MECHANISM OF MATRIX METALLOPROTEINASE-14 (MMP-14) REGULATION DURING ATHEROSCLEROSIS

A Dissertation

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

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DECEMBER 2006

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

MECHANISM OF MATRIX METALLOPROTEINASE-14 (MMP-14)

REGULATION DURING ATHEROSCLEROSIS

presented by Arvind Shakya

a candidate for the degree of Doctor of Philosophy

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ABSTRACT

Atherosclerosis is the most common cause of morbidity and mortality in the developed countries including the US. Atherosclerosis is characterized by the narrowing of the arterial lumen due to formation of the plaque within the intimal layer of the arterial wall resulting in the impaired flow of blood to the target organs. Most severe consequences of atherosclerosis arise due to the rupture of the atherosclerotic plaque, which is the cause of stroke, myocardial infarction, etc. Rupture of the plaque occurs due to the thinning of the fibrous cap of the plaque. Increased expression of matrix metalloproteinases (MMPs), including MMP-14, the enzymes which can cause degradation of many components of the extracellular matrix, is one of the causes leading to thinning and rupture of the fibrous cap of the atherosclerotic plaque. Increased expression of MMP-14 is due to its transcriptional upregulation during inflammatory conditions such as atherosclerosis. In a recent study, we have shown that serum amyloid A-activating factor-1 (SAF-1) is involved in the increased expression of MMP-14 in the atherosclerotic lesion area. This discovery is a major breakthrough in understanding the regulation of MMP-14 gene expression during inflammatory conditions. However, we still do not have a clear understanding of the regulatory processes that are involved in the expression of MMP-14

during physiological and pathological conditions. Therefore, to gain a better understanding of the molecular events involved in the regulation of MMP-14 during atherosclerosis, this study was designed to understand the induction mechanism of MMP-14 during atherosclerosis and to assess the role of other regulatory transcription factors involved. One such transcription factor, octamer binding protein-1 (Oct-1) may be involved in the regulation of MMP-14 due to close proximity of Oct-1 binding site to the SAF-1 binding site in the MMP-14 promoter.

Towards this goal, the first aim of this study was to assess the expression pattern of these two transcription factors in the unaffected and atherosclerotic affected arteries which are most likely the stage IV plaque according to classification given by Stary et al. (Stary et al., 1995a; Stary et al., 1995b). Using immunohistochemical analysis, we here show that the expression of MMP-14 and SAF-1 increases several folds in the atherosclerotic lesion area, but the expression of Oct-1 is very low or at the basal level. By immunohistochemical analysis we have also shown that the macrophage cells present in the atherosclerotic plaque area were the major cell types expressing MMP-14 and SAF-1. We further investigated the relative importance of these transcription factors in the transcriptional regulation of MMP-14 gene expression. Transient transfection assays using wild type MMP-14 promoter indicated that SAF-1 increases the expression of MMP-14 gene in a dose dependent manner. We also showed that ectopic expression of Oct-1 along with SAF-1, decreases the SAF-1 mediated expression of MMP-14, suggesting the antagonistic role of Oct-1 in MMP-14 gene expression. Our next aim was to study the interaction of SAF-1 and Oct-1 with the MMP-14 promoter. Electrophoretic

mobility shift assays (EMSA) showed that both these proteins were able to interact with the MMP-14 promoter. SAF-1 interacts very strongly with the MMP-14 promoter, but Oct-1 interacts very faintly. Using chromatin immunoprecipitation (ChIP), we show that these two transcription factors can interact with the MMP-14 promoter under *in vivo* conditions. Our next aim was to assess if there is any physical interaction between these two transcription factors. By co-immunoprecipitation (co-IP) we show that SAF-1 and Oct-1 can interact with each other, under both *in vitro* and *in vivo* conditions. By transient transfection assays using yeast GAL4 CAT reporter plasmid, we show that Oct-1 is involved in inhibiting the transactivation potential of SAF-1.

Taken together, these results demonstrate that there is physical interaction between SAF-1 and Oct-1, and Oct-1 may be involved in controlling SAF-1 mediated expression of MMP-14 gene. The crucial finding that Oct-1 is a downreguator of MMP-14 gene, may be an important target for developing new therapeutic measures for the treatment of atherosclerosis.

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LIST OF ABBREVIATIONS

AHA	American heart association
AP-1	Activator protein-1
CAT	Chloramphenicol acetyl transferase
CHD	Coronary heart disease
ChIP	Chromatin immunoprecipitation
CVD	Cardiovascular disease
EMSA	Electrophoretic mobility shift assay
HDL	High density lipoprotein
ICAM-1	Intercellular cell adhesion molecule-1
IL-1	Interleukin-1
LDL	Low density lipoprotein
LP-A	Lipoprotein –A
MAPK	Mitogen activated protein kinase
mm-LDL	minimally modified low density lipoprotein
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-kappa B
NO	Nitric oxide
Oct-1	Octamer binding protein-1
ox-LDL	Oxidized low density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet derived growth factor
PKA	Protein kinase A

PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear
PPAR	Peroxisome proliferator-activated receptor
SAA	Serum amyloid A
SAF-1	Serum amyloid A activating factor-1
Sp-1	Specific protein-1
SRA	Scavenger receptor-A
TGF-β	Tissue growth factor-beta
TNFα	Tumor necrosis factor-alpha
VCAM-1	Vascular cell adhesion molecule-1
VSMC	Vascular smooth muscle cell

CHAPTER 1

INTRODUCTION

Atherosclerosis is a chronic inflammatory condition involving the arteries. It is characterized by the occlusion of arteries due to formation of atherosclerotic plaque. The two important determinants in the progression of atherosclerosis are inflammation and oxidative stress (Alexander, 1995; Libby, 2002). The inflammatory process is critically involved in the progression and major complications of atherosclerosis such as stroke and myocardial infarction, which are most important causes of morbidity and mortality due to the rupture of the plaque. The plaque rupture occurs as a result of destruction of extracellular matrix by the action of proteases including matrix metalloproteinases (MMPs) (Hiller et al., 2000). MMPs belong to a family of 28 endopeptidases that are structurally related zinc and calcium dependent enzymes and they are capable of degrading all components of extracellular matrix with varying degree of specificity (Nagase and Woessner, 1999). MMP-14 is an important member of this family of proteases. It is believed to be an important protease involved in the disruption of the plaque. We have also shown the co-localization of SAF-1 and MMP-14 in the atherosclerotic plaque and increased responsiveness of the MMP-14 promoter to increased expression of SAF-1 (Ray et al., 2004). MMP-14 is a cell-associated membrane bound MMP (Sato et al., 1994) and is involved in the activation of MMP-2 (Strongin et al., 1995) and MMP-13 (Hernandez-Barrantes et al., 2002). The increased expression of MMP-14 in the inflammatory conditions is due to its transcriptional upregulation (Dahi et al., 2005; Ray et al., 2004). The promoter of MMP-14 contains

binding sites for several transcription factors such as Sp-1, Egr-1, AP-4 and NF-κB (Lohi et al., 2000).

One of the major risk factors contributed to the pathogenesis of atherosclerosis is hyperlipidemia (Boullier et al., 2001; Libby, 2002). High circulating levels of lipids leads to formation of lipid engorged macrophages known as foam cells. These foam cells are involved in the pathogenesis of atherosclerosis by producing various inflammatory cytokines leading to altered gene expression in the cells involving the plaque i.e., foam cells, vascular smooth muscle cells and endothelial cells (Fowler et al., 1985; Itabe and Takano, 2000; Kruth, 2001).

In a recent study it has been shown that an inflammation responsive transcription factor SAF-1 (serum amyloid A activation factor-1) is also involved in the transcriptional regulation of MMP-14 gene during atherosclerosis (Ray et al., 2004). SAF-1 is a member of zinc finger family of transcription factors. SAF-1 was originally identified as a regulator of serum amyloid A gene that is expressed at a very high level under inflammatory conditions (Ray and Ray, 1998). The DNA binding activity of SAF-1 is induced by multiple signaling pathways including PKC (Ray et al., 2000), PKA (Ray et al., 2002a) and MAPK (Ray et al., 2002b) mediated phosphorylation. SAF-1 has been shown to interact with other transcription factors such as AP-1 and Sp-1 to regulate the expression of MMP-1, MMP-9 and SAA (Ray et al., 2005; Ray et al., 2003a; Ray et al., 1999a), which are activated by the inflammatory cytokines such as IL-1.

The inflammatory cytokines regulate various cellular processes by activating cytoplasmic signal transduction pathways by converging the information from various extracellular stimuli to the gene promoters via the transcription factors such as AP-1 and NF-κB. These transcription factors are activated via different pathways and can function as gene expression enhancers and repressors (Figure 1) and regulate the gene transcription by binding to their respective binding site on the gene promoter. Transcriptional cross-regulation by these transcription factors also depend on the direct protein-protein interaction among themselves (Wang et al., 2004). Such orchestrated actions of transcription factors are quite common in the transcription of many, if not all, genes. Thus, it is possible that different transcription factors may function in concert to regulate MMP-14 gene expression. In the atherosclerotic plaque, we have earlier observed the involvement of SAF-1, which may also recruit other transcription factors for optimum expression of MMP-14. Indeed, the MMP-14 promoter contains a binding site for a ubiquitously expressed transcription factor Oct-1, just proximal to the SAF-1 binding site. The presence of Oct-1 like binding site suggests a possible role of Oct-1 in the regulation of MMP-14 gene transcription.

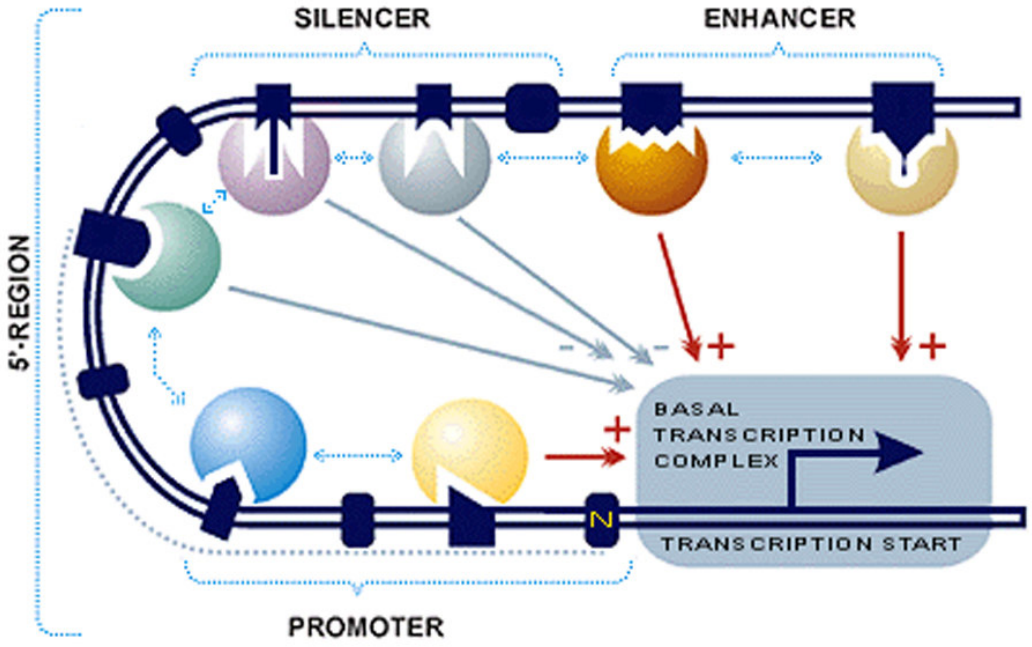

Figure 1: Transcriptional machinery of the eukaryotic genes. The model depicting the transcriptional machinery of the eukaryotic gene is shown here. The eukaryotic gene expression is regulated by many transcription factors, according to the requirements of the cell. The promoter region contains several binding sites for the transcription factors that interact with the basal transcription complex to drive the transcription. These transcription factors can act as either transcriptional enhancers or repressors.

The expression of the MMPs, including MMP-14 is regulated primarily at the level of transcription (Dahi et al., 2005). Though several studies have been conducted to elucidate the mechanism by which MMP-14 is regulated, relatively less information is available regarding the transcriptional control of MMP-14 expression in the atherosclerotic plaque. MMP-14 promoter contains consensus binding sites for Sp-1, Egr-1 and NF-κB, but not any AP-1 or AP-2 sites. Transcription factors Sp1 and Egr-1 have been reported to be involved in the transcriptional regulation of MMP-14 gene (Khachigian et al., 1996; Lohi et al., 2000). One study has also reported that MMP-14 gene expression is induced by phorbol 12-myristate 13-acetate (Dimmeler et al., 1996; Lohi et al., 1996), but not through AP-1, raising the possibility of involvement of another PMA responsive transcription factor. One such transcription factor is SAF-1, which is activated by PKC pathway (Ray et al., 2000) and is also induced under inflammatory conditions, and has been recently shown to play an important role in MMP-14 gene expression under the conditions of atherosclerosis. PMA has also been shown to modulate Oct-1 activity in the human B lymphoblastoid (Daudi) cells (Dent et al., 1991), which suggests that Oct-1 and SAF-1 either independently or in combination regulate MMP-14 gene expression.

Understanding of the molecular events that results in increased expression of MMP-14 during chronic inflammatory conditions such as atherosclerosis, may help in designing therapeutic measures for the control of atherosclerosis. Thus, the objective of the present study was to understand the mechanism by which these transcription factors interact to regulate the expression of MMP-14. **We hypothesize that the increase in the MMP-14 expression is due to the increase in the expression of the transcription factors that**

are activated during inflammatory conditions. We also hypothesize that the increased expression of these inflammation responsive transcription factors overcomes the mechanism by which expression of MMP-14 is under control during physiological conditions.

To test our hypothesis, we have designed the following specific aims:

- Determine the expression patterns of the inflammation responsive transcription factor SAF-1 and ubiquitous transcription factor Oct-1 in the atherosclerotic plaque.
- Determine the functional effects of SAF-1 and Oct-1 expression on MMP-14 transcriptional activity.
- Determine the possible interaction of SAF-1 and Oct-1 with the MMP-14 promoter.
- Determine the mechanism by which SAF-1 and Oct-1 regulate the expression of MMP-14 gene.

These studies will provide insights into the possible mechanism of MMP-14 gene expression during inflammatory conditions such as atherosclerosis. These findings may lead to future studies directed towards the development of therapeutic measures for the control of MMP-14 gene expression and thus control the severity of atherosclerosis.

CHAPTER 2

LITERATURE REVIEW

Atherosclerosis is a pathological condition that is responsible for several important adverse vascular events including coronary artery disease, stroke and myocardial infarction. It is responsible for most of the cardiovascular morbidity and mortality in the developed countries of the world. According to the American Heart Association (Felton et al., 1997) atherosclerosis is defined as "the process in which deposits of fatty substances, cholesterol, cellular waste products, calcium and other substances build up in the inner lining of an artery, known as plaque and it usually affects large and medium-sized arteries with some hardening of arteries occurring in the older people."

Major sites affected with atherosclerosis

The vulnerability of the arteries for the development of atherosclerotic lesions varies, some parts being more vulnerable than others. The abdominal aorta is more susceptible to atherosclerosis than the thoracic aorta. In the thoracic aorta, the lesions are predominantly found in the ascending aorta and the aortic arch. In the abdominal segment, the lesions are found between the renal arteries and the iliac bifurcation. Certain medium sized arteries such as coronary arteries and peripheral arteries of the limbs are more severely affected as compared to the others including internal mammary artery and inferior epigastric arteries (Sons et al., 1993).

Epidemiology of atherosclerosis

Cardiovascular diseases are the leading cause of death in US adults since 1900. According to AHA, "nearly 2500 Americans die of CVD each day, an average of 1 death every 35 seconds. CVD claims more lives each year than the next 4 leading causes of death combined, which are cancer, chronic lower respiratory diseases, accidents, and diabetes mellitus." (Thom et al., 2006) (Heart Disease and Stroke Statistics—2006 Update). Atherosclerosis is the most important cause of morbidity and mortality in the US and Europe.

Risk factors

The disease starts developing during late adolescence and early adulthood (Mohler et al., 1991), though the clinical signs are evident only after the age of 45 years. Most important risk factors for atherosclerosis includes age, gender, hypercholesterolemia, hypertriglyceridemia, decreased high density lipoprotein (HDL), diabetes mellitus, arterial hypertension, tobacco use, alcohol use and physical inactivity.

Lipids: Higher levels of cholesterol are related to the development of atherosclerosis. In a landmark study, when more than 300,000 middle-aged men were screened for the Multiple Risk Factor Intervention Trial (MRFIT), higher cholesterol levels led to an increased risk of coronary heart disease (CHD) death (Martin et al., 1986). High density lipoprotein (HDL) cholesterol is the major determinant of cholesterol in plasma and is an important determinant of risk for CHD and myocardial infarction. It has been reported that myocardial infarction is positively related to total cholesterol levels and inversely to

HDL cholesterol level (Abbott et al., 1988). Elevated levels of low density lipoproteins (LDL) are one of the major risk factors for the development of atherosclerosis. The smaller, denser LDL particles have been associated with greater risk (Gardner et al., 1996). Lipoprotein A (LP-A), a moiety of LDL is an important determinant of atherosclerosis. Levels of LP-A have been reported to be higher in Africans and African-Americans than in Whites (Gidding et al., 1996).

Hypertension: The non-Hispanic blacks have a higher rate of hypertension compared to Whites and Hispanics. Moreover, in all ethnic groups, the prevalence of hypertension was higher in men than in women (Thom et al., 2006). The risk of CVD is highly related to blood pressure and levels of systolic pressure are more highly associated with the development of CVD than the levels of diastolic pressure (Kannel, 2000). According to a study conducted in 2002, the thiazide diuretics and angiotensin-converting enzyme (Basbaum and Werb, 1996) inhibitors were effective as the initial therapy to reduce CVD risk (Trial, 2002).

Tobacco use: Cigarette smoking increases the risk of CVD outcomes. Cessation of smoking have been reported to reduce the risk to half 1-2 years after quitting (Ockene et al., 1990). Moreover, passive smoking has been related to an increased risk of CVD than for non-smokers (Steenland, 1992).

Obesity: Obesity contributes to the development of several atherosclerotic risk factors including hypertension, diabetes mellitus, low HDL cholesterol, elevated triglycerides

and elevated levels of inflammatory markers. Obesity accounts for approximately 23% of CHD in men and 15% in women (Wilson et al., 2002).

Diabetes mellitus: The risk of atherosclerosis increases among men with type II diabetes mellitus and metabolic syndrome. Several risk factors such as hypertension, increased triglycerides and decreased HDL cholesterol occur at a higher frequency in people suffering from diabetes mellitus (Lakka et al., 2002).

Inflammation: Inflmmatroy markers such as C-reactive protein are highly related to increased risk of atherosclerosis (Ridker et al., 1998). Blood levels of the amino acid homocystein have been reported to contribute to the elevated risk of atherosclerosis (Bostom et al., 1999).

Layers of arterial wall

The cardiovascular system is composed of four main types of blood vessels. These are arteries, veins, capillaries and sinusoids. Atherosclerosis is the disease which affects the major conduit arteries of the body. The arterial wall is composed of different cell types and the connective tissue. These are arranged in three zones or tunicae: the intima, media and adventitia, which are illustrated in figure 2.

The intima: In normal arteries, the intima consists of an endothelial layer, a subendothelial layer and a basement membrane. The intima is the narrow region bounded on the luminal side by a single continuous layer of endothelial cells and peripherally by

elastic fibers known as the internal elastic lamina. The endothelial cells form a monolayer that lines the entire vascular system. In the subendothelial layer, smooth muscle cells and various components of extracellular matrix are present.

The media: It is mainly formed of smooth muscle cells, connective tissue containing elastic and collagen fibers. The vascular smooth muscle cells are capable of proliferation after injury and certain pathological conditions e.g the vascular smooth muscle cells present in the tunica media are responsible for many functions of the arterial wall, such as vasodilation or constriction, synthesis of various types of collagen, elastin and proteoglycans.

The adventitia: Tunica adventita is a poorly defined layer of connective tissue. It also contains some smooth muscle cells, nerve fibers and fibroblasts.

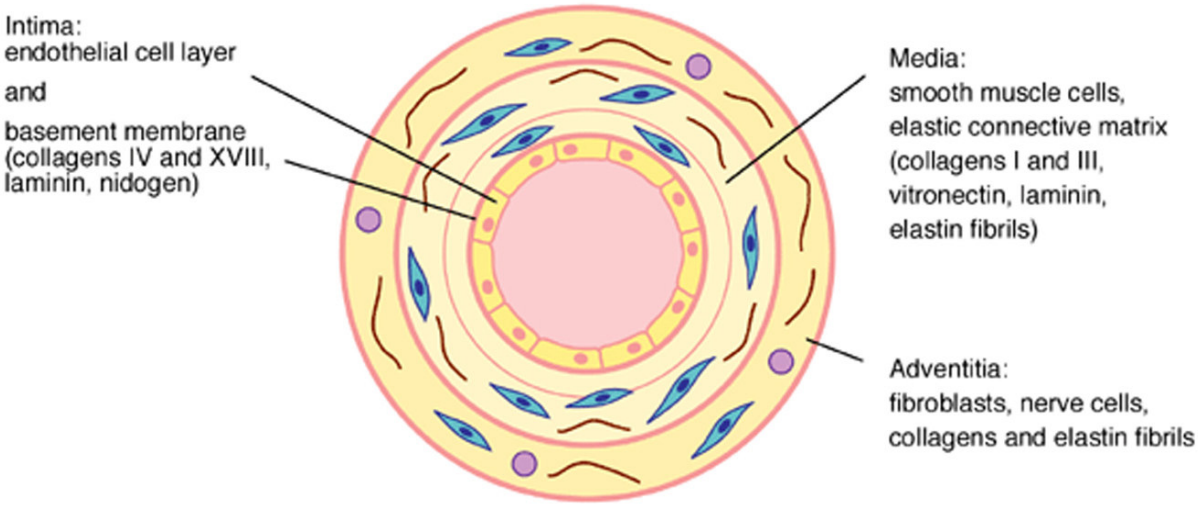

Figure 2: Schematic view of an arterial wall cross-section. The arterial wall has three distinct layers consisting of different cell types. The intima of the artery is mainly composed of the endothelial cells and associated basement membrane. The media consists of vascular smooth muscle cells and the elastic connective tissue matrix. The adventitia layer consists mainly of the fibroblasts and associated connective tissue.

