1 on monocyte recruitment. Transwell chemotaxis assay was performed by using J774 macrophage like cell line. Conditioned mediums collected from PL and 5-HT stimulated CFs, obviously induced chemotaxis of J774 cells.

We should keep in mind that the cytokine and chemokine concentrations in myocardial tissue, which is exposed to various stimuli arose from leukocytes and cardiomyocytes, may be higher than the levels in our *in vitro* systems for isolated

CFs where cytokines and chemokines will be diluted in culture medium. Therefore to conclude about the exact contributions of PL and 5-HT stimulation on cardiac tissue *in vivo* studies should be performed.

These data provided novel insights into the effect of 5-HT on CFs and also put emphasis on the relevant role of 5-HT in platelet mediated effects. They also showed that most of these effects was observed via 5-HT_{2A} receptors. However, further *in vivo* studies will be needed to determine the exact participation of platelets and platelet-derived 5-HT in the initiation of critical events leading to inflammation and fibrosis in the injured and failing myocardium.

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