Cells of the atherosclerotic plaque

Nearly all the cells present in the atherosclerotic plaque area contribute to the development of atherosclerosis. A schematic depicting the cellular changes in the arterial wall during atherosclerosis is shown in figure 3. These cells include the resident cells such as vascular smooth muscle cells and the inflammatory cells including marcophages and the mast cells.

Vascular smooth muscle cells: Due to injury to the arterial wall, there is proliferation of VSMCs in response to stimulation by various growth factors such as platelet derived growth factor (PDGF) (Murry et al., 1997) and insulin like growth factors (Bayes-Genis et al., 2000).

Endothelial cells: Endothelial cells forms a monolayer covering the inner surface of the blood vessels. Endothelial cell activation occurs due to the presence of inflammatory cytokines and oxidized forms of LDL (Cines et al., 1998). These activated endothelial cells express various adhesion molecules such as vascular cell adhesion molecule-I (VCAM-I) and intracellular cell-adhesion molecule-I (ICAM-I), which are responsible for the adherence of macrophages to the luminal surface and recruitment in the atherosclerotic plaque (Rubanyi, 1993).

Macrophages: Macrophages and the foam cells, which are lipid-laden macrophages, are one of the most important cell types present in the atherosclerotic plaque. The activated monocytes get attached to the endothelial cells and accumulate in the sub-endothelial

layer of the artery. In addition to the accumulation of monocytes from circulation, these macrophages proliferate in the atherosclerotic plaque under the influence of ox-LDL, macrophage growth factor and colony stimulating factor-1 (Hamilton et al., 1999).

Macrophages express certain ox-LDL receptors on their surface such as scavenger receptor A (SRA), scavenger receptor B1 (SRB1), CD36, CD68 (Li and Glass, 2002). These receptors are involved in the uptake of ox-LDL particles into the macrophages and convert them into foam cells. These macrophages and foam cells produce a variety of biologically active molecules such as prostaglandins (Narumiya et al., 1999), leukotrienes (Samuelsson, 2000), various growth factors, cytokines, oxygen radicals and proteolytic enzymes.

Mast cells: Mast cells secrete certain proteases including chymase, tryptase and carboxypeptidase A. They also produce a variety of inflammatory cytokines such as TNFα, TGF-β, IL-4, IL-5, IL-6 and IL-13 (Metcalfe et al., 1997). The number of mast cells has been reported to be increased in the atherosclerotic plaques (Kaartinen et al., 1996) and are involved in the recruitment of inflammatory cells, foam cell formation and destabilization of the atherosclerotic plaque (Kelley et al., 2000).

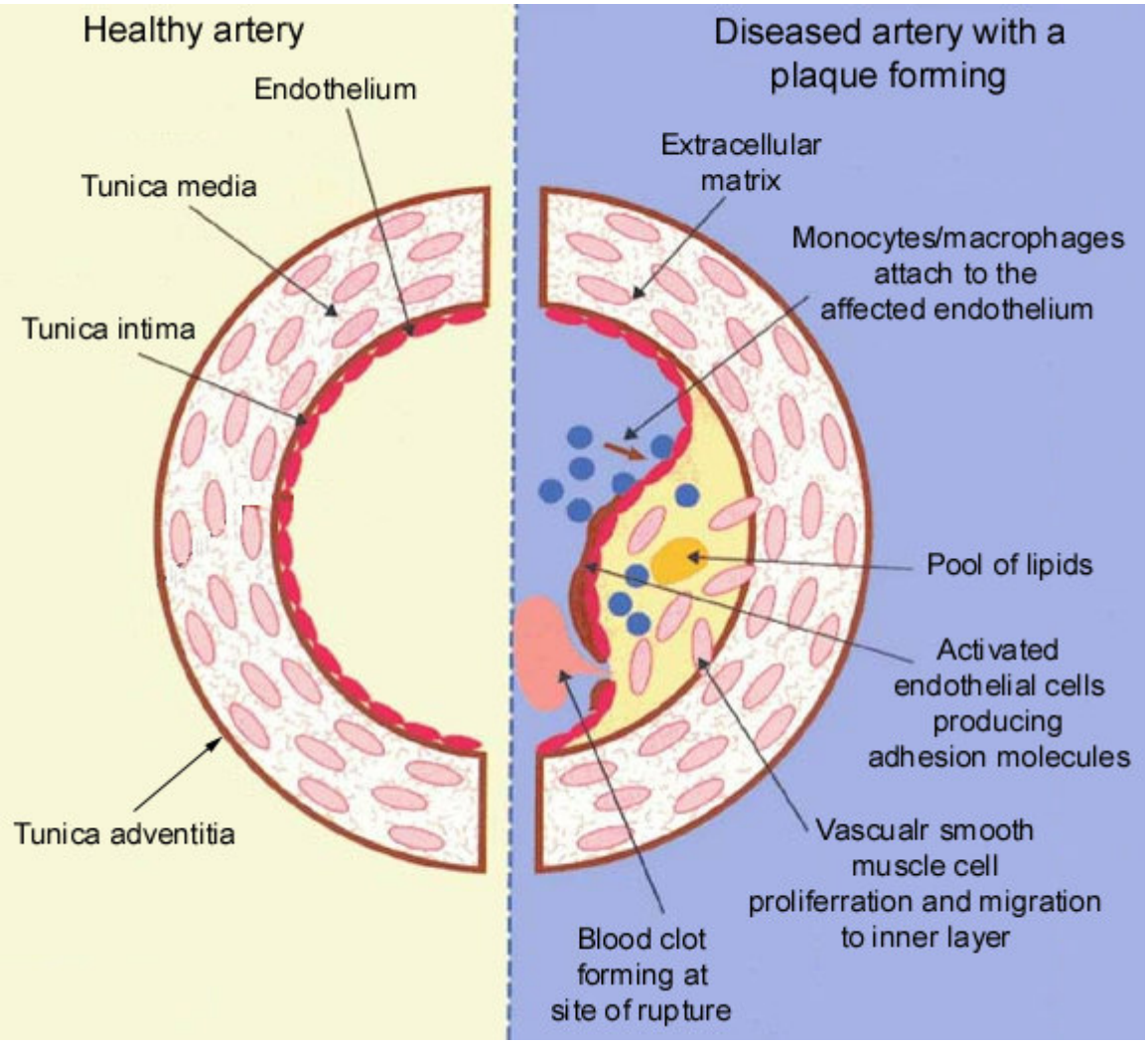

Figure 3: Histological changes in the arterial wall during atherosclerosis. During pathogenesis of atherosclerosis, there is the build up of lipids, foam cells and necrotic debris in the intimal layer of the artery. These events subsequently lead to the rupture of the atherosclerotic plaque at the shoulder region of the plaque and the formation of the blood clots.

Hemodynamics of blood flow

Blood vessels are constantly subjected to mechanical forces in the form of stretch, cyclic mechanical strain due to the pulsatile nature of blood flow and shear stress. Stretch on the blood vessels is due to the blood pressure and the shear stress is due to the friction of blood against the vessel wall.

The blood flow patterns play a very important role in the localization of atherosclerotic plaque. Plaques tend to form in regions of low, disturbed or oscillating blood flow, such as arterial branches and bifurcations, whereas the parts of blood vessels exposed to steady blood flow tend to be lesion free (Malek et al., 1999). The mechanisms by which the vessel wall is protected under physiological conditions is by the shear stress induced production of NO (Dimmeler et al., 1996) and shear stress-induced inhibition of MAP kinases (Yamawaki et al., 2003) . These mechanisms are protective as they are able to attenuate the action of TNF mediated stimulation of vascular cell adhesion molecule (VCAM) expression and possibly prevents the development of atherosclerosis.

Role of inflammation in Atherosclerosis

Inflammation is a homeostatic mechanism that has evolved as a response to cellular injury. Vascular injury such as infarction, trauma or infection initiates a cascade of inflammatory processes. The major cell types involved in inflammation are circulating leukocytes, including monocytes, polymorphonuclear neutrophils (PMN) and B and T lymphocytes. These cells interact with one another and with vascular endothelial cells through various cell surface receptors and inflammatory cytokines.

Atherosclerosis is now considered a chronic inflammatory condition which involves circulating monocytes (which differentiate into macrophages) and T lymphocytes that are recruited to the site of injury.

The endothelial cells mediate adherence and migration of leukocytes from circulation to the site of injury. The activation of these endothelial cells results in the presentation of adhesion molecules on the luminal surface that support leukocyte adhesion (Luscinskas and Gimbrone, 1996). Expression of these molecules occurs both constitutively and inducibly, and is regulated at the level of transcription by NF-κB (De Martin et al., 2000). These adhesion molecules include selectins (E-selectin, P-selectin), integrins and immunoglobulin family members such as ICAM-1 and VCAM-1 (Ruberg et al., 2002). Leukocytes bind to these molecules through specific receptors that mediate rolling, adherence and diapedesis. Endothelial cell activation and the expression of these adhesion molecules occurs under variety of conditions including exposure to inflammatory mediators such as TNFα, IL-1β and oxidative stress (Cines et al., 1998).

Cellular responses to injury

The first atherosclerotic lesion formed is known as fatty streak (Restrepo and Tracy, 1975) formed due to hypercholesterolemia, which has been associated with the progression of atheosclerosis. The later atherosclerotic lesions are characterized by formation of atheroma (lipid core with a dense fibrous cap), reduced luminal diameter and impaired blood flow. The histologic evaluation of the atheroma has provided evidence regarding the involvement of chronic inflammatory cells including

macrophages and T lymphocytes. These inflammatory cells bind to the endothelial cells, due to expression of the adhesion molecules on the surface of activated endothelial cells in response to hyperlipidemia (Cybulsky and Gimbrone, 1991). Along with the abnormal expression of adhesion molecules, other events leading to the progression of atherosclerosis are abnormal expression of NO and dysregulated release of hemostatic substrates such as decreased tissue plasminogen activator (tPA) and increased plasminogen activator inhibitor-I (PAI-I) (Ruberg and Loscalzo, 2002).

Role of lipids in atherosclerosis

Low density lipoprotein (LDL) cholesterol particles diffuse through the endothelial cell membrane and pass through into the subintimal region as depicted in the figure 4. High density lipoprotein (HDL) particles exert their athero-protective action by reverse diffusion of LDL cholesterol back into the circulation. The trapped LDL is subjected to the reduced oxygen environment in the subintimal region and is oxidized to minimally modified LDL (mm-LDL) or oxidized LDL (ox-LDL) (Keaney, 2000). This oxidation of LDL is mediated via a variety of reactions with substances such as metal ions, reactive oxygen species, macrophage oxidation enzymes such as myeloperoxidase and phospholipase A_2 (Libby et al., 2002). This oxidation of LDL is fundamental to the initiation of development of atherosclerosis and inflammatory cascade. These oxidized forms of LDL can induce inflammatory cytokines such as IL-1β and TNFα. These oxidized forms of LDL also stimulate the macrophages to accumulate lipids to form "foam cells" through the scavenger receptor A (SRA) and CD-36 (Libby, 2002).

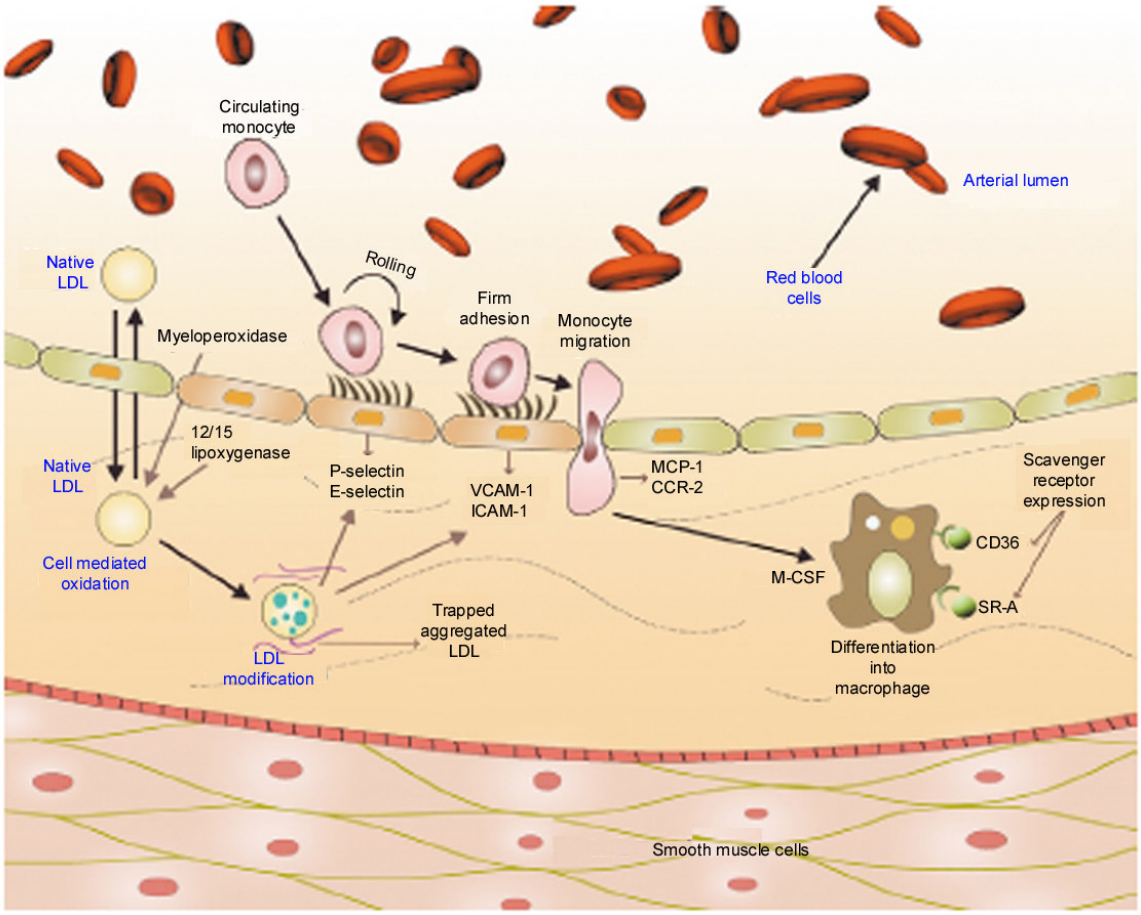

Figure 4: Monocyte recruitment to the subendothelial space. Low-density lipoproteins (LDL) particles migrate into the vessel wall are trapped as a result of oxidative modification. Myeloperoxidase and 12/15 lipoxygenase enzymes have been proposed as being responsible for these modifications including oxidation. Circulating monocytes attach to vascular endothelial cells and roll into the vascular intima. Monocytes that have migrated into the subendothelial space differentiate further into macrophages under the influence of monocyte colony stimulating factor and express receptors that specifically recognize oxidized LDL, scavenger receptor A and CD36 (Kutuk and Basaga, 2003).

Lesions of atherosclerosis

The earliest lesion of atherosclerosis is “fatty streak”. It is formed by the aggregation of lipid-laden macrophages or foam cells and the T-lymphocytes in the intima of the arterial wall. These fatty streaks subsequently lead to the formation of “intermediate lesions”. These intermediate lesions are composed of much more infiltration of foam cells and smooth muscle cells. These types of lesions advance into the formation of fibrous plaques. These fibrous plaques grow in size towards the arterial lumen to obstruct the flow of blood through it. These fibrous plaques are composed of the necrotic core with numerous foam cells and covered by a dense connective tissue cap.

According to the American Heart Association, the atherosclerotic plaque can be graded into six types depending upon its histological features such as constituent cells, composition and architecture (Stary et al., 1995b; Stary et al., 1995c). The Roman numerals I-VI are used to classify the lesions in order of their appearance and progression (Figure 5).

Type I lesions: This is the initial lesion formed of small infiltration of foam cells in the intimal layer of the artery. These lesions are generally present at the sites of adaptive thickening.

Type II lesions: These lesions are commonly known as “fatty streaks” and are composed of foam cells and vascular smooth muscle cells.

Type III lesions: In these types of lesions, there is increased accumulation of lipid droplets and vascular smooth muscle cells. These types of lesions may develop into type IV lesions which are capable of producing clinical disease.

Type IV lesions: The type IV lesions are also known as atheroma and these lesions are characterized by the formation of the "lipid core" and they do not have any increase in the fibrous tissue. This type of lesion is the first lesion which is considered pathologic in nature.

Type V lesions: Type V lesions are the lesions where fibrous tissue starts to form over the plaque and the calcification of the plaque starts. These type of lesions start to occlude the arteries and are considered "clinically relevant".

Type VI lesions: This type of lesion is considered to be mainly responsible for most of the morbidity and mortality associated with atherosclerosis. These type of lesions are "complicated lesions" and are associated with the disruption of the plaque known as the unstable lesions.

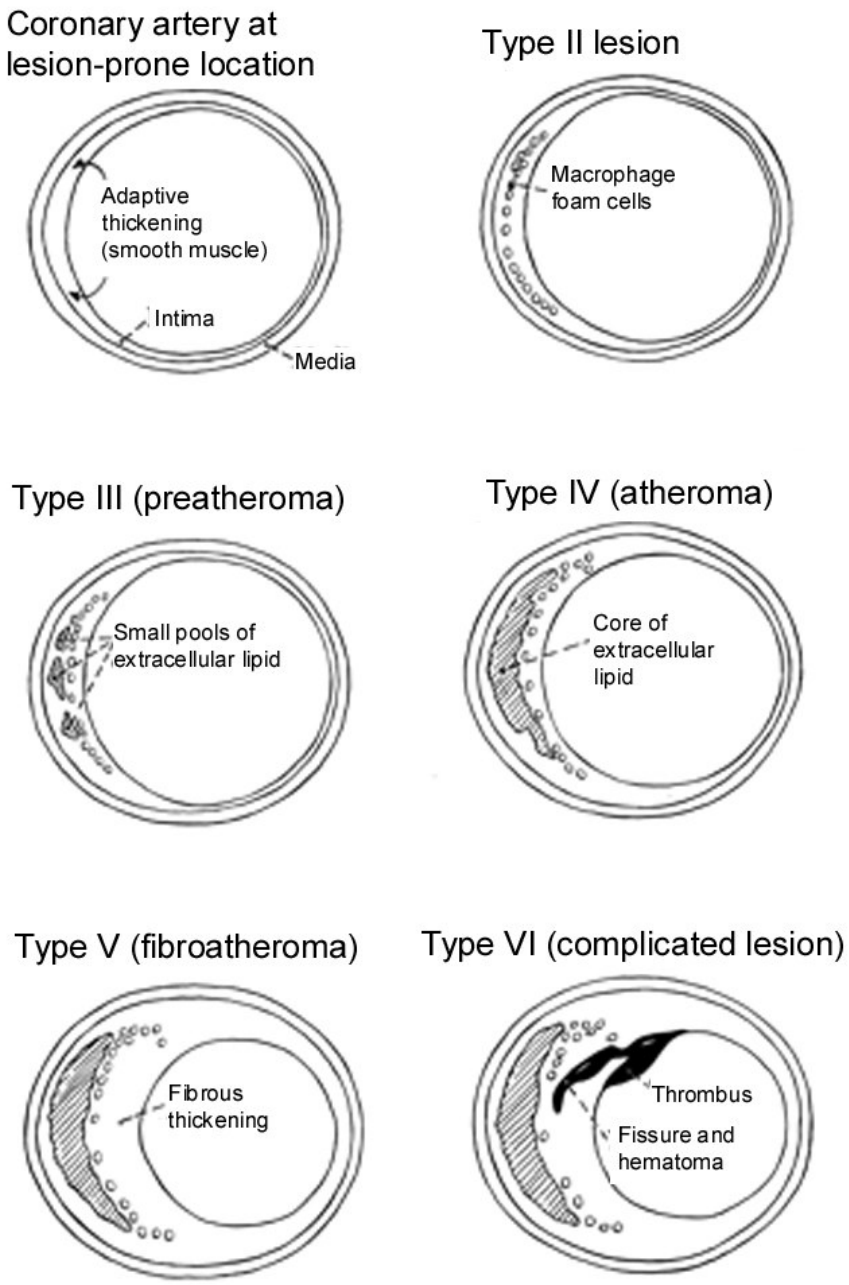

Figure 5: The schematic diagram showing various stages of atherosclerotic lesion development. The atherosclerotic lesions have been divided into six types depending of the severity of the disease. Type I, II and III lesions are clinically silent. Type IV, V and VI lesions are considered to be clinically relevant (Stary et al., 1995c).

Properties of unstable plaque

The unstable plaque is highly vulnerable to rupture leading to complications such as ulceration, hemorrhage, hematoma formation and thrombosis. Thinning and rupture of the wall of the cap of atherosclerotic plaque is the most important cause of severe clinical consequences of atherosclerosis. One of the important reason for the rupture of the atherosclerotic plaque is the lipid content and the distribution of these lipids in the plaque (Felton et al., 1997). The plaques with more lipid content are more prone to the rupture. Among various cell types present in the atherosclerotic plaque, macrophages are the major cells responsible for the plaque disruption as they are the cells which are responsible for uptake of lipoproteins, secretion of growth factors and production of matrix degrading enzymes. This rupture of the unstable plaque disrupts the endothelial cover and the blood comes in direct contact with the highly thrombogenic atheromatous material leading to activation and aggregation of platelets (Ambrose, 1992).

Matrix metalloproteinses

Matrix metalloproteinases (MMPs) are a class of structurally related proteinases that are collectively responsible for the degradation of extracellular matrix proteins (Nagase and Woessner, 1999; Sternlicht and Werb, 2001). These are zinc and calcium dependent enzymes and they have been shown to play an important role in both physiological and pathological processes. They are involved in normal tissue remodeling processes such as wound healing, embryogenesis and angiogenesis. These proteolytic enzymes are also involved in the pathological processes including atherosclerosis, arthritis, cancer

metastasis and tissue ulceration (Woessner, 1998).There are 25 known MMPs that are found in vertebrates, of which 24 are found in humans.

All the MMPs consist of five domains. These are signal peptide, a propeptide, a catalytic domain, a hinge region and a hemopexin-like domain (Visse and Nagase, 2003). All MMPs except MMP-23, are maintained in their zymogen form (Kinoh et al., 1996) by a cysteine switch motif PRCGXPD in the propeptide. The catalytic domain contains the zinc-binding motif HEXGHXXGXXH, and is responsible for the proteolysis (Visse and Nagase, 2003). The catalytic domain of MMPs contains a zinc ion and 2-3 calcium ions that are required for the stability and the enzymic activity of MMPs (Nagase and Woessner, 1999). MMPs have been classified into collagenases, gelatinases, stromelysins, matrilysins and membrane type- matrix metalloproteinases based on substrate specificity, sequence similarity, and domain organization.

Activation and regulation of MMPs

The activity level of all the MMPs is mostly regulated at the level of transcription (Basbaum and Werb, 1996). A majority of the MMPs are secreted as zymogens and require activation in order to be enzymatically functional. These MMPs are activated through the removal of the prodomain through proteolytic cleavage or through urokinase generated plasmin (Carmeliet et al., 1997). Pro-MMP-2 and pro-MMP-13 have also been demonstrated to be activated by membrane-type MMPs (MT-MMPs) (Cowell et al., 1998).

As these MMPs are capable of degrading many of the proteins, there are mechanisms by which their activity is regulated under physiological conditions. These activated MMPs can be inhibited by the plasma proteinase inhibitor α2-macroglobulin and by tissue inhibitors of metalloproteinases (TIMPs).

Among the MMPs, MMP-14 has been reported to be overexpressed by the macrophages and smooth muscle cells which are associated with the unstable atherosclerotic plaque (Rajavashisth et al., 1999b). Inflammatory cytokines such as IL-1α, TNF-α and Ox-LDL increase the expression of MMP-14 in the macrophage and smooth muscle cells (Rajavashisth et al., 1999a; Ray et al., 2004).

Transcriptional regulation of MMPs

Transcription of mammalian genes is the function of interaction between various transcription factors with the proximal promoter region of the gene. Likewise, the transcription of MMPs is also controlled by various transcription factors. All the secreted MMPs, except MMP-2 and MMP-11, have a TATA box and AP-1 binding site. This AP-1 site has been reported to be critical for the transcription of MMP genes (Benbow and Brinckerhoff, 1997), but other transcription factors such as PEA3/Ets, STAT-1 and NF-κB are required for the integration of various signals to increase the transcription of MMPs.

The promoter of membrane type MMPs including MMP-14, does not have a TATA box and several transcription start sites. The Sp-1 site has been reported to be essential for the transcriptional activity (Lohi et al., 2000).

MMP-14

MMP-14 protein is highly expressed under inflammatory conditions and inflammatory cytokines are believed to mediate the upregulation of MMP-14 gene expression. MMP-14 can degrade many extracellular matrix components including collagen type I, II and III, fibronectin, vitronectin, tenascin, laminin-1 and aggrecan (d'Ortho et al., 1997; Ohuchi et al., 1997). MMP-14 is known to be expressed in fibroblasts, smooth muscle cells and endothelial cells under various physiological and pathological conditions. MMP-14 is expressed along with MMP-2 in many tissues, suggesting a functional relationship between these two proteinases (Apte et al., 1997; Kinoh et al., 1996). MMP-14 is expressed at high levels by various cancer cells (Seiki, 1999), during wound healing (Madlener, 1998) and during vascular injury (Jenkins et al., 1998).

MMP-14 gene promoter sequence has the recognition sites for many transcription factors including Sp-1, Egr-1, AP-4, NF-κB and Nkx-2 (Lohi et al., 2000). There is no TATA box and no consensus sequence for CCAAT box (Figure 6). In addition, its promoter does not have AP-1 and AP-2 recognition sites. MMP-14 gene promoter has multiple transcription start sites as determined by the RNAse and S1 nuclease protection assays and by primer extension. There are four major start sites and numerous putative start

sites. The most down stream start site is designated as +1. Other major transcription start sites are located at -12, -28 and -35 position (Lohi et al., 2000).

MMP-14 gene expression is known to be induced by IL-1α, IL-1β and TNF- α (Imai et al., 1997). Also phorbol esters (Birkedal-Hansen, 1993) and Concavalin A (ConA) (Brown et al., 1990; Overall and Sodek, 1990) are known to stimulate the expression of MMP-14. The expression of MMP-14 by macrophages is highly increased during atherosclerosis during the later stages of the disease. Also, the chronic inflammatory condition results in increased number of macrophages in the plaque. Activation of the macrophages leads to the release of cytokines, chemokines and growth factors. Various pro-inflammatory cytokines are known to be involved in the upregulation of these MMPs in response to the stimulus from lipopolysaccharides and oxidized lipids.

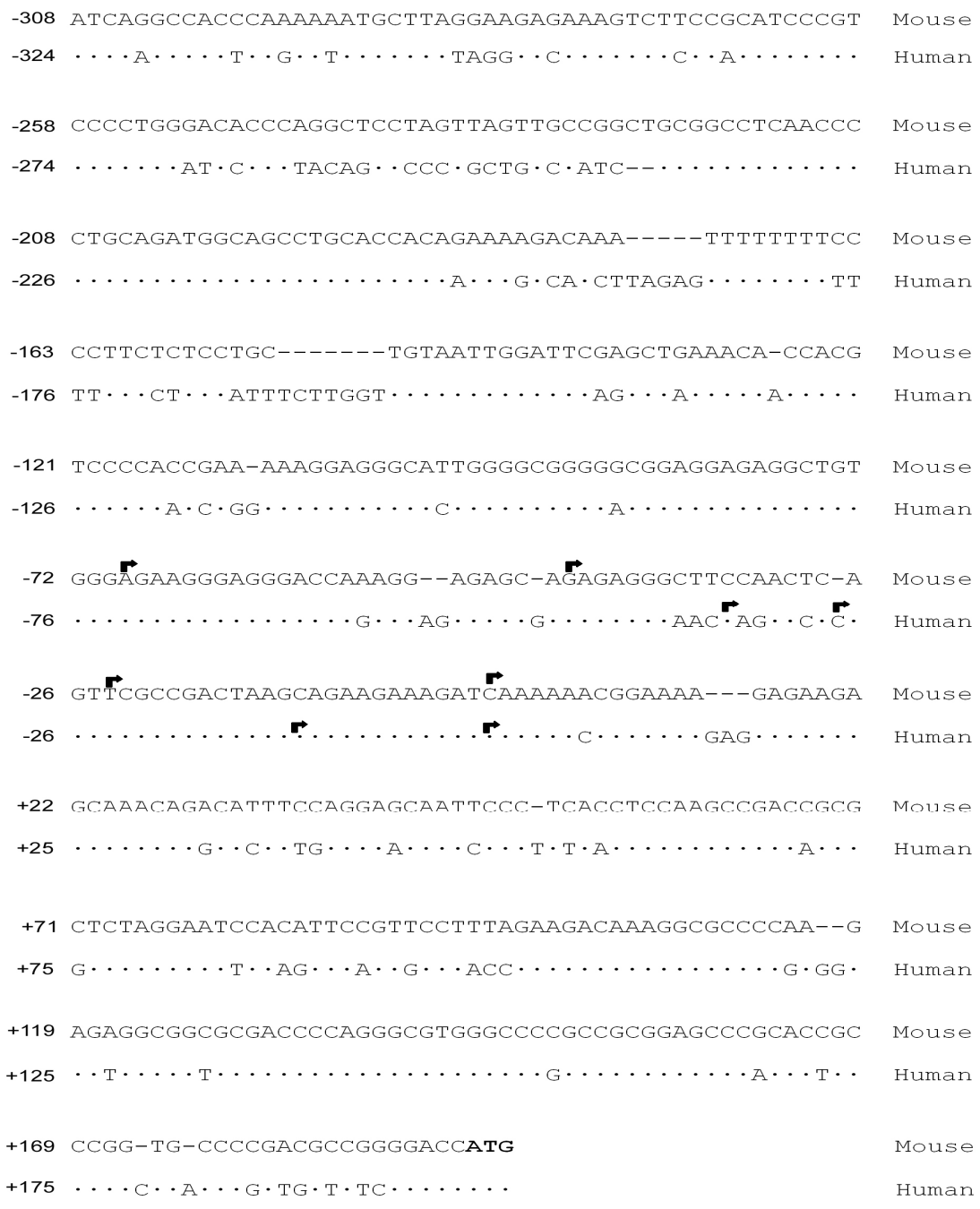

Figure 6: Sequence alignment of mouse and human MMP-14 promoter sequences. The sequence alignment of mouse and human MMP-14 promoter shows considerable homology between mouse and human MMP-14 promoters. The transcription start sites for mouse and human promoters is shown by the bent arrows and the ATG codon is depicted in bold.

Serum Amyloid A Activating Factor-1 (SAF-1)

SAF is a family of inducible transcription factors. The members of this family contain a multiple Cys2-His2-type zinc fingers at the carboxyl terminal end (Ray and Ray, 1998). SAF-1, a member of this family of transcription factor has a high degree of resemblance to MAZ/Pur-1 family of transcription factors (Bossone et al., 1992; Kennedy and Rutter, 1992).

SAF-1 is highly induced under the influence of inflammatory cytokines including IL-1β, IL-6 (Ray et al., 1999a), LPS (Ray and Ray, 1997a) and MM-LDL (Ray et al., 1999b). SAF-1 is also reported to be involved in the activation of SAA gene in the macrophage/monocyte cells in response to LPS (Ray and Ray, 1997c). SAF-1 has been shown to interact synergistically with Sp-1 for the induction of SAA gene in the macrophage/monocyte cells (Ray and Ray, 1997b).

SAF-1 is an inflammation responsive transcription factor. Phosphorylation by protein kinase C is an important event in the activation of SAF-1 (Ray et al., 2000). It has also been reported to be activated in response to various inflammatory cytokines such as IL-1 by cyclic AMP dependent signaling pathway. Cyclic AMP regulates the effects of many transcription factors by activation of protein kinases which phosphorylate various transcription factors (Lalli and Sassone-Corsi, 1994). Activation of SAF-1, mediated by the catalytic (C) subunit of protein kinase A (PKA), is involved in its increased DNA-binding and transactivation potential (Ray et al., 2002a). Phoshorylation by PKA induces a conformational change in SAF-1 protein such that its DNA binding domains are

unmasked (Ray et al., 2003b). Mitogen activated protein (MAP) kinases are a family of protein serine/threonine kinases that are activated in response to various extracellular stimuli such as IL-1and IL-6. MAP kinases are also involved in the phosphorylation of SAF-1 resulting in increased DNA-binding activity and transactivation potential (Ray et al., 2002b).

Octamer binding protein (Oct-1)

Oct-1 is a member of the POU (Pit-1, Oct-1 and Oct-2, Unc-86) domain family of transcription factors which bind to the octamer motif (5´-ATGCAAAT-3´) (Falkner and Zachau, 1984). These transcription factors were initially identified in mammals (Pit-1, Oct-1 and Oct-2) and in *Caenorhabditis elegans* (Herr et al., 1988). The POU domain consists of a bipartite DNA-binding motif, POU specific and POU homeodomain, connected by a linker domain of about 20 amino acids (Sturm and Herr, 1988). Oct-1 is expressed ubiquitously and is present in most cell types during all the stages of cell cycle (Carbon et al., 1987). The mechanism by which Oct-1 regulates gene expression is by interacting with the other transcription factors or co-activators which are activated in response to the specific stimulus (Dailey et al., 1994; Groenen et al., 1992). Oct-1 has been reported to interact with several other transcription factors by helix-turn-helix motif that is responsible for both DNA binding and interacting with other transcription factors for regulating the expression of the target genes (Nakshatri et al., 1995). Such an interaction has been reported to either activate or repress the expression of the target genes. Some of the genes positively regulated by Oct-1 include both house keeping and tissue specific genes such as histone H2B (Fletcher et al., 1987), small nuclear RNAs

(Murphy et al., 1992) and immunoglobulin genes (Sturm et al., 1988). The genes which are downregulated by Oct-1 such as von Willebrand factor (Schwachtgen et al., 1998) and prolactin gene (Subramaniam et al., 1998).

CHAPTER 3

DETERMINATION OF THE EXPRESSION PATTERNS OF MMP-14 AND INFLAMMATION RESPONSIVE TRANSCRIPTION FACTOR SAF-1. ROLE OF SAF-1 IN THE ATHEROSCLEROTIC PLAQUE

Abstract

The degradation of the fibrous cap of the unstable plaques is considered to be the most threatening event in the pathogenesis of atherosclerosis. Several proteases are believed to be involved in the process of degradation of the extracellular matrix components ultimately leading to the rupture of the atherosclerotic plaque. Among these, members of the matrix metalloproteinase (MMP) family are most important enzymes. One of the most important members of this family is MMP-14 that is overexpressed in the atherosclerotic plaques. It is unique as it is able to cause focal degradation of extracellular matrix and due to its ability to activate other matrix metalloproteinases. The increase in the MMP-14 expression is due to its transcriptional induction. The promoter of MMP-14 gene contains binding sites for several transcription factors including Sp-1 and PEA3. Examination of the proximal promoter region of MMP-14 gene for the presence of DNA sequences that may represent binding sites for known transcription factors revealed that it contains an element for the possible binding of ubiquitous transcription factor, Octamer binding protein-1 (Oct-1). We have recently reported that an inflammation responsive transcription factor SAF-1 is expressed at a very high level in the atherosclerotic plaques and the proximal promoter region contains a binding site for this transcription factor. To

address what effect these two transcription factors may have on MMP-14 gene expression, in this study we have analysed the expression of SAF-1 and Oct-1 with relation to the expression of MMP-14 in the atherosclerotic plaques by immunohistochemical analysis. The results of this study indicate that the cells of the atherosclerotic plaque express MMP-14 and SAF-1 at a very high level. Relative to SAF-1, expression of Oct-1 is very limited. The results also indicate that these proteins are mostly present in macrophage cells in the atherosclerotic lesions.

Introduction

The role of matrix metalloproteinases such as MMP-14 has been well established now in the process of degradation of the fibrous cap of the atherosclerotic plaque. MMP-14 is responsible for the degradation of extracellular matrix components including collagen type I, II and III, gelatin, fibrin, fibronectin, laminin-1 and 5, tenascin, aggrecan etc (Hernandez-Barrantes et al., 2002; Toth et al., 2003). MMP-14 has also been reported to be involved in the activation of other MMPs such as MMP-2 (Strongin et al., 1995) and MMP-13 (Hernandez-Barrantes et al., 2002). Under physiological conditions, the expression of MMP-14 is very low and is balanced by its natural inhibitor called tissue inhibitor of metalloproteinase-2 (TIMP-2). However, under pathological conditions such as atherosclerosis, the balance between MMP-14 and TIMP-2 is disturbed as a result of increased expression of MMP-14 due to its increased transcription. Several studies have shown that there is increased expression of MMP-14 in the atherosclerotic plaques. We have recently reported by immunohistochemical analysis of the atherosclerotic plaque

that the level of MMP-14 is highly increased in the atherosclerotic plaque region of the affected arteries as compared to the non-affected regions of the same artery which showed almost no staining for MMP-14 (Ray et al., 2004).

The increase in MMP-14 expression is mainly due to its transcriptional induction. It has been reported that inflammatory cytokines and growth factors are involved in the signaling pathways for its transcriptional induction (Lohi et al., 1996). Since transcription is regulated by several transcription factors that bind to the promoter of the target gene, a number of studies have focused on the proximal promoter region of MMP-14. Analysis of the promoter region of MMP-14 gene has shown the presence of several transcription factor binding sites including Sp-1, PEA3 (Lohi et al., 2000) and SAF-1 (Ray et al., 2004).

The inflammation responsive transcription factor SAF-1 has been implicated in MMP-14 induction during atherosclerosis. SAF-1 was identified as an activator of serum amyloid A gene during amyloidosis (Ray and Ray, 1997c). Subsequently, it has been found to play an important role in inflammatory diseases such as atherosclerosis (Ray et al., 2004) and osteoarthritis (Ray et al., 2003a). The expression and activity of SAF-1 is highly increased by the signaling pathways initiated by inflammatory cytokines such as IL-1 and IL-6 (Ray and Ray, 1996). During atherosclerosis, increased expression of SAF-1 is both at the level of mRNA and protein (Ray et al., 2004). Immunohistochemical as well as Western blotting revealed that SAF-1 expression was increased during atherosclerotic conditions (Ray et al., 2004).

The promoter of MMP-14 gene contains an Oct-1 binding site like sequences just upstream of the SAF-1 binding site, suggesting that this Oct-1 site may be involved in the regulation of MMP-14 gene expression. Thus, we were interested in investigating the role of Oct-1 in the regulation of MMP-14 gene and the possibility of the physical interaction between SAF-1 and Oct-1. The critical question that arises is whether MMP-14, SAF-1 and Oct-1 are co-expressed in the atherosclerotic plaque. To answer this question, we have analyzed the presence of these regulatory proteins in the cells of the atherosclerotic plaques.

Material and methods

Atherosclerotic plaque specimens

The atherosclerotic plaques were obtained from Yucatan pigs (n=16) fed with high fat diet comprising of mini-pig chow enriched with 2% cholesterol, 17.1% coconut oil, 2.3% corn oil and 0.7% sodium cholate. The pigs were euthanized at the end of 20 weeks and samples of their carotid arteries were fixed in buffered 10% formalin and embedded in paraffin. All animals were handled according to the protocols and guidelines approved by the Animal Care and Use Committee at the University of Missouri. Comparisons were performed using Student's t-tests. P values< 0.05 were considered statistically significant.

Immunohistochemical analysis

Immunohistoschemical analysis was done on the 4μm thick carotid artery sections from the high fat diet fed pigs to stimulate the atherosclerotic conditions. The sections were

deparaffinized by dipping the slides with the carotid artery sections in Xylene for 10 minutes and rehydrated by dipping them in graded alcohol with decreasing ethanol concentration i.e. 100%, 95%, 70%, 50% for two minutes each and finally in IHC buffer I (50mM Tris-HCl, pH7.6, 150mM NaCl) for two times for five minutes each. The endogenous peroxide activity was quenched using 3%H_2O_2 in methanol for 45 minutes at room temperature with continuous shaking. The slides were then washed with the IHC buffer I two times each for 5 minutes. Antigen retrieval was done by heating the rehydrated carotid artery sections with 10mM citrate buffer preheated to 100^{o}C for 30 minutes. The slides were then allowed to cool down to room temperature. The non-specific binding was blocked using a blocking solution containing 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS). The sections were incubated with the 3% BSA solution at 37 ^{0}C for 30 minutes. The blocking solution was then removed and the primary antibody was applied and kept overnight at 4 ^{0}C. Dilutions of the primary antibodies were made in Antibody dilution buffer (50mM Tris-HCl, pH7.6, 150mM NaCl, 1% BSA). Next day, primary antibody was removed and the sections were washed two times with IHC buffer II (50mM Tris-HCl, pH7.6, 150mM NaCl, 0.05% Tween-20). Horseradish peroxidase (HRP)-labeled antibody was then applied to the sections and incubated at room temperature for 30 minutes. The sections were then washed with IHC buffer II, two times for five minutes each. The color reaction was carried out using the DAKO liquid DAB (3, 3′-diaminobenzidine) substrate-chromogen system (DAKO Corporation, Carpinteria, CA) for equal times for the control and antibody treated sections. The color reaction was stopped by dipping the slides in distilled water. The sections were then stained with hematoxylin solution (Sigma, St. Louis, MO) for 10

minutes. The sections were then washed two times with distilled water to remove excess stain. The sections were dehydrated using graded ethanol i.e. 50%, 70%, 95% and three times in 100% ethanol and finally for two times in xylene. The sections were finally mounted using permount.

For immunohistochemical staining for MMP-14, goat polyclonal anti-MMP-14 antibody (Santa Cruz Biotechnology, Inc, Santa cruz, CA) against C- terminal was used as primary antibody and affinity purified and HRP-labeled rabbit anti-goat antibody (Calbiochem, La Jolla, CA) was used as secondary antibody. Rabbit anti-SAF-1 antibody for SAF-1 staining and rabbit anti-Oct-1 antibody (Santa Cruz Biotechnology, Inc, Santa cruz, CA) for Oct-1 staining were used as primary antibodies. For the immunohistochemical identification of macrophages anti-Scavenger receptor A (SRA) antibody was used the primary antibody. Pre-immune serum was used as control during all the immunohistochemical studies.

Results

Immunohistochemical staining for MMP-14 in the atherosclerotic plaque

The results from the immunohistochemical analysis of atherosclerotic plaques using anti MMP-14 antibody revealed that MMP-14 was highly expressed in the atherosclerotic plaque region, especially in the shoulder region of the plaque (figure 7). Adjacent regions of the artery not affected by the plaque were not immunoreactive suggesting a local mechanism for upregulation of MMP-14 in the atherosclerotic plaque region.

Immunohistochemical staining for SAF-1 in the atherosclerotic plaque

Increased expression of MMPs in the atherosclerotic plaque is due to their transcriptional upregulation and many inflammation responsive transcription factors including SAF-1 are believed to play a central role. The immunohistochemical staining with anti-SAF-1 antibody revealed that cells in the area of the artery affected by the atherosclerotic plaque were highly immunoreactive for SAF-1 (figure 8). These regions correspond to the areas of intense infiltration of inflammatory cells, predominantly macrophages. In contrast, the regions of the same arterial tissue not affected by the atherosclerotic plaque did not show any expression of SAF-1. The increase in the expression of SAF-1 in the atherosclerotic plaque region suggests that SAF-1 may be involved in the expression of the genes in that local region only. Our results using immunohistochemical analysis with MMP-14 antibody also showed the expression of MMP-14 in the same regions where SAF-1 is being expressed raising the possibility of SAF-1 having a regulatory role in the overexpression of MMP-14.

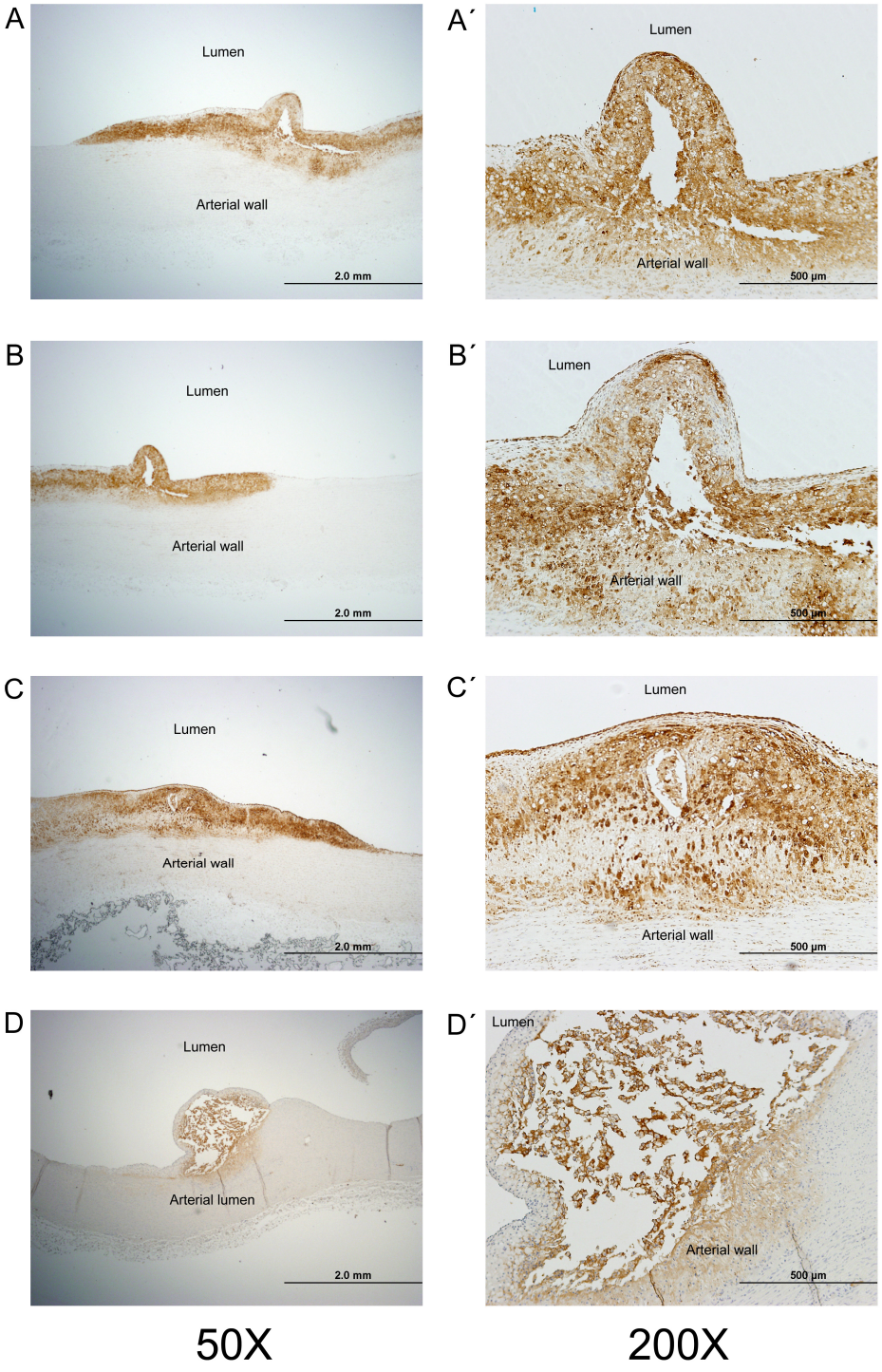

Figure 7: Expression of MMP-14 in the atherosclerotic plaque. The atherosclerotic plaque sections from the Yucatan pigs fed high fat diet for 20 weeks for the development of atherosclerosis were probed with anti-MMP-14 antibody. Several representative plaques are shown here in low (Panel A, B, C and D) and high magnification (Panel A´, B´, C´ and D´). These plaques revealed the increased expression of MMP-14 protein in the atherosclerotic plaque regions of the affected arteries.

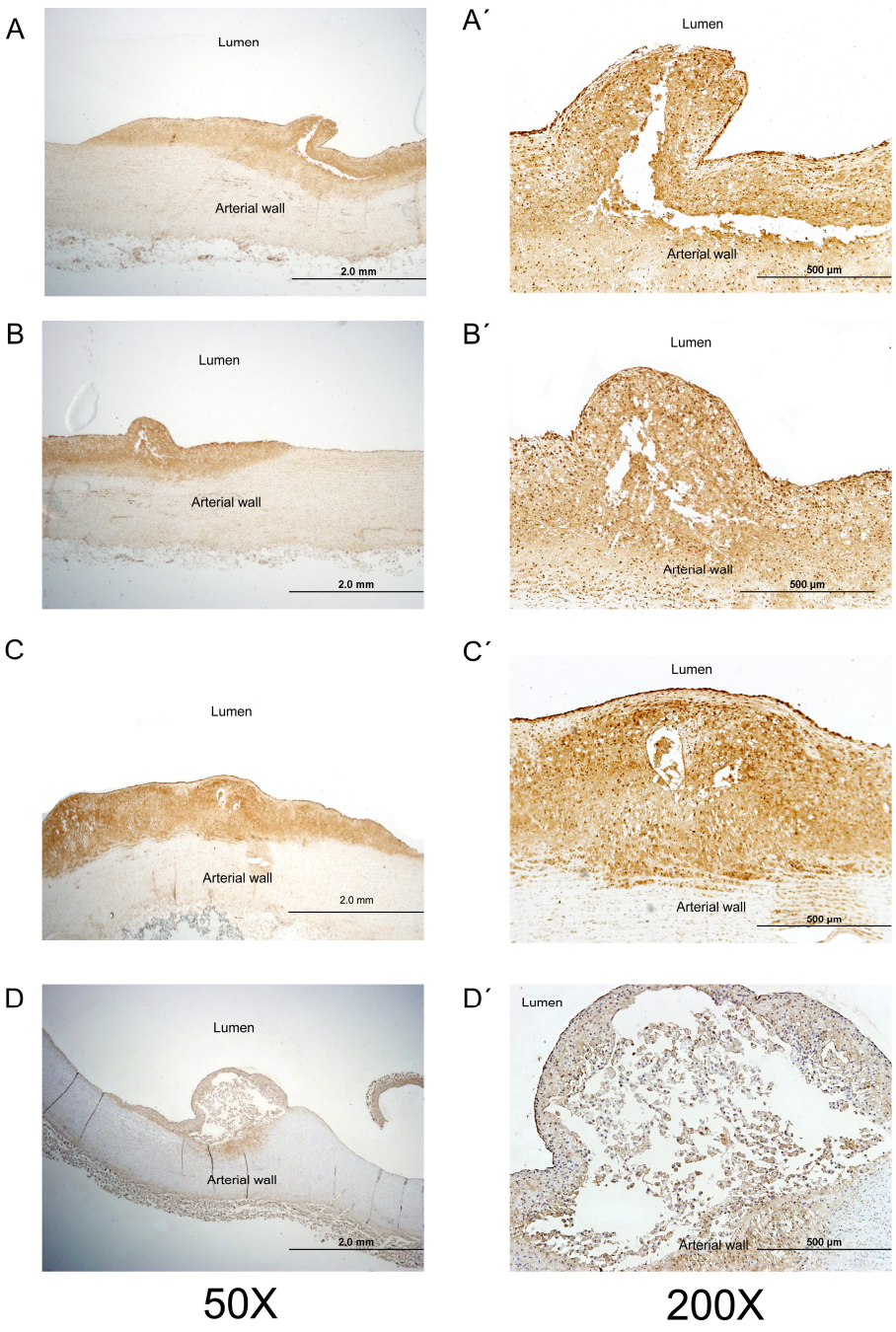

Figure 8: Expression of Serum Amyloid A Activating Factor-1 (SAF-1) in the atherosclerotic plaque. The atherosclerotic plaque sections from the Yucatan pigs fed high fat diet for the development of atherosclerosis were probed with anti-SAF-1 antibody. Several representative plaques at low (Panel A, B, C and D) and high magnification (Panel A´, B´, C´ and D´) of these plaques are shown.

Immunohistochemical analysis for the characterization of the cells expressing SAF-1

Immunohistochemical analysis of carotid artery sections from high fat diet-fed pigs indicated that SAF-1 was highly expressed in the atherosclerotic plaque. To exactly identify the cells over-expressing SAF-1 under the atherosclerotic conditions, immunohistochemistry of the arterial sections was performed using anti-SAF-1 and anti-SRA antibodies (Figure 9). Scavenger receptors are the proteins that are involved in endocytosis and accumulation of lipoproteins in the atherosclerotic lesions. Scavenger receptor A (SRA) is predominantly found on the surface of macrophages (Krieger et al., 1993). The results revealed that SAF-1 was expressed in the same cells that were expressing SRA suggesting that macrophages are the type of cells that predominantly express SAF-1 during atherosclerosis.

Co-localization of MMP-14 with SAF-1 in the atherosclerotic plaque

The presence of high level of SAF-1 in the atherosclerotic plaques raises the possibility that MMP-14 gene expression is regulated by SAF-1 transcription factor. The immunohistochemical analysis of the serial sections of the atherosclerotic plaque also revealed that both MMP-14 (figure 7) and SAF-1 (figure 8) proteins were expressed in the same region of the atherosclerotic plaque indicating that SAF-1 may be involved in the upregulation of MMP-14 gene under atherosclerosis.

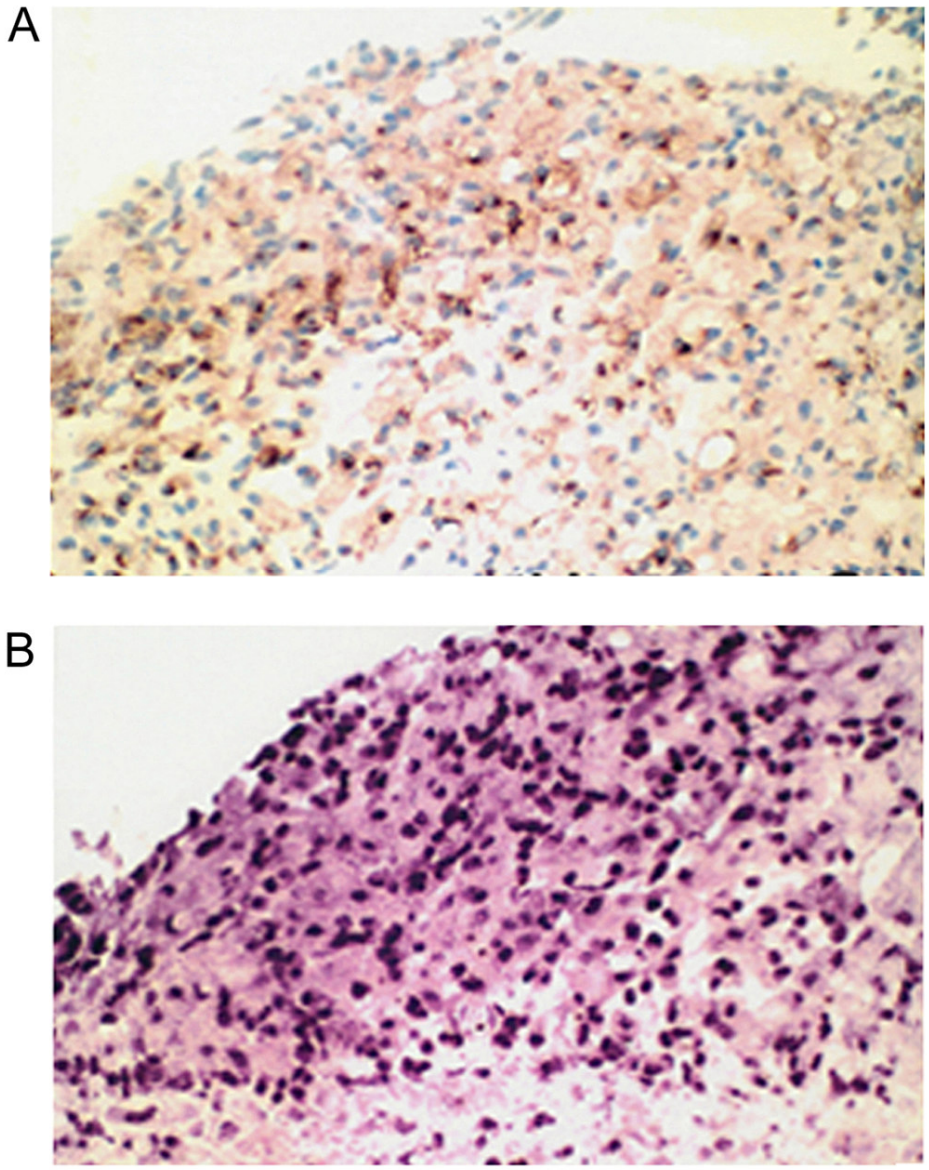

Figure 9: SAF-1 expression in the atherosclerotic plaque heavily infiltrated with macrophages. Immunohistochemical analysis of serial sections of the atherosclerotic plaque with anti-SAF-1 antibody (400X) (Panel A) and macrophage specific anti-SRA antibody (400X) (Panel B) revealed that macrophage cells present in the area were the cell type expressing SAF-1.

Mapping of the SAF-1 responsive element in MMP-14 promoter

The results of our immunohistochemical analysis suggested that the macrophage cells present in the atherosclerotic plaque region overexpresses both MMP-14 and SAF-1. The co-expression of SAF-1 and MMP-14 in the atherosclerotic plaque suggested that SAF-1 may be the regulator of MMP-14 gene expression in the macrophages present in the area. To test this possibility, we carried out the transient transfection assays using progressively deleted MMP-14 CAT reporter constructs to determine the SAF-1 responsive element in the MMP-14 promoter. 1.7MMP-14 CAT and 0.2MMP-14 CAT reporter plasmids show very little expression of reporter gene, whereas the expression of reporter gene is increased several folds when 0.13MMP-14 CAT reporter plasmid was used. The results of our transient transfection assays suggested that the SAF-1 responsive element is present between -134 to +1 region of MMP-14 promoter (Figure 15). Moreover, it also suggest the presence of some negative regulatory elements between -208 and -134 region of MMP-14 promoter which is able to suppress the expression of MMP-14 gene, but is not able to completely abolish its expression.

SAF-1 is a positive regulator of MMP-14 gene transcription

Co-expression of SAF-1 protein and MMP-14 in the atherosclerotic plaque and high levels of active SAF-1 in THP-1 cells that binds efficiently to the MMP-14 promoter indicate the possibility of a role of SAF-1 in MMP-14 gene expression. To determine the effect of SAF-1 on the expression of MMP-14, human monocyte derived THP-1 cells were transfected with 0.3MMP-14-CAT reporter plasmid and different concentrations of an expression plasmid containing wild type SAF-1 cDNA . The 0.3MMP-14-CAT

contains full length CAT cDNA whose transcription is driven by the 0.3-kb fragment of the MMP-14 gene promoter. The expression of SAF-1 increased the MMP-14 promoter activity in a dose dependent manner. Transfection of cells with empty expression plasmid did not increase the expression of reporter gene (Figure 16). These results indicated that SAF-1 has the ability to up-regulate the expression of MMP-14 in the human monocyte cells. The transient transfection assays using progressively deleted constructs suggests the SAF-1 responsive element may be located within -134 bp of the MMP-14 promoter.

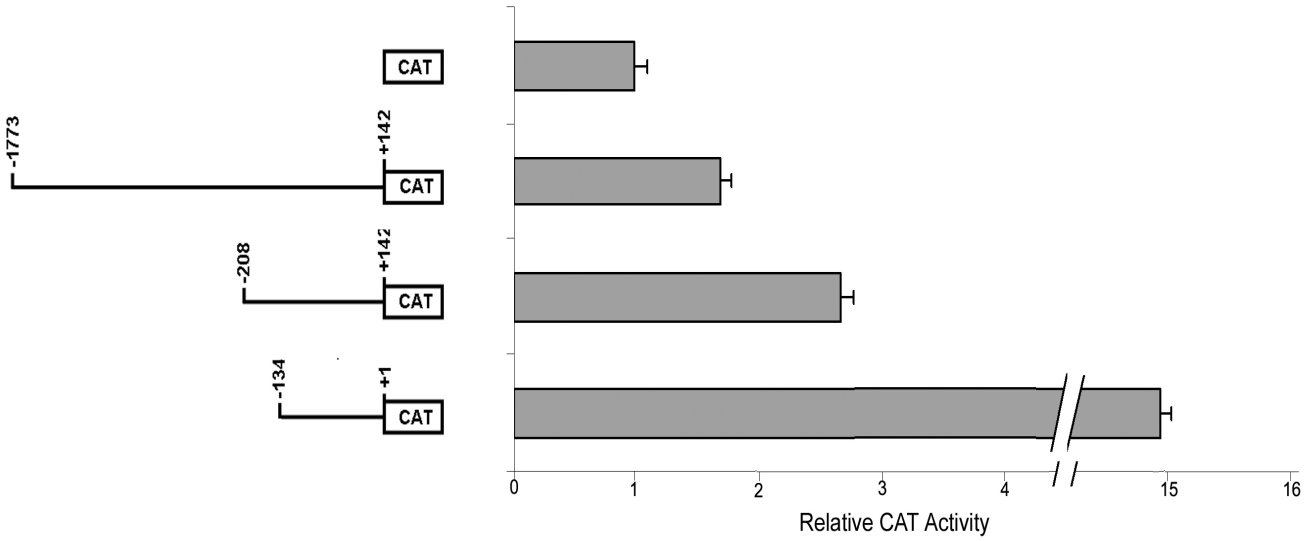

Figure 10: SAF-1 responsive element is located in within the -134 nucleotides upstream of transcription start site. Progressively deleted MMP-14 CAT reporter constructs were co-transfected with SAF-1 expression plasmid to determine the SAF-1 responsive element in MMP-14 promoter. These results suggest that the SAF-1 responsive element of the MMP-14 promoter is located with the -134 bp of the MMP-14 promoter.

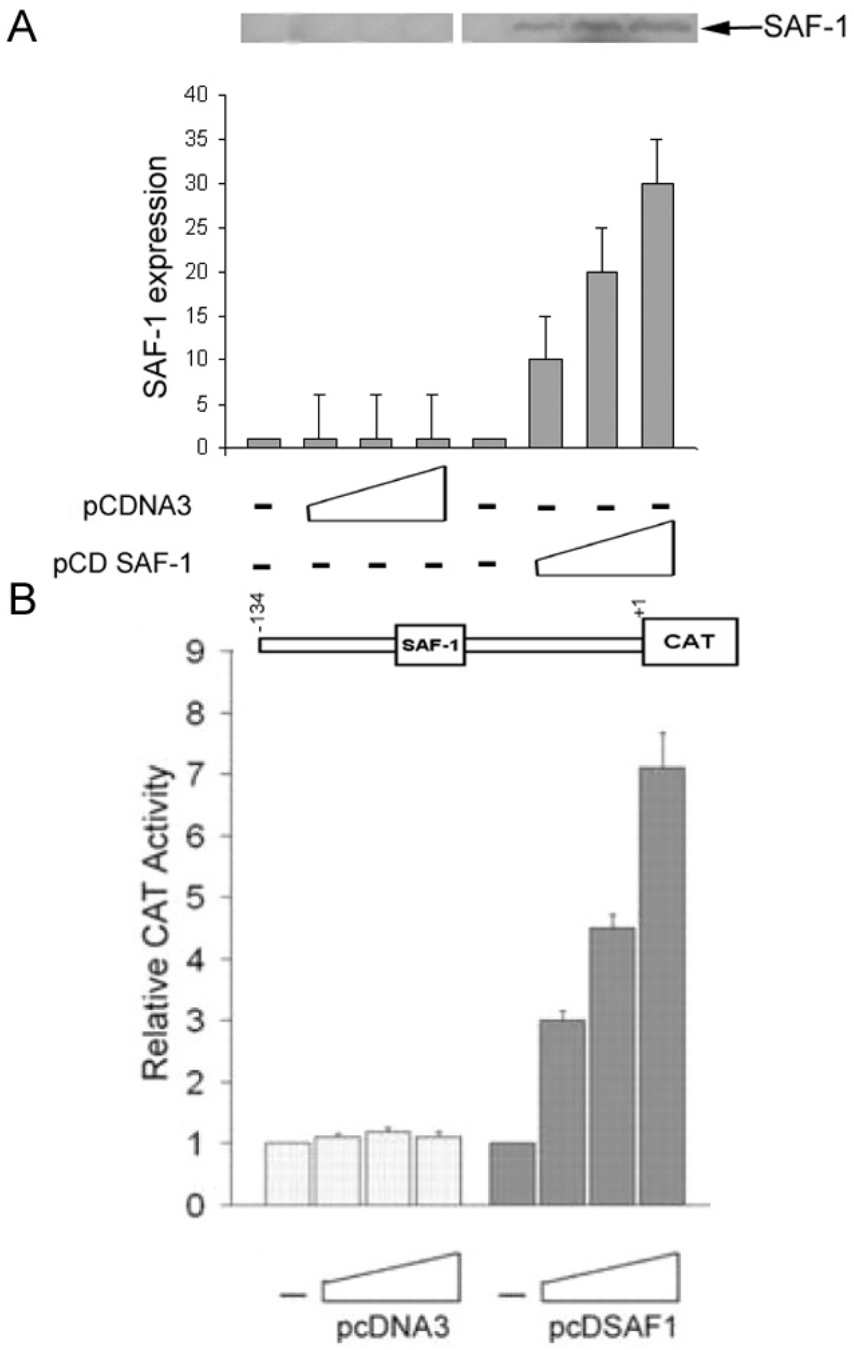

Figure 11: SAF-1 increases the expression of MMP-14 gene in a dose dependent manner. 0.13MMP-14 CAT3 reporter plasmid was co-transfected with the increasing doses of SAF-1 expression in the THP-1 cells. Panel A: The expression of empty pCDNA3 plasmid and pCD SAF-1 plasmid is shown. There was increase in expression of SAF-1 in the transfected cells. Panel B: The results indicate that SAF-1 is able to increase the expression of the reporter gene in a dose dependent manner, whereas the empty plasmid fails to increase its expression.

Discussion

In this study we have identified the expression of MMP-14 and the inflammation responsive transcription factor SAF-1 in the atherosclerotic plaque. This study also helped us to determine the expression of Oct-1 transcription factor in the atherosclerotic plaque. We have also correlated the expression levels of SAF-1 and MMP-14 in these atherosclerotic plaques. We have shown that higher levels of SAF-1 expression in the macrophage foam cells present in the lesion area expressing MMP-14. We have also shown that Oct-1 is expressed by the macrophage cells present in the shoulder region of the plaque, which is the site of entry of the macrophages into the lesion area. Most of the macrophage cells present in the core of the plaque do not express Oct-1, which can be explained by the fact that the macrophage cells in the core lesion area undergo apoptosis, which form most of the dead cells present in the core of the plaque (Leonarduzzi et al., 2006). Moreover, it has been shown that the factors such as PMA which increase the expression of SAF-1 on one hand decrease the DNA binding acticity of Oct-1 (Dent et al., 1991). This raises the possibility of impaired inhibition of SAF-1 activity during the induction of MMP-14 gene expression.

Interestingly, the nuclear extracts prepared from cultured monocyte cells stimulated with the atherosclerotic agents such as Ox-LDL show a slight increase in the level of Oct-1 protein. It has been recently reported that PPARγ ligands can increase the levels of Oct-1 protein, though they do not induce the expression of Oct-1 mRNA (Bruemmer et al., 2003) suggesting some post-translational mechanism for its activation. More recently, investigators have reported that though the amount of Oct-1 increases in the nuclear

fraction, there is no increase in the levels of Oct-1 in the whole cell extracts (Lopez-Bayghen et al., 2006). These findings are in agreement with our immunohistochemical analysis where we do see the translocation of Oct-1 from the cytoplasm to the nucleus (Figure 14, panel B), and the Western blot findings that the amount of Oct-1 increases in the nuclear extracts prepared from the Ox-LDL THP-1 cells.

The MMP-14 gene expression is mainly regulated at the transcriptional level. Here we have provided evidence that SAF-1 is co-expressed at a higher level in the atherosclerotic lesion area. SAF-1 interacts with the MMP-14 promoter during activation of MMP-14 upregulation. Unlike SAF-1, increase in the cellular level of Oct-1 is limited at best. Our data (figures 7 and 8) show that a limited number of macrophage cells contain higher levels of Oct-1. This finding suggests that Oct-1 is likely to exert some yet unknown effect on the MMP-14 expression.

Question remains whether Oct-1 has any direct effect on MMP-14 gene expression via its possible interaction with the Oct-1 like binding element. Alternatively, it is possible that Oct-1 binding site due to its proximity to SAF-1 binding site in MMP-14 promoter may have an indirect effect through its interaction with SAF-1. The Oct-1 transcription factor is involved in a various biological process such as regulation of housekeeping genes and immunoglobulin genes. It has been postulated that for the proper functioning of Oct-1, it must be post-translationally modified, such as its interaction with other regulatory proteins (Sytina and Pankratova, 2003).

Translocation of Oct-1 from cytoplasm to nucleus in the activated macrophage cells (Figure 9) suggests that Oct-1 is most likely activated during macrophage stimulation, commonly found in the atherosclerotic tissue. It is not known whether such translocation event is the result of an activation process such as phosphorylation or other form of post-translational events commonly associated with nuclear transport (Lopez-Bayghen et al., 2006). To date, there is no report of Oct-1 activation in atherosclerotic plaque. It would be interesting to know the mechanism of Oct-1 transport in the macrophage cells in atherosclerotic plaques.

This is the first evidence where for a co-expression of SAF-1 and MMP-14 in the atherosclerotic plaque. Furthermore, we show, for the first time, that Oct-1 is translocated to the nucleus in the atherosclerotic plaques. Further studies involving the interaction of SAF-1 and Oct-1 transcription factors will provide evidence for the understanding of the possible mechanism of MMP-14 gene regulation.

CHAPTER 4

DETERMINATION OF THE EFFECTS OF SAF-1 AND OCT-1 EXPRESSION ON MMP-14 TRANSCRIPTIONAL ACTIVITY

Abstract

The mammalian gene expression is the function of many transcription factors acting on the promoter region of the gene. The expression of MMP-14 is upregulated during inflammatory conditions and is regulated by the inflammation responsive transcription factors. These transcription factors interact with their respective binding sites on the MMP-14 promoter to regulate its expression. The proximal promoter region of MMP-14 contains binding sites for both SAF-1 and Oct-1. We have seen by immunohistochemical and Western analysis that the level of SAF-1 increases several folds which may be due to increase in the nuclear localization of Oct-1. Transient transfection assays using MMP-14 promoter constructs indicated that Oct-1 antagonizes the SAF-1 mediated expression of MMP-14. Deletion of Oct-1 site was not able to inhibit the action of Oct-1, suggesting a direct physical interaction between SAF-1 and Oct-1. Our results with the transient transfection assays also indicated that Oct-1 alone is not able to inhibit the SAF-1 mediated expression of MMP-14 suggesting the role of other co-repressors. Together these results suggest that the expression of MMP-14 is regulated by SAF-1 under atherosclerotic conditions and Oct-1 can partially block the expression of MMP-14 by directly interacting with SAF-1 and inhibiting its action.

Introduction

The role of MMP-14 enzyme in the degradation of extracellular matrix is now well established. MMP-14 is responsible for the degradation of various extracellular matrix components such as collagen type I, II and III, fibronectin, vitronectin, tenascin, laminin-1 and aggrecan. Under normal physiological conditions, the expression of MMP-14 is very low. However, under pathological conditions such as atherosclrosis, the expression of MMP-14 increases several folds due to its transcriptional upregulation. Several studies involving human and animal models have revealed that the expression of MMP-14 is more in the areas of arteries affected with atherosclerosis compared to the unaffected areas of the same arteries (Ray et al., 2004).

The increase in the expression of MMP-14 is mainly due to its transcriptional induction. Several inflammatory cytokines including IL-1 and oxidized LDL are involved in the signaling cascade leading to increased expression of MMP-14 (Rajavashisth et al., 1999a). We have recently reported that the expression of both MMP-14 mRNA and protein is increased many folds under the influence of ox-LDL (Ray et al., 2004). The increase in the expression due to the transcriptional induction is due to the interaction of transcription factors with the gene promoter. The promoter of MMP-14 has binding sites for several transcription factors including SAF-1 (Ray et al., 2004), Sp-1 (Lohi et al., 1996) and PEA3 (Lohi et al., 2000).

SAF is a family of inducible transcription factors. The members of this family contain a multiple Cys2-His2-type zinc fingers at the carboxyl terminal end (Ray and Ray, 1998).

SAF-1, a member of this family of transcription factor has a high degree of resemblance to MAZ/Pur-1 family of transcription factors (Bossone et al., 1992; Kennedy and Rutter, 1992).

SAF-1 is highly induced under the influence of inflammatory cytokines including IL-1β, IL-6 (Ray et al., 1999a), LPS (Ray and Ray, 1997c) and MM-LDL (Ray et al., 1999b). SAF¬-1 is also reported to be involved in the activation of SAA gene in the macrophage/ monocyte cells in response to LPS (Ray and Ray, 1997c). SAF-1 has been shown to interact synergistically with Sp-1 for the induction of SAA gene in the macrophage/monocyte cells (Ray and Ray, 1997b).

SAF-1 is an inflammation responsive transcription factor. Phosphorylation by protein kinase C is an important event in the activation of SAF-1 (Ray et al., 2000). It has also been reported to be activated in response to various inflammatory cytokines such as IL-1 by cyclic AMP dependent signaling pathway. Cyclic AMP regulates the effects of many transcription factors by activation of protein kinases which phosphorylates various transcription factors (Lalli and Sassone-Corsi, 1994). Activation of SAF-1, mediated by the catalytic (C) subunit of protein kinase A (PKA), is involved in its increased DNA-binding and transactivation potential (Ray et al., 2002a). Phoshorylation by PKA induces a conformational change in SAF-1 protein such that its DNA binding domains are unmasked (Ray et al., 2003b). Mitogen activated protein (MAP) kinases are a family of protein serine/threonine kinases that are activated in response to various extracellular stimuli such as IL-1and IL-6. MAP kinases are also involved in the phosphorylation of

SAF-1 resulting in increased DNA-binding activity and transactivation potential (Ray et al., 2002b).

Oct-1 is expressed ubiquitously and is present in most cell types during all the stages of cell cycle (Carbon et al., 1987). The mechanism by which Oct-1 regulates gene expression is by interacting with the other transcription factors or co-activators which are activated in response to the specific stimulus (Dailey et al., 1994; Groenen et al., 1992). Oct-1 has been reported to interact with several other transcription factors by helix-turn-helix motif that is responsible for both DNA binding and interacting with other transcription factors for regulating the expression of the target genes (Nakshatri et al., 1995). Such an interaction has been reported to either activate or repress the expression of the target genes.

Close proximity of the binding sites, as shown in figure00, of these two transcription factors suggested the possible role of these two transcription factors interacting with each other for MMP-14 gene regulation. In this study we show that though SAF-1 is responsible for the increase in MMP-14 gene expression, Oct-1 does not have any effect on its expression. Interstingly, Oct-1 is able to antagonize the action of SAF-1 and is thus able to downregulate expression of MMP-14.

GGCTGCGGCCTCAACCCCTGCAGATGGCAGCCTGCACCACAGAAAAGACAAATTTTT

TTTCCCCTTCTCTCCTGCTGTAATTGGATTCGAGCTGAAACACCACGTCCCCACCGAA

AAAGGAGGGCATTGGGGCGGGGGCGGAGGAGAGGCTGTGGGAGAAGGGAGGGAC

CAAAGGAGAGCAGAGAGGGCTTCCAACTCAGTTCGCCGACTAAGCAGAACTAAGCAG

AAGAAAGATCAAAAAACGGAAAAGAGAAGAGCAAACAGACATTTCCAGGAGCAATTCC

CTCACCTCCAAGCCGACCGCGCCTCTAGGAATCCACATTCCGTTCCTTTAGAAGACAA

AGGCGCCCCAAGAGAGGCGGCGCGACCCCAGGGCGTGGGCCCCGCCGCGGAGCC

CGCACCGCCCGGTGCCCCGACGCCGGGGACC**ATG**

Figure 12: The proximal promoter sequence of MMP-14 gene. The proximal promoter sequence of MMP-14 is shown here. The SAF-1 binding site of MMP-14 promoter is shown in red color. The putative Oct-1 binding site is shown in blue color. The major transcription start site is depicted by the bent arrow and the ATG codon is shown in bold.

Materials and methods

Cell culture and transfection

The human monocyte cell line THP-1 were obtained from the American Type Culture Collection and maintained in suspension in RPMI-1640 medium containing 10% fetal bovine serum (FBS). No antibiotic was added to the growth medium. For transient transfection of THP-1 cells, 2 X 10^7 cells were transfected with 10µg of plasmid DNA by the DEAE-dextran method (Sambrook et al., 1989). After the transient transfection, the THP-1 cells were incubated in a humified CO_2 incubator for 48 hours. After that period, the cells were harvested by centrifugation at 4^0C at 2500 rpm for 10 minutes and the supernatant containing the entire growth medium was discarded. The cells were washed once with 1X PBS. The cells were then resuspended in 100µl of 0.25M Tris-HCl, pH 7.5. The cells were lysed by three freeze-thaw cycles. Endogenous acetylase was inactivated by heating the cell lysate at 60^0C for 10 minutes. Protein amount was determined by measuring OD at 228.5nm and 234.5nm and using the following formula:

$$\text{Protein amount (mg/ml)} = (A_{228.5nm}-A_{234.5nm}) \times 317 \times 10^{-3} \times \text{Dilution factor}$$

The reaction mixture included 7µl of 4mM acetyl CoA, 0.5µl of [^{14}C] chloramphenicol (25µCi/500µl), protein equivalent amount of cell extract and adequate volume of 0.25M Tris-HCl, pH 7.5 per assay. The reaction mixture was incubated at 37^0C for several hours. Reaction was stopped by the addition of 500µl of ethyl acetate and quickly vortexing. The reaction products were extracted by vortexing for 1 minute each, followed by centrifugation for 5 minutes and collecting the solvent layer in a fresh eppendorf tube.

The reaction products were dried in a vacuum drier, resuspended in 15µl of ethyl acetate and spotted on a TLC plate (Whatman flexible plates). After spotting the TLC plates were placed in a chromatography chamber that had been presaturated with 50ml of chloroform/methanol (11.5:1) and chromatographed until the solvent reached the top of the TLC plate. After drying in air, the TLC plates were exposed to Fuji films. Spots corresponding to the acetylated products were excised from the TLC plate and counted by liquid scintillation analyzer for quantization of CAT activity.

Plasmids

CAT reporter plasmids containing progressive deletions of the mouse MMP-14 promoter were constructed by PCR amplification and cloning into pBLCAT3 vector. 1.7MMP-14 CAT reporter plasmid contains -1773 to +142 of MMP-14 promoter. Various deletion constructs including 0.2MMP-14 CAT (-208/+142) and 0.13MMP-14 CAT (–134/+1) were constructed by removal of the upstream sequences by restriction digestion of the 1.7MMP-14 CAT reporter plasmid. pcDSAF-1 plasmid contains full-length SAF-1 cDNA in pcDNA3 vector (Invitrogen).

Results

Deletion of Oct-1 binding site does not overcome the Oct-1 mediated inhibition of MMP-14 promoter activity

For further investigation into the mechanism of inhibition of MMP-14 promoter activity by Oct-1, we designed CAT reporter plasmids containing only SAF-1 binding site by

deleting the putative Oct-1 binding site. THP-1 cells were transiently transfected using these deletion constructs. The MMP-14 SAF-1 CAT reporter plasmid displayed increasing CAT reporter activity in a dose dependent manner. Ectopic expression of Oct-1 decreased the SAF-1 mediated induction of MMP-14 promoter activity even though the Oct-1 binding site was deleted (Figure 17).

Together these results suggests that SAF-1 site is critical for the MMP-14 promoter function and Oct-1 inhibits the SAF-1 mediated induction of MMP-14 gene by binding to its binding site in the MMP-14 promoter. These results also suggest that Oct-1 is able to inhibit SAF-1 mediated expression of MMP-14 without having to interact with the MMP-14 promoter. Together, these results suggest that SAF-1 and Oct-1 transcription factors may be interacting with each other, either directly or indirectly in the expression of MMP-14.

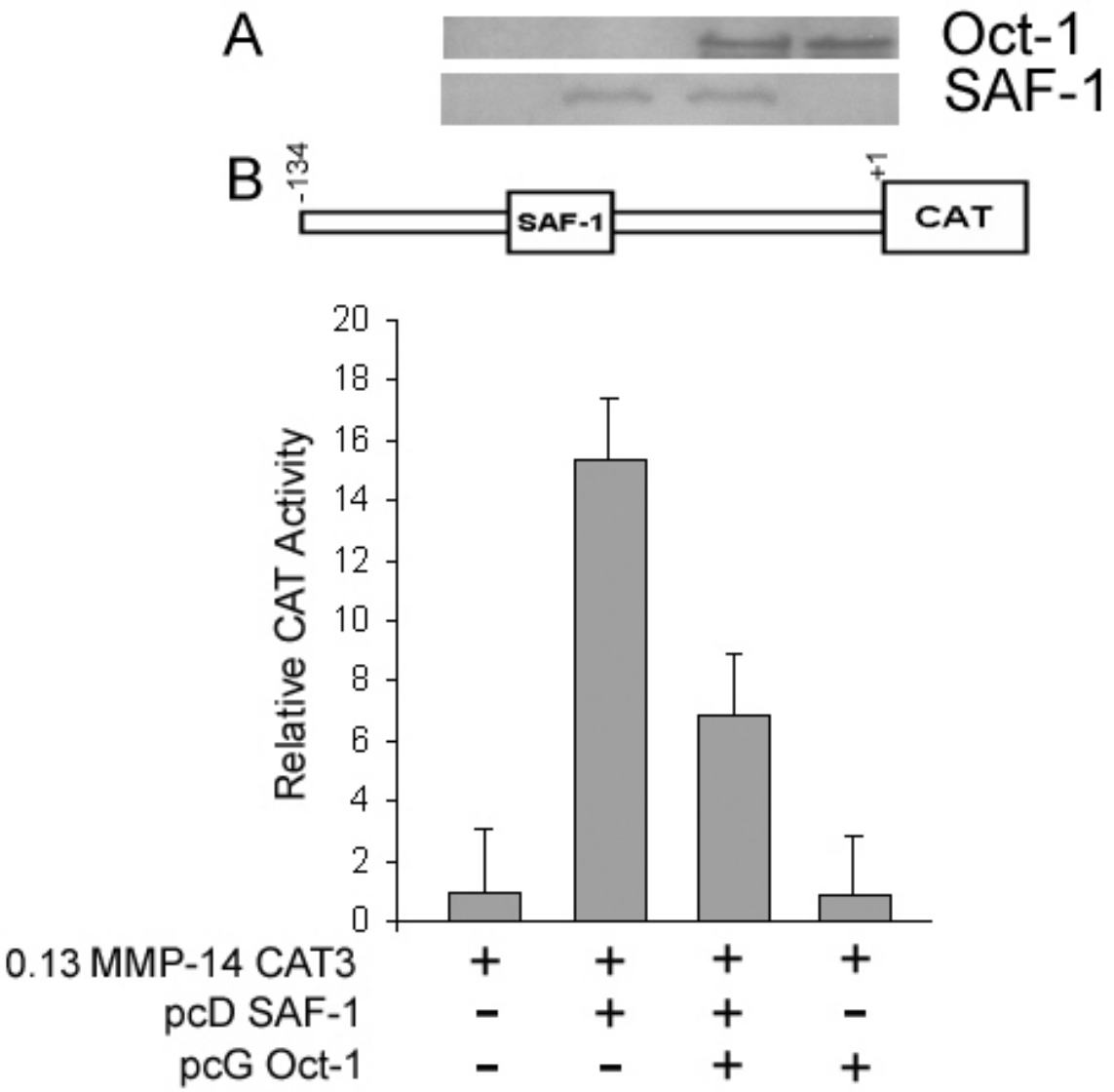

Figure 13: Oct-1 antagonizes SAF-1 activity. The THP-1 cells were co-transfected with 0.13MMP-14 CAT3 reporter plasmid along with either SAF-1 expression plasmid or Oct-1 expression plasmid or both. Panel A: The expression of SAF-1 and Oct-1 expression plasmids is shown by Western blotting. Panel B:The SAF-1 expression plasmid increases the expression of reporter gene around sixteen folds whereas the Oct-1 expression plasmid has no effect on the expression of reporter gene. When the SAF-1 and Oct-1 expression plasmids are co-transfected along with the reporter construct, expression of Oct-1 is able to decrease the expression of SAF-1 induced expression of reporter gene.

Immunohistochemical analysis of the atherosclerotic plaque to assess the expression of Oct-1

The close proximity of the Oct-1 binding site to the SAF-1 binding site in the MMP-14 promoter prompted us to investigate the possible role of Oct-1 in the regulation of MMP-14 gene expression. Thus to observe the level of Oct-1 in the atherosclerotic plaques, we performed immunohistochemical analysis using anti-Oct-1 antibody. The results revealed that Oct-1 is not overexpressed in the atherosclerotic plaque region, as the SAF-1 and MMP-14 proteins. The Oct-1 protein, being an ubiquitous transcription factor, is expressed only at the basal levels (Figure 10).

Since we did not see any change in the expression level of Oct-1 in cells of the atherosclerotic plaque, we wanted to investigate if the atherosclerotic stimulus Ox-LDL was able to alter the expression pattern of Oct-1 in the cultured monocyte cells using Western blotting.

Immunohistochemical analysis of normal artery sections for expression of MMP-14, SAF-1 and Oct-1

In atherosclerotic plaque regions of the affected arteries, we observed an increase in the expression of MMP-14 and SAF-1 proteins, whereas the expression of Oct-1 was at a basal level. To determine the expression of these proteins in normal arteries and unaffected regions of the arteries with the atherosclerotic regions, we performed immunohistochemical analysis of these regions (Figure 11). Serial sections of normal arteries were probed with anti-MMP-14, anti-SAF-1 and anti-Oct-1 antibodies. The

results revealed that there is almost no expression of MMP-14 and SAF-1 in the normal arteries, whereas Oct-1 is expressed by all cells present in the artery (Figure 11, panel C). These results suggest that the expression of MMP-14 is increased in atherosclerotic plaque when the level of SAF-1 is induced in the region of the plaque.

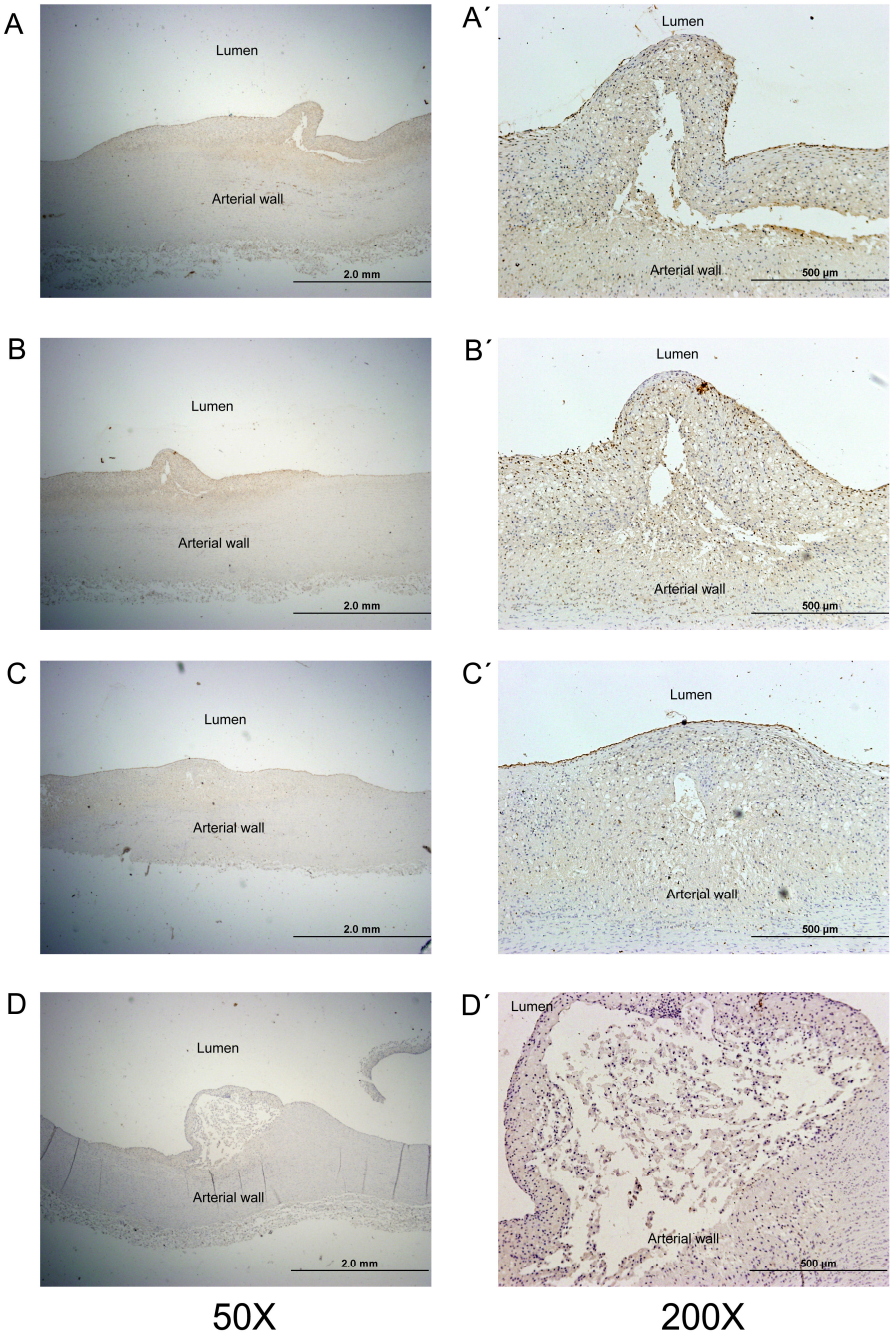

Figure 14: Expression of Octamer binding protein-1 (Oct-1) in the atherosclerotic plaque. The atherosclerotic plaque sections from the Yucatan pigs fed high fat diet for the development of atherosclerosis were probed with anti-Oct-1 antibody. Several representative atherosclerotic plaques are depicted here in low (Panel A, B, C and D) and high magnification (Panel A´, B´, C´ and D´).

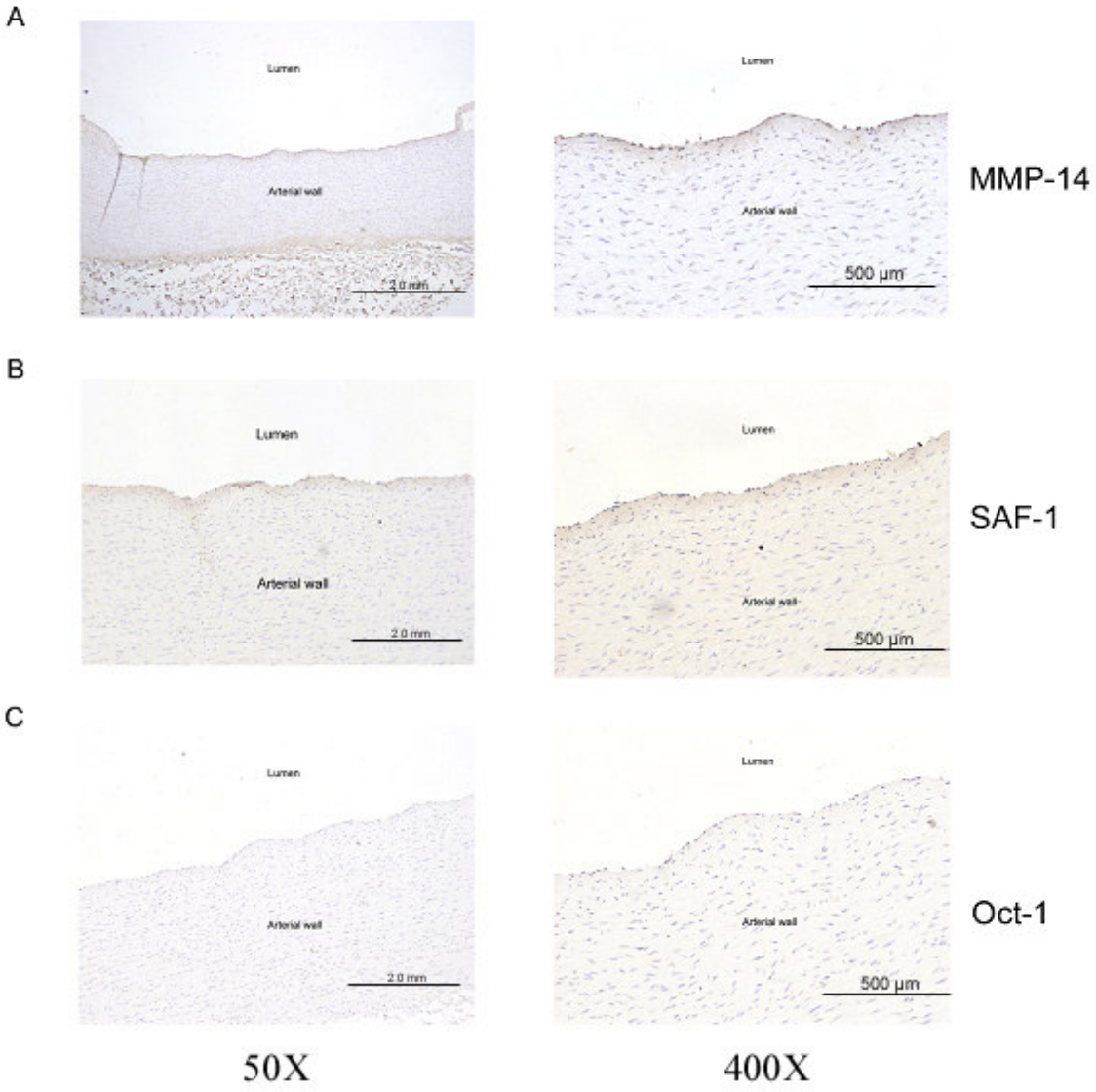

Figure 15: Expression of MMP-14, SAF-1 and Oct-1 in arteries not affected with atherosclerosis. The unaffected arteries from the Yucatan pigs fed high fat diet for the development of atherosclerosis were probed with anti MMP-14 (Panel A), anti SAF-1 (Panel B) and anti-Oct-1 antibody (Panel C). Several representative sections are depicted here in low and high magnification. We do not observe any expression of MMP-14 and SAF-1 in these sections, but Oct-1 is expressed at the basal level.

Expression of SAF-1 and Oct-1 in the monocyte cells

The human monocyte/macrophage THP-1 cells were stimulated with the atherosclerotic agent such as Ox-LDL and the nuclear extracts were prepared from both unstimulated and Ox-LDL stimulated THP-1 cells. Equal amounts of the proteins were fractionated on an 11% SDS-PAGE gel, transferred onto the nitrocellulose membrane and probed with either anti-SAF-1 or anti-Oct-1 antibody (Figure 12). The results revealed that the levels of SAF-1 increases by several folds in the nuclear extract, whereas the increase in the levels of Oct-1 was only two folds.

The results of our histochemical analysis of the atherosclerotic plaque suggested us that there is only a basal level of expression of Oct-1 in the macrophage cells present in the lesion area. At high magnification (1000X), we were able to see that the macrophage cells present in the shoulder region of the plaque show the expression of Oct-1 (Figure 13). In contrast only a few macrophage cells present in the core of the lesion showed the presence of Oct-1, as compared to SAF-1 and MMP-14, which are expressed at a very high level by these cells. By immunohistochemical analysis of the atherosclerotic plaque, we have also seen that the Oct-1 protein migrates from the cytoplasmic compartment of the macrophage cells to the nuclear compartment (Figure 14). This nuclear localization of the Oct-1 protein may be the possible reason for the increase in the Oct-1 levels we observed by Western blotting of the nuclear extracts prepared from the uninduced and ox-LDL induced THP-1 macrophage cells.

Together, these results suggest that the levels of SAF-1 and MMP-14 proteins increase at a very high level, the level of the Oct-1 protein remain at the basal level, though there is slight increase in the nuclear transport of Oct-1 protein.

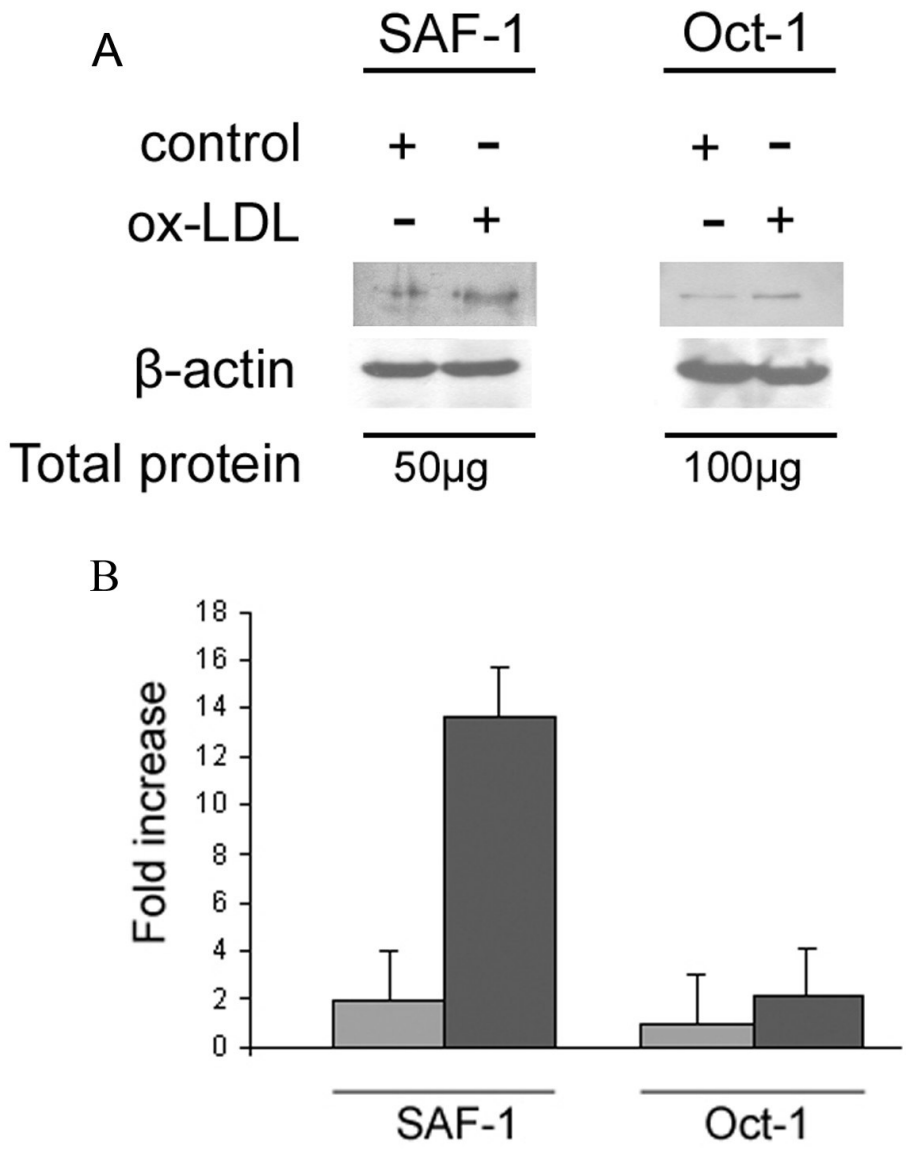

Figure 16: Expression of SAF-1 and Oct-1 in the uninduced and ox-LDL induced THP-1 cells. The nuclear extracts from the uninduced and ox-LDL induced treated cells were fractionated on an 11% SDS-PAGE gel, transferred onto a nitrocellulose membrane and probed with either anti-SAF-1 or anti-Oct-1 antibody. Panel A: The results revealed that the expression of SAF-1 was increased several folds as compared to Oct-1 whose expression was increased only marginally. Panel B: Densitometric analysis of the Western blot quantifying the increased expression of SAF-1 in ox-LDL induced THP-1 cells compared to the uninduced cells.

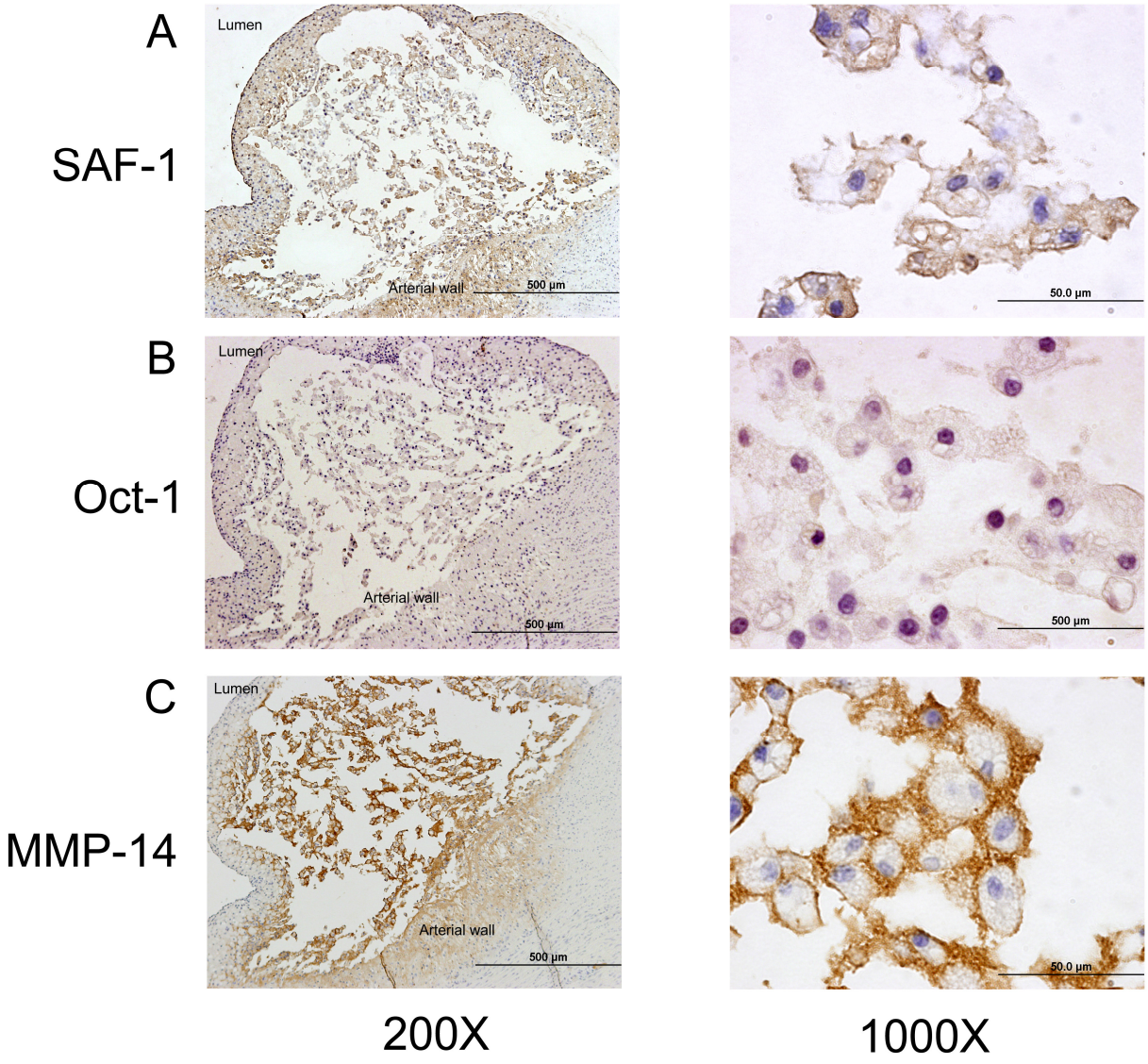

Figure 17: Expression of SAF-1, MMP-14 and Oct-1 in the macrophage cells present in the atherosclerotic plaque. The serial sections of the atherosclerotic plaques were probed using anti-SAF-1, anti-MMP-14 and anti-Oct-1 antibodies to determine the relative expression of these proteins in the macrophage cells present in the lesion area. The results reveal that SAF-1 is expressed abundantly in the macrophage cells and is present both in the cytoplamic and the nuclear compartments (Panel A). The MMP-14 protein is also expressed at the high levels on the cell membrane of the macrophage cells (Panel B). The expression of Oct-1 was restricted to only a few macrophage cells in the plaque region. The macrophage cells expressing Oct-1 are shown by red arrow heads and the cells not expressing Oct-1 are shown by black arrow heads (Panel C).

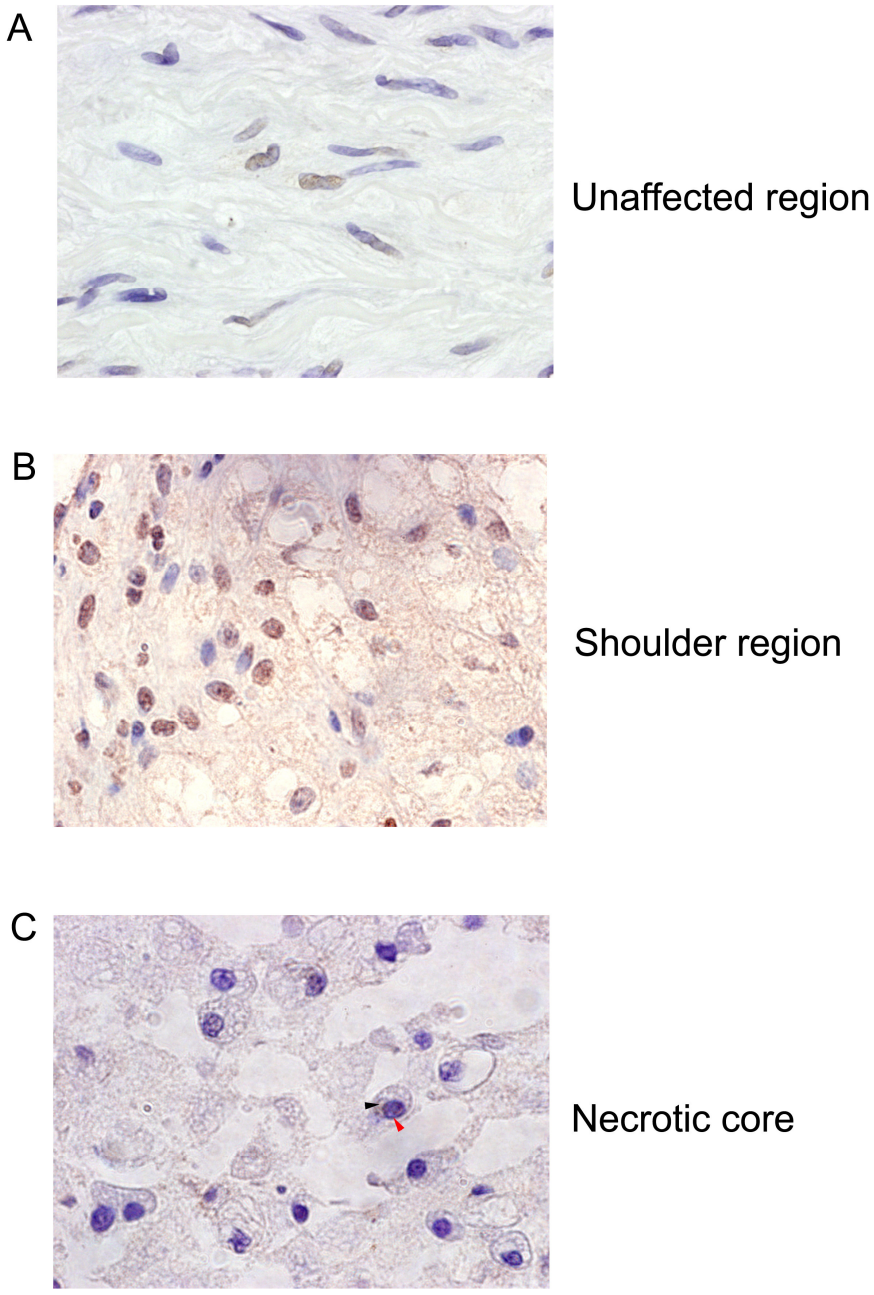

Figure 18: The expression of Oct-1 in the macrophage cells. Panel A: Expression of Oct-1 in the unaffected regions of the artery is at the basal levels in the vascular smooth muscle cells. Panel B: Oct-1 is expressed in many of the macrophage cells present in the shoulder region of the atherosclerotic plaque, suggesting that the expression of Oct-1 in the macrophage cells goes down during atherosclerosis. Panel C: Only a few macrophage cells present in the necrotic core show the expression of Oct-1. It also reveals that the Oct-1 transcription factor transports itself from the cytoplasmic compartment of the macrophage cells to the nuclear compartment during atherosclerosis. By immunohistochemical analysis, here we show that Oct-1 protein present in the cytoplasm (black arrow head) is migrating to the nucleus (red arrow head).

Discussion

The molecular mechanism by which the MMP-14 expression is controlled under physiological conditions is disturbed during atherosclerosis. This results in overexpression of MMP-14 leading to the degradation of the extracellular matrix components of the atherosclerotic cap. We have recently reported that an inflammation responsive transcription factor SAF-1 is expressed at a high level in the macrophage foam cells of the atherosclerotic plaque (Ray et al., 2004). Here we have shown that another ubiquitously expressed transcription factor Oct-1 may be responsible for antagonizing the action of SAF-1 and may be responsible to control the expression of MMP-14 during normal physiological conditions. It has been reported by several investigators that Oct-1 is involved in the downregulation of several genes including von Willebrand factor gene (Schwachtgen et al., 1998) and prolactin gene (Subramaniam et al., 1998). Ectopic expression of Oct-1 along with SAF-1 in the THP-1 cells was able to decrease the MMP-14 promoter function, but could not reduce it to the basal levels. This observation suggests that Oct-1 alone is not able to fully antagonize the action of SAF-1 mediated expression of MMP-14 gene. In other words, some other inhibitor molecules may be required, along with Oct-1, to completely repress the action of SAF-1 on the MMP-14 promoter activity. Our results also suggest that Oct-1 may not be interacting with the MMP-14 promoter directly though its element in the MMP-14 promoter. Since Oct-1 binding to MMP-14 promoter is not necessary for the inhibition of SAF-1 mediated inhibition of MMP-14 promoter activity, further experiments need to be done to investigate the exact nature of the interaction of Oct-1 with SAF-1 and MMP-14 promoter.

CHAPTER 5

DETERMINATION OF THE SAF-1 AND OCT-1 BINDING TO THE MMP-14 PROMOTER

Abstract

During gene transcription, various transcription factors interact with the promoter of the gene with their respective binding sites to direct the transcription. These transcription factors then interact with the basal transcription factors to induce or suppress the transcription according to the cellular requirements. There are certain transcription factors that can regulate the transcription which do not interact with the promoter, but can still regulate gene transcription by interacting with other transcription factors. The purpose of this study was to determine if SAF-1 and Oct-1 transcription factors can interact with the MMP-14 promoter. By electrophoretic mobility shift assays, we here show that the DNA binding activity of SAF-1 and Oct-1 is induced by the atherosclerotic agents. To determine if these transcription factors can interact with MMP-14 promoter under *in vivo* conditions, we performed chromatin immunoprecipitation (ChIP) assay. The results revealed that these two transcription factors can interact with the MMP-14 promoter. The results of our ChIP assays also suggested the potential physical interaction between SAF-1 and Oct-1.

Introduction

The transcription factors are the proteins that bind with the promoter of a gene and regulate its expression under physiological and pathological conditions. There are transcription factors that bind directly with the promoter of the gene, and there are several transcription factors which may not interact directly with the promoter but influence the transcription indirectly by interacting with the transcription factors involved in the formation of the basal transcriptional machinery. We have recently reported that the inflammation responsive transcription factor, SAF-1 positively influences the MMP-14 gene transcription by directly binding with the MMP-14 promoter under *in vitro* conditions by DNaseI footprint analysis (Ray et al., 2004). Our studies involving the transient transfection assays have suggested that another ubiquitously expressed transcription factor Oct-1 may be involved in antagonizing the action of SAF-1 on the MMP-14 promoter activity. Upon examining the promoter of MMP-14 gene, we have found that the binding site for Oct-1 transcription factor in the MMP-14 promoter is located in close proximity to the SAF-1 binding site. This observation suggested us that the Oct-1 mediated inhibition of the MMP-14 promoter activity may be due to the interaction of these two transcription factors on the promoter of MMP-14 gene. The possible mechanisms by which Oct-1 can inhibit the SAF-1 mediated MMP-14 gene expression may include that 1) Oct-1 inhibits the binding of SAF-1 to its binding site in the MMP-14 promoter or 2) causing such conformational changes in the SAF-1 protein that it is unable to positively regulate the expression of MMP-14.

Here we have studied the molecular mechanisms by which Oct-1 is influencing the MMP-14 transcription. These studies have helped us to understand the general mechanism by which these two transcription factors may be involved in the MMP-14 gene regulation. We have performed DNA binding assays to test the possibility if Oct-1 directly interacts with MMP-14 promoter. To test the possibility that whether Oct-1 directly interacts with the MMP-14 promoter *in vivo* and whether Oct-1 inhibits the interaction of SAF-1 with the MMP-14 promoter, we have performed the chromatin immunoprecipitation assays. These experiments have helped us to determine the mechanism by which Oct-1 may be involved in the MMP-14 transcription.

Materials and methods

Preparation of Nuclear extracts

The human monocyte THP-1 cells were stimulated with Ox-LDL for 48 hours. Nuclear extracts were prepared both from Ox-LDL induced and uninduced cells as described previously (Ray and Ray 1997). The cells were harvested by centrifugation at 1500 rpm for 5 minutes and washed 3 times with sterile phosphate-buffered saline (PBS). The cells were then resuspended in buffer A (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.5 µg/ml each of leupeptin, antipain, pepstatin, 0.1 µg/ml chymostatin, 0.3 TIU/ml aprotinin, 0.5 mg/ml benzamidine) and incubated on ice for 15 minutes. The cells were vortexed vigorously for 10 seconds and centrifuged at 3000 rpm for 5 minutes to collect the nuclei. The supernatant was collected as Post-nuclear extract. To the nuclei, buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 0.5µl /ml each of leupeptin, antipain, pepstatin,

0.1µl /ml chymostatin, 0.3 TIU/ml aprotinin, 0.5 mg/ml benzamidine) was added and incubated on ice for 30 minutes with occasional vortexing. The supernatant was collected as nuclear extract after centrifugation at 7000 rpm for 5 minutes. Protein concentrations were measured using the method previously described (Bradford, 1976).

Preparation of radiolabeled probe

The promoter region of MMP-14 corresponding to Oct-1 binding site was cloned in the pTZ19U plasmid vector to be used for the preparation of probe in electrophoretic mobility shift assays. The plasmid DNA was digested with restriction enzymes to create the 3´ recessed ends and isolated from the agarose gel. The isolated DNA fragment was purified by passing through Sephadex G-50 (coarse) mini-column and used for radiolabeling. About 100 ng DNA was incubated with [α^{32}P]dCTP and 5 units of klenow fragment of DNA polymerase (Promega, Madison, WI) in presence of DNA polymerase buffer and incubated at room temperature for 1 hour. 5mM dNTP's were added to the reaction mixture and further incubated for 15 minutes at room temperature. The reaction was stopped by heating at 75^{0}C for 1 minute. The labeled DNA probe was then purified from free [α^{32}P]dCTP by passing it through Sephadex G-50 (coarse) mini-column and used for EMSA or DNaseI footprint assays or stored at -20^{0}C until used.

Electrophoretic mobility shift assay

EMSA were performed using equal protein amounts of the nuclear extracts prepared from the uninduced and Ox-LDL induced THP-1 cells. The DNA fragments corresponding to base pairs -00 to -00 for MMP-14 were end-labeled using [α^{32}P] dCTP and used as the

probe in EMSA reactions. The nuclear extracts were mixed with the binding buffer (10mM HEPES, pH 7.9, 60mM KCl, 5mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, 0.1 mM $ZnCl_2$), 10% (v/v) glycerol, 1μg of poly (dI•dC), $MgCl_2$, and 0.05% (v/v) Nonidet P-40 for 15 minutes at room temperature. 5 X 10^4cpm probe was added to the reaction mixture and further incubated at room temperature for 30 minutes. In some assays, non radioactive competitor double stranded oligonucleotide that contains the canonical Oct-1 binding sequence was added in some assays to assess the specificity of the DNA-protein complexes. For antibody interaction assays, polyclonal anti-Oct-1 antibody raised in mouse was added to the reaction mixture during a preincubation period of 30 minutes on ice. Reaction mixtures were fractionated on a 6% nondenaturing polyacrylamide gel with recirculation of running buffer (7mM Tris-HCl, pH 7.5, 3mM Sodium acetate, 1mM EDTA). The gels were dried and autoradiographed at -80^0C with intensifying screen.

Chromatin Immunoprecipitation Assay

To further characterize the role of SAF in the regulation of MMP-14 gene expression *in vivo*, Chromatin Immunoprecipitation (ChIP) analysis was performed. For this, the THP-1 cells were grown in suspension and protein-DNA complexes were cross-linked by addition of 1% formaldehyde solution. To quench the cross-links, glycine was added to fixed culture. The cells were harvested by centrifugation, washed and lysed in RIPA buffer (10mM Tris-HCl, pH8; 140mM NaCl, 0.025%NaN_3, 1% Triton X-100, 0.1% SDS, 1% Deoxycholic acid) with protease inhibitors. The cell suspension was then aliquoted and sonicated to shear the chromatin to the average size of 500bp. Protein A sepharose beads were added to the sonicated mixture and incubated on a rotation wheel for 2 hours

at 4^0C. The samples were then centrifuged and the supernatant was collected in a fresh tube as preclear extracts. The anti-SAF-1 antibody at appropriate dilution was added to the preclear extracts and incubated on ice for 3 hours. Protein A sepharose beads were added and incubated on a rotation wheel for 1 hour at 4^0C. The samples were then centrifuged and the sample was collected for sizing DNA. The beads were washed with RIPA-500 (10mM Tris-HCl, pH8; 500mM NaCl, 0.025%NaN_3, 1% Triton X-100, 0.1% SDS, 1% Deoxycholic acid) and LiCl/detergent solution (0.5% Deoxycholic acid,1mM EDTA, 250mM LiCl, 0.5% NP-50, 10mM Tris-HCl, pH8).

To elute the immunoprecipitates, 1%SDS/ 1X TE buffer was added and incubated at 65^0C for 10 min, and centrifuged briefly. The elute was transferred to a fresh tube and the beads were washed with 0.67%SDS/1X TE buffer. Reversal of the cross-links was achieved by incubating the immunoprecipitates and total extracts for 6 hrs at 65^0C. Proteinase K solution (1µl 20µl/µl glycogen, 5µl of 20µg/µl Proteinase K, 244.5µl 1X TE, pH7.6) was added and further incubated at 37^0C for 2 hrs. The DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The DNA was then analyzed by PCR. The results of the PCR with the immunoprecipitated DNA and using MMP-14 promoter specific primers will amplify the target sequence, confirming the binding of SAF-1 to the MMP-14 promoter region.

Results

SAF-1 interacts with the MMP-14 promoter

To determine whether SAF-1 can interact with the MMP-14 promoter, we performed some electrophoretic mobility shift assays using the nuclear extracts prepared from uninduced and Ox-LDL induced THP-1 cell nuclear extracts and radiolabeled MMP-14 promoter fragment with SAF-1 binding site as the probe. The results showed that there is formation of five DNA-protein complexes (Figure 18). To characterize these DNA-protein complexes formed, we used competition assays using consensus SAF-1 element, cold MMP-14 SAF-1 DNA and anti-SAF-1 antibody were used. All the DNA-protein complexes were inhibited when the cold probe was added to the reaction mixture, while the non-specific oligonucleotide failed to inhibit the formation of these compleaxes. The addition of the SAF-1 specific oligonucleotide in the reaction mixture inhibited the formation of complexes a, d and e indicating that these complexes were formed due to the interaction of SAF-1 with the MMP-14 promoter. Similarly, the complexes a, d and e were inhibited when anti-SAF-1 antibody was included in the reaction, further suggesting the involvement of SAF-1 in these complexes.

Taken together, these results indicate that SAF-1 can directly interact with the MMP-14 promoter. Also, SAF-1 forms multiple complexes with the MMP-14 promoter, suggesting that either SAF-1 is forming multimeric homo-complexes or it is interacting with other transcription factors. Further investigations regarding the expression level SAF-1 in the atherosclerotic plaques are required to characterize the role of SAF-1 in the MMP-14 gene regulation.

Oct-1 binds to the MMP-14 promoter at Oct-1 binding site

To determine whether Oct-1 can interact with the MMP-14 promoter, we performed some electrophoretic mobility shift assays using the nuclear extracts prepared from uninduced and Ox-LDL induced THP-1 cell nuclear extracts and radiolabeled MMP-14 promoter fragment with Oct-1 binding site as the probe. The results showed that there is formation of several DNA-protein complexes (Figure 19). To characterize these DNA-protein complexes formed, we used competition assays using consensus Oct-1 element as competitor oligonucleotide. The results revealed that two of these complexes were inhibited as shown by the arrows. Similar results were obtained when anti-Oct-1 antibody was used in the competition assays, which also inhibited the formation of these two DNA-protein complexes. These results suggest that these two complexes are formed due to interaction of Oct-1 with MMP-14 promoter. The identity of the other DNA-protein complexes formed is not yet known.

Taken together, these results indicate that Oct-1 can directly interact with the MMP-14 promoter. Also, the DNA-binding activity of Oct-1 is increased in response to stimulation by Ox-LDL. Further investigations regarding the expression level Oct-1 in the atherosclerotic plaques are required to characterize the role of Oct-1 in the MMP-14 gene regulation.

A

-140

TCGAGCTGAAACACCACGTCCCCACCGAAAAAGGAGGGCATTGGGGCGGGGGCGGAGGAGAGGCTGTGGG

AGAAGGGAGGGACCAAAGGAGAGCAGAGAGGGCTTCCAACTCAGTTCGCCGACTAAGCAGAAGAAAGATC +1

B

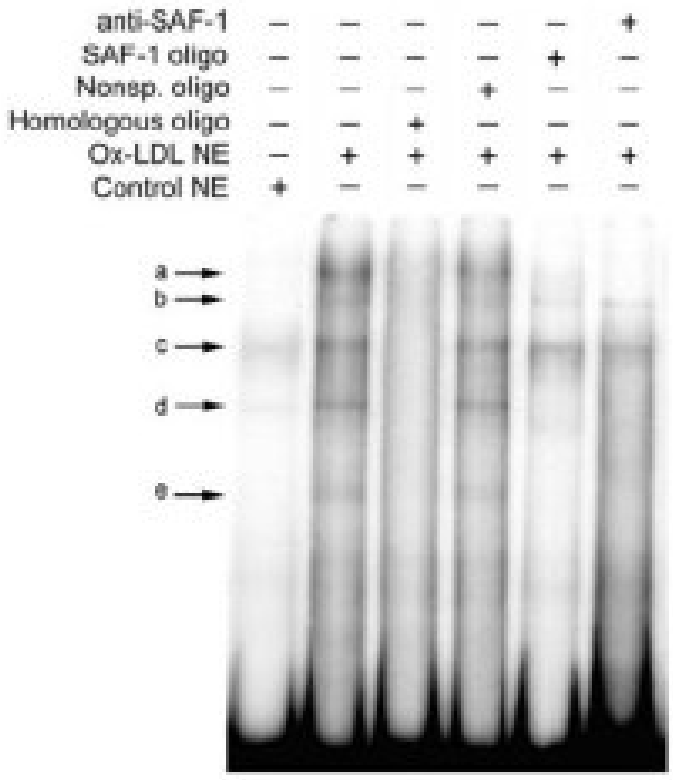

Figure 19: SAF-1 interacts with the MMP-14 promoter. Radiolabeled MMP-14 DNA from -140 to +1 was used as probe in the DNA binding assays using uninduced and ox-LDL induced THP-1 cell nuclear extracts (Panel A). The probe formed several DNA-protein complexes which were characterized using cold homologous oligo, non-specific oligo, SAF-1 specific oligo and SAF-1 antibody (Panel B).

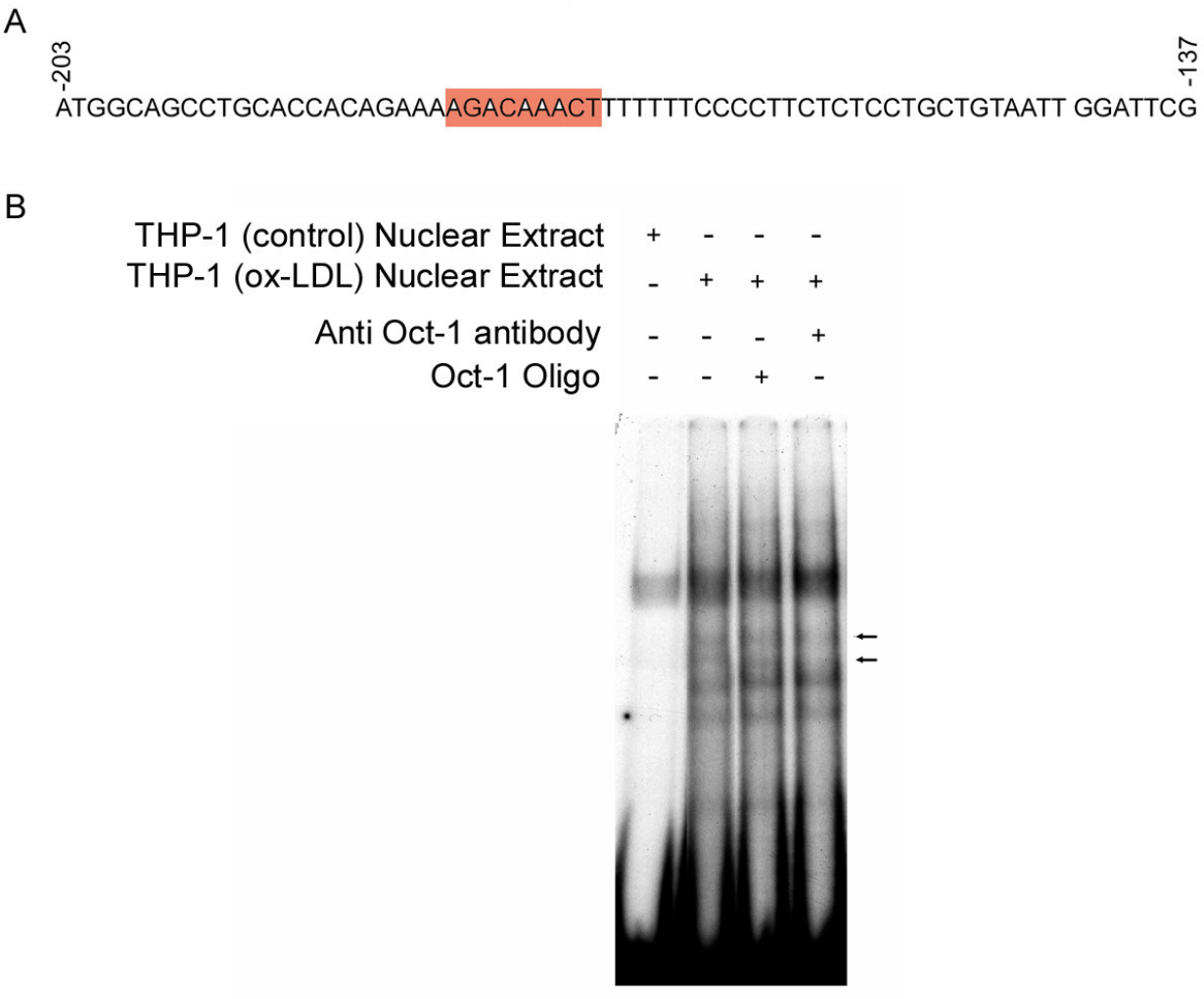

Figure 20: Oct-1 interacts with the MMP-14 promoter. Radiolabeled MMP-14 DNA from -203 to -137 was used as probe in the DNA binding assays using uninduced and ox-LDL induced THP-1 cell nuclear extracts (Panel A). The probe formed several DNA-protein complexes which were characterized using Oct-1 specific oligo and Oct-1 antibody (Panel B).

SAF-1 and Oct-1 interact with the MMP-14 promoter under in vivo conditions

The results of the electrophoretic mobility shift assays have suggested that both SAF-1 and Oct-1 can interact with the MMP-14 promoter through their respective binding sites. To determine whether such an interaction of these proteins with MMP-14 promoter occurs under *in vivo* conditions, we have performed the chromatin immunoprecipitation assay. The uninduced and Ox-LDL induced THP-1 cells were used for the chromatin immunoprecipitation by either anti-SAF-1 or anti-Oct-1 antibody. The immunoprecipitated DNA was identified by PCR amplification of the region of MMP-14 promoter encompassing both SAF-1 and Oct-1 binding sites (Figure 20).

The results show that in the uninduced THP-1 cells, both the anti-SAF-1 and anti-Oct-1 antibodies failed to immunoprecipitate any DNA. Whereas, in the THP-1 cells induced by Ox-LDL, the anti-SAF-1 antibody was able to immunoprecipitate the MMP-14 DNA, as identified by PCR amplification. Similar results were obtained when anti-Oct-1 antibody was used for immunoprecipitation.

These results suggest that both SAF-1 and Oct-1 can interact with the MMP-14 promoter. Although the close proximity of the SAF-1 and Oct-1 binding sites raises the possibility of the physical interaction between these two transcription factors, our present results do not resolve this issue. Investigation of such an interaction during MMP-14 gene regulation is important for deciphering the exact mechanism of MMP-14 gene regulation. Further experiments such as co-immunoprecipitation assays to determine their physical interaction will be performed to investigate this possibility.

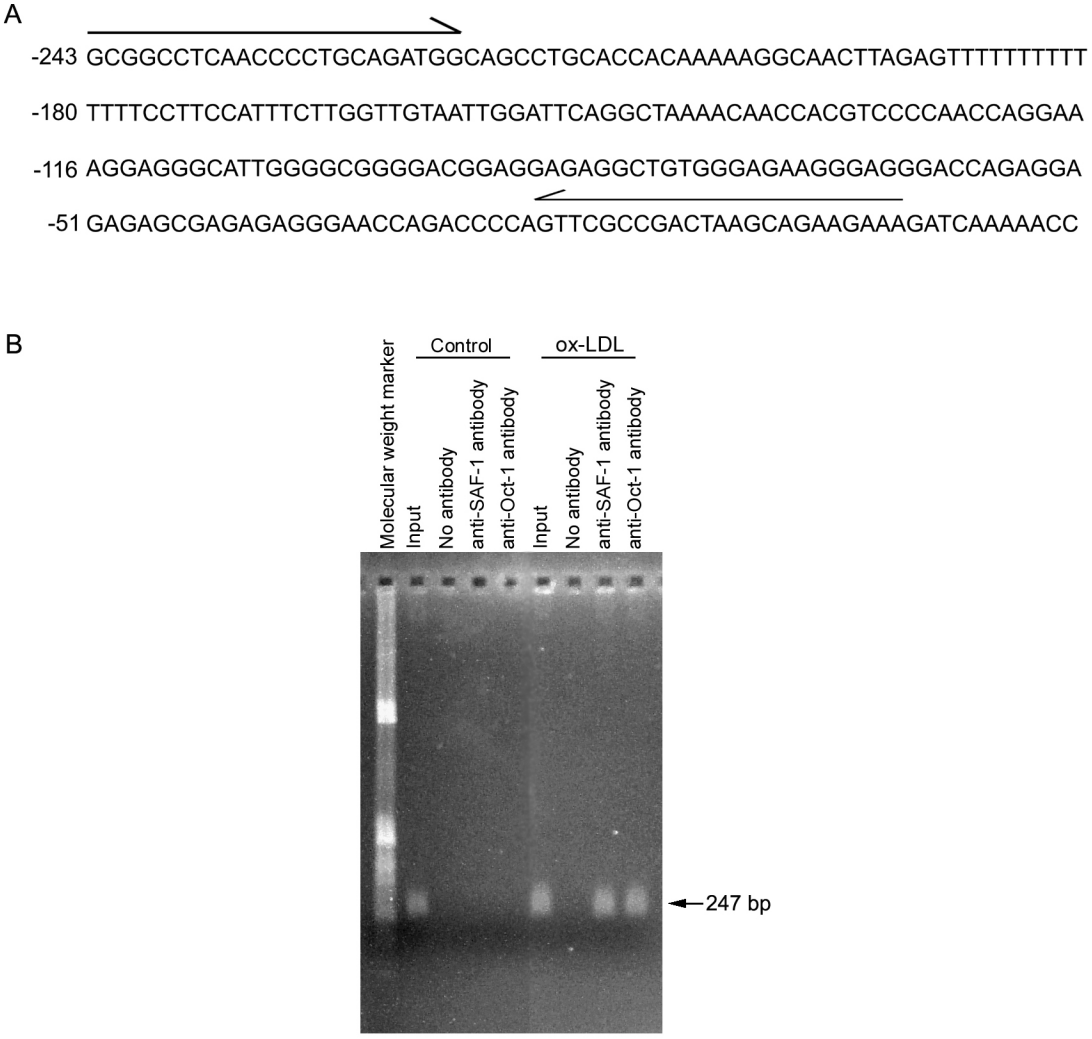

Figure 21: SAF-1 and Oct-1 interact with MMP-14 promoter *in vivo*. Chromatin Immunoprecipitation (ChIP) was performed on the uninduced and ox-LDL induced THP-1 cells with either anti-SAF-1 antibody or anti-Oct-1 antibody to immunoprecipitate the DNA frangment bound with these transcription factors. The immunoprecipitated DNA was amplified using MMP-14 specific primers encompassing both SAF-1 and Oct-1 DNA binding sites of the MMP-14 promoter (Panel A). The PCR products were run on a 1.5% agarose gel and the expected PCR product of size 247 bp was amplified (Panel B).

Discussion

The eukaryotic gene expression is the function of several transcription factors and co-activators interacting with the gene promoter. These transcription factors form a complex on promoter/enhancer regions in order to form the functional transcriptional units to control the expression of the target gene. The promoter regions of the genes have several transcription factor binding sites. These transcription factors not only interact with their binding sites on the promoter, but also with other transcription factors to form a transcriptional machinery that can interact with the basal transcriptional apparatus to drive the transcription. It has been reported that these transcription factor complexes are essential for the activity of the RNA polymerase enzyme (Carey, 1998).

Oct-1, which is a POU domain transcription factor regulate transcription by interacting with other proteins. These POU domain transcription factors can interact with a variety of proteins including DNA-binding transcriptional activators, coregulators, basal factors, and replication factors (Herr and Cleary, 1995). Oct-1 has been reported to interact with other transcription factors to regulate gene transcription, both synergistically and antagonistically. It has been reported to interact with the transcription factors such as Sp1 and AP-1 to synergistically upregulate the expression of human snRNA gene (Strom et al., 1996) and IL-2 (de Grazia et al., 1994) respectively. On the other hand, it also interact with the transcription factors such as retinoid X receptor (RXR) and C/EBPβ to downregulate the expression of promoters containing thyroid hormone response element (Kakizawa et al., 1999; Trial) and immunoglobulin genes (Hatada et al., 2000).

CHAPTER 6

DETERMINATION OF THE MECHANISM BY WHICH SAF-1 AND OCT-1 REGULATE THE EXPRESSION OF MMP-14 GENE

Abstract

Mammalian gene transcription engages a battery of transcription factors, both constitutively expressed and inducible transcription factors. These transcription factors can act as either transcriptional activators or repressors. As we have shown in our previous studies, the MMP-14 gene transcription is regulated by SAF-1 and Oct-1 transcription factors. Our studies have also shown that the transcription of MMP-14 gene is Oct-1 represses the expression of MMP-14 by blocking the action of SAF-1. These transcription factors may interact directly or indirectly with each other during the regulation of MMP-14 gene expression. This study was designed to understand the mechanism by which these transcription factors regulate the expression of MMP-14 gene in the monocyte/macrophage cells. By electrophoretic mobility shift assays, we here show that the heteromeric DNA-protein complex containing SAF-1 and Oct-1 can interact with MMP-14 promoter. We further show here that SAF-1 and Oct-1 can physically interact with each other at SAF-1 or Oct-1 binding sites on the MMP-14 promoter. The physical interaction between these two transcription factors can occur both under *in vivo* and *in vitro* conditions, as we demonstrate here by co-immunoprecipitation assays. We also here show by GAL4AD-DBD system that Oct-1 antagonizes the action of SAF-1 by decreasing its transctivation potential. Together, these results show that there is a direct interaction between these two transcription factors, which is responsible

for the decrease in the transactivation potential of SAF-1, thus decreasing the expression of MMP-14 gene.

Introduction

The eukaryotic gene expression is a complex series of events involving the interaction of various transcription factors with the promoter region of the gene. MMP-14 gene expression, which is a member of matrix metalloproteinase family of proteases, is regulated by several transcription factors including Sp-1, PEA3 and SAF-1 (Lohi et al., 2000; Ray et al., 2004). The co-expression of SAF-1 and MMP-14 enzyme has been reported in the atherosclerotic plaque and SAF-1 has been shown to positively regulate its expression under atherosclerotic conditions (Ray et al., 2004). We have also shown that another transcription factor, Oct-1 is also involved in the regulation of MMP-14 gene. Oct-1 is capable of downregulating SAF-1 mediated expression of MMP-14. Examination of the proximal promoter region of MMP-14 reveals that there is an Oct-1 binding site like sequence present upstream of SAF-1 binding site. This raises the possibility that binding of Oct-1 may be the event that downregulates SAF-1 induced expression of MMP-14. Close proximity of Oct-1 binding site to the SAF-1 binding site also raises the possibility of the interaction between these two transcription factors.

The members of POU family of transcription factors including Oct-1 have been reported to interact with other transcription factors in the regulation of target genes. Moreover SAF-1, which is a zinc finger transcription factor, has also been reported to be interact

with several other transcription factors to regulate the expression of the target genes including SAA, MMP-1 and MMP-9. Moreover, SAF-1 has structural motifs such as two poly-alanine and a single poly-proline tracts which are potential interacting domains for the interaction with other proteins.

The close proximity of SAF-1 and Oct-1 elements in MMP-1 promoter and their antagonistic role in MMP-14 gene transcription suggests that these two proteins might interact physically with each other. In this study we have provided evidence that SAF-1 interacts with Oct-1, both *in vivo* and *in vitro*.

Materials and methods

Protein expression

The BL-21(DE3) pLysS cells were transformed with the pRSET vector (Invitrogen) with the coding sequence for SAF-1 or Oct-1 cDNA. A few colonies were grown next day in the 2X YT culture medium with ampicillin in the culture tubes in the shaker incubator at 37^0C. IPTG was added at the final concentration of 0.1mM to the rapidly growing culture when the OD at 280nm was around 0.5 and allowed to grow at 37^0C in shaker incubator for 4-5 hours. At the end of 4-5 hours, the cells were harvested by centrifugation at 2500 rpm for 5 minutes and the supernatant was discarded. The cells were resuspended in the buffer with no urea. The cells were lysed by 3 freeze-thaw cycles and centrifuged at 14000 rpm for 5 minutes. The supernatant was collected as the soluble fraction. To the membrane pellet, the buffer with urea was added. The membrane pellet was resuspended in the buffer using a pipette tip followed by brief sonication. The suspension was then

centrifuged at 14000 rpm for 5 minutes and the supernatant was collected as the membrane fraction. The soluble and membrane fractions were fractionated by SDS-PAGE to know the degree of expression of the recombinant SAF-1 protein.

The recombinant SAF-1 protein was then purified by the Ni-agarose (Invitrogen) column affinity chromatography following manufacturer's protocol.

Preparation of Nuclear Extracts

The human monocyte THP-1 cells were stimulated with Ox-LDL for 48 hours. Nuclear extracts were prepared both from Ox-LDL induced and uninduced cells as described previously (Ray and Ray 1997). The cells were harvested by centrifugation at 1500 rpm for 5 minutes and washed 3 times with sterile phosphate-buffered saline (PBS). The cells were then resuspended in buffer A (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.5 µg/ml each of leupeptin, antipain, pepstatin, 0.1 µg/ml chymostatin, 0.3 TIU/ml aprotinin, 0.5 mg/ml benzamidine) and incubated on ice for 15 minutes. The cells were vortexed vigorously for 10 seconds and centrifuged at 3000 rpm for 5 minutes to collect the nuclei. The supernatant was collected as Post-nuclear extract. To the nuclei, buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 0.5µl /ml each of leupeptin, antipain, pepstatin, 0.1µl /ml chymostatin, 0.3 TIU/ml aprotinin, 0.5 mg/ml benzamidine) was added and incubated on ice for 30 minutes with occasional vortexing. The supernatant was collected as nuclear extract after centrifugation at 7000 rpm for 5 minutes. Protein concentrations were measured using the method previously described (Bradford, 1976).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA were performed using equal protein amounts of the nuclear extracts prepared from the uninduced and Ox-LDL induced THP-1 cells. The DNA fragments corresponding to base pairs -210 to -5 of MMP-14 promoter was end-labeled using [α^{32}P] dCTP and used as the probe in EMSA reactions. The nuclear extracts were mixed with the binding buffer (10mM HEPES, pH 7.9, 60mM KCl, 5mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, 0.1 mM $ZnCl_2$), 10% (v/v) glycerol, 1µg of poly (dI•dC), $MgCl_2$, and 0.05% (v/v) Nonidet P-40 for 15 minutes at room temperature. 5 X 10^4cpm probe was added to the reaction mixture and further incubated at room temperature for 30 minutes. In some assays, non radioactive competitor double stranded oligonucleotide that contains the SAF binding sequence element of Serum Amyloid A (SAA) promoter from -254 to -226 was added in some assays to assess the specificity of the DNA-protein complexes. Cold probe was included in the reaction mixture as competitor oligonucleotides. For antibody interaction assays, polyclonal anti-SAF-1 antibody raised in rabbit or polyclonal anti-SAF-1 antiserum from mouse was added to the reaction mixture during a preincubation period of 30 minutes on ice. Reaction mixtures were fractionated on a 6% nondenaturing polyacrylamide gel with recirculation of running buffer (7mM Tris-HCl, pH 7.5, 3mM Sodium acetate, 1mM EDTA). The gels were dried and autoradiographed at -80^{0}C with intensifying screen.

Co-immunoprecipitation Assay

The nuclear extracts or the bacterially expressed purified pure proteins, prepared by the above described methods were used for the co-immunoprecipitation assays. The proteins

(nuclear extracts or purified proteins) were incubated with protein G-agarose in presence of IP buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.1% SDS, 3mM Sodium orthovanadate, 0.5 mg/ml Benzamidine, 2.5mM PMSF) at 4^0C for 1 hour with rotory motion. The pre-cleared proteins was first incubated either with anti Oct-1 antibody or anti SAF-1 antibody in IP buffer at 4^0C for 12 hours with rotory motion followed by incubation with protein G-agarose beads for 2 hours at 4^0C with rotory motion. The unbound proteins were removed by washing the beads with three times with ten column volumes of IP buffer. The beads were boiled with Laemmli sample buffer and proteins were fractionated on an 11% SDS-PAGE gel. The proteins were then transferred onto a nitroclellulose membrane. The co-precipitated proteins were detected by Western blotting with anti SAF-1 or anti Oct-1 antibodies.

Construction of GAL4DBDSAF-1 Plasmids—Plasmid RSV-GAL4DBD (Flemington et al., 1993), encoding the DNA-binding domain (DBD) located within amino acids 1–147 of the yeast GAL4 gene, was used to prepare the GAL4DBDSAF-1 construct. Full length SAF-1 cDNA was ligated in frame C-terminal to the GAL4 DNA-binding domain (GAL4DBD).

Cell culture and transfection assays

THP-1 human monocyte cells were used for transient transfection assays using DEAE-dextran method (Sambrook et al., 1989). The amount of DNA in each transfection was kept constant using a carrier DNA. Reporter plasmids and the eukaryotic expression vectors containing GAL4DBD, wild type Oct-1 and GAL4DBDSAF-1AD cDNA were

transfected along with pSV-β galactosidase (Promega). The pSV-β galactosidase plasmid was used as an internal control for measuring transfection efficiency. Cells were harvested 24 hours post-transfection and the CAT activity in the cell extracts was determined as described in previous chapters. β galactosidase activity was assayed with the substrate *o*-nitrophenyl-β-D-galactopyranoside as described (Sambrook et al., 1989). Cell extracts containing equal amounts of β galactosidase activity were used for CAT assay. Prior to CAT assay, the cell extracts were heated at 60^0C for 10 minutes to inactivate endogenous acetylase activity. All transfection assays were performed at least three times.

Results

SAF-1 interacts with Oct-1 at SAF-1 element of MMP-14 promoter

The close proximity of the SAF-1 and Oct-1 binding sites on the MMP-14 promoter and their mutually antagonistic actions on the MMP-14 gene expression raises the possibility that there is a physical interaction between SAF-1 and Oct-1, either directly or indirectly. To investigate the interaction between these two transcription factors, electrophoretic mobility shift assay was performed using bacterially expressed pure SAF-1 and Oct-1 proteins. Radiolabeled MMP-14 probe containing only SAF-1 binding site was incubated with SAF-1 and Oct-1 proteins. We observed the formation of a DNA-protein complex (Figure 21). The formation of this complex was inhibited by the addition of either Oct-1 specific or SAF-1 specific oligonucleotides in the reaction mixture. Moreover, we observed a supershift in DNA-protein complex when anti Oct-1 antibody was added

along with in the reaction mixture. These results indicated that there is a physical interaction between SAF-1 and Oct-1 in the presence of DNA.

In vivo interaction of SAF-1 and Oct-1

To determine the interaction between SAF-1 and Oct-1 under *in vivo* conditions, we used co-immunoprecipitation assay. We used the THP-1 cell nuclear extracts prepared from uninduced and Ox-LDL induced cells. When the proteins were co-immunoprecipitated with anti Oct-1 antibody and Western blotted with anti Oct-1 antibody, the results indicated that SAF-1 and Oct-1 can interact with each other (Figure 22). Similar results were obtained when the proteins were co-immunoprecipitated with anti SAF-1 antibody and Western blotted with anti Oct-1 antibody. These results indicate that SAF-1 can interact with Oct-1 in the absence of DNA.

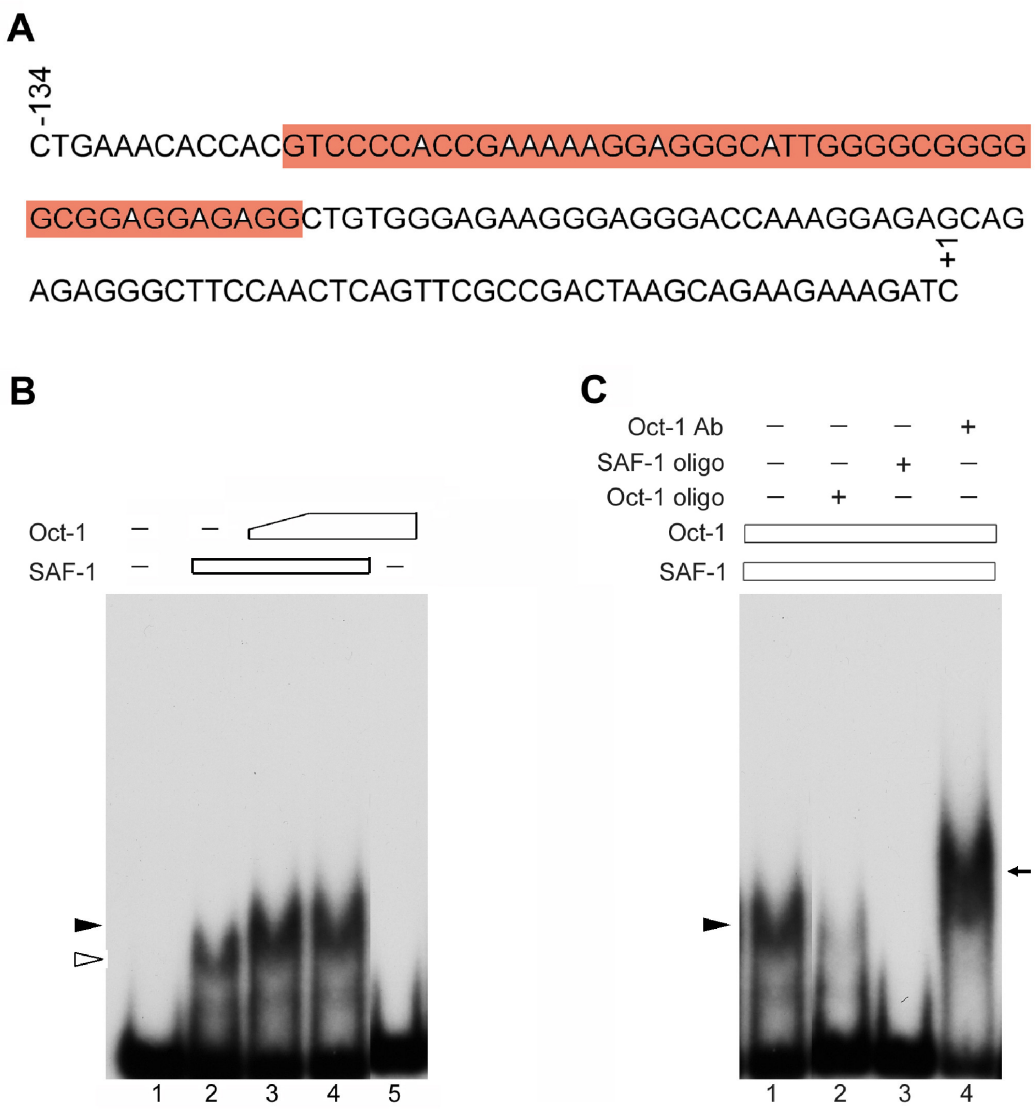

Figure 22: Oct-1 interacts physically with SAF-1. Panel A: DNA sequence of the MMP-14 promoter used as the probe. The SAF-1 binding site is highlighted. Panel B: Binding of purified SAF-1 and Oct-1 proteins to radiolabeled MMP-14 DNA. Lanes 2-4 contains 1 µg of SAF-1 protein. Lanes 3, 4 and 5 contains 0.5, 1 and 1 µg of Oct-1 protein respectively. Open arrowhead indicate the migration position of SAF-1 derived complex and the closed arrowhead indicates SAF-1-Oct-1 complex. Panel C: Characterization of DNA-protein complex. 1 µg each of purified SAF-1 and Oct-1 proteins were incubated in presence of Oct-1 specific (lane 2), SAF-1 specific (lane 3) oligonucleotides and anti Oct-1 antibody (lane 4). Oct-1 and SAF-1 specific oligonucleotides inhibited the complex formation indicated by closed arrowhead while anti Oct-1 antibody supershifted the complex indicated by closed arrow.

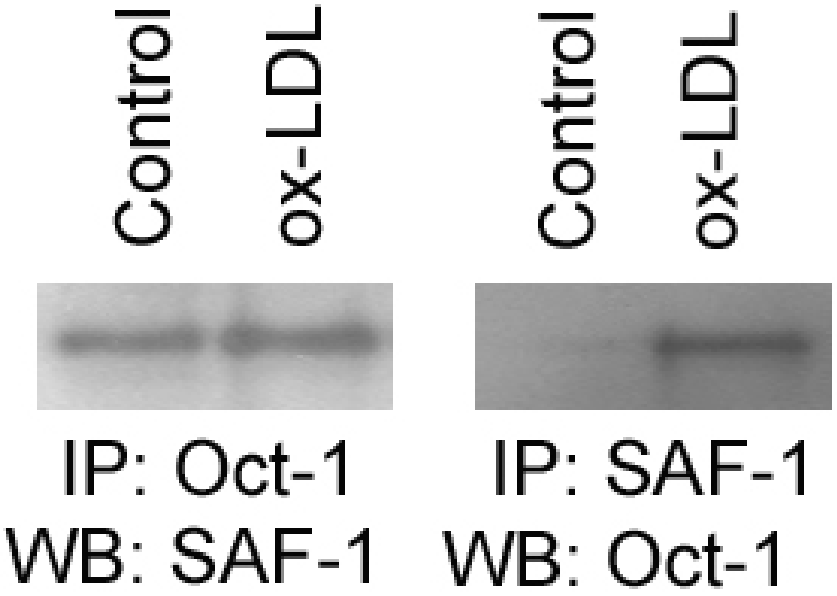

Figure 23: SAF-1 and Oct-1 interact with each other *in vivo*. Co-Immunoprecipitation of the SAF-1 protein by using anti-Oct-1 antibody and the co-immunoprecipitation of Oct-1 protein using anti-SAF-1 antibody suggests the *in vivo* interaction between SAF-1 and Oct-1 proteins.

SAF-1 and Oct-1 interact physically under in vitro conditions

To investigate the possibility that SAF-1 and Oct-1 can interact with each other in the absence of DNA under *in vitro* conditions, we studied the protein-protein interaction using the co-immunoprecipitaion assay using bacterially expressed purified proteins. The proteins were incubated with each other in the absence of any DNA or in the presence of either SAF-1 specific, Oct-1 specific or non-specific DNA. When the proteins were co-immunoprecipitated with anti Oct-1 antibody and Western blotted with anti SAF-1 antibody, the results indicated that SAF-1 and Oct-1 are able to interact with each other in the presence of either SAF-1 specific or Oct-1 specific DNA (Figure 24). Interestingly, SAF-1 and Oct-1 proteins failed to interact with each other in the absence of any DNA or in presence of non-specific DNA. Similar results were obtained when anti SAF-1 antibody was used for co-immunoprecipitation followed by Western blotting with anti Oct-1 antibody (Figure 23).

These results indicate that SAF-1 and Oct-1 interact with each other in the presence of the SAF-1 or Oct-1 specific DNA.

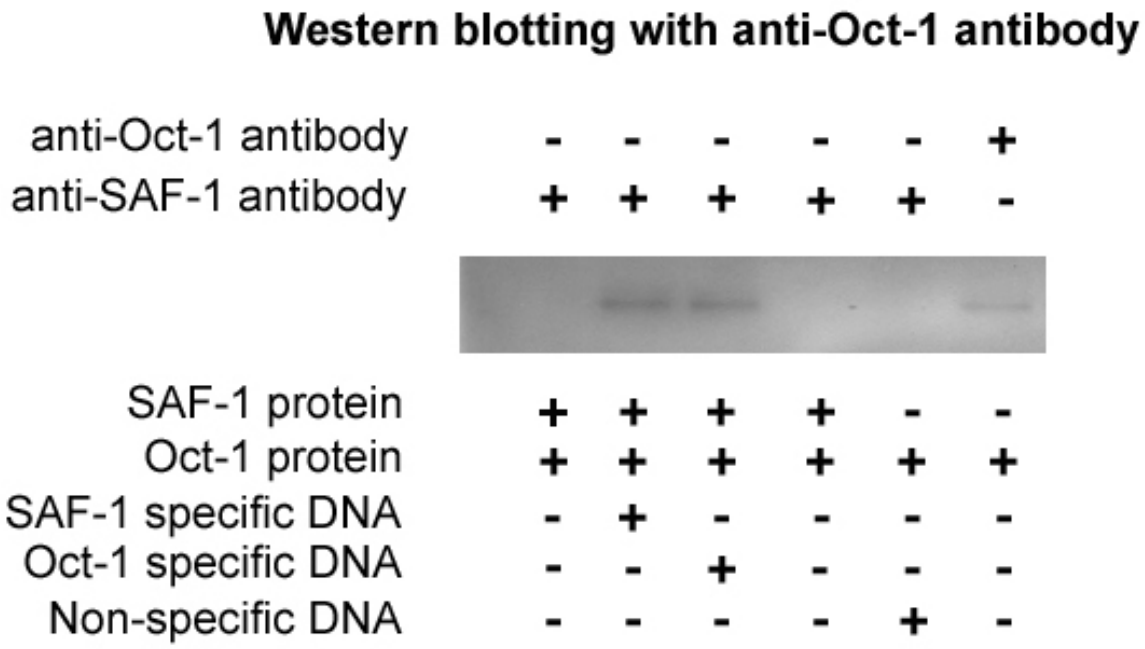

Figure 24: Oct-1 antibody immunoprecipitates SAF-1 protein *in vitro*. Bacterially expressed and purified SAF-1 and Oct-1 proteins were used for the co-immunoprecipitation assay to determine the interaction between these transcription factors. The results show that the interaction between SAF-1 and Oct-1 can occur only if either SAF-1 or Oct-1 proteins are bound to their respective binding sites. Absence of DNA or presence of unrelated DNA failed to facilitate the interaction between these two transcription factors.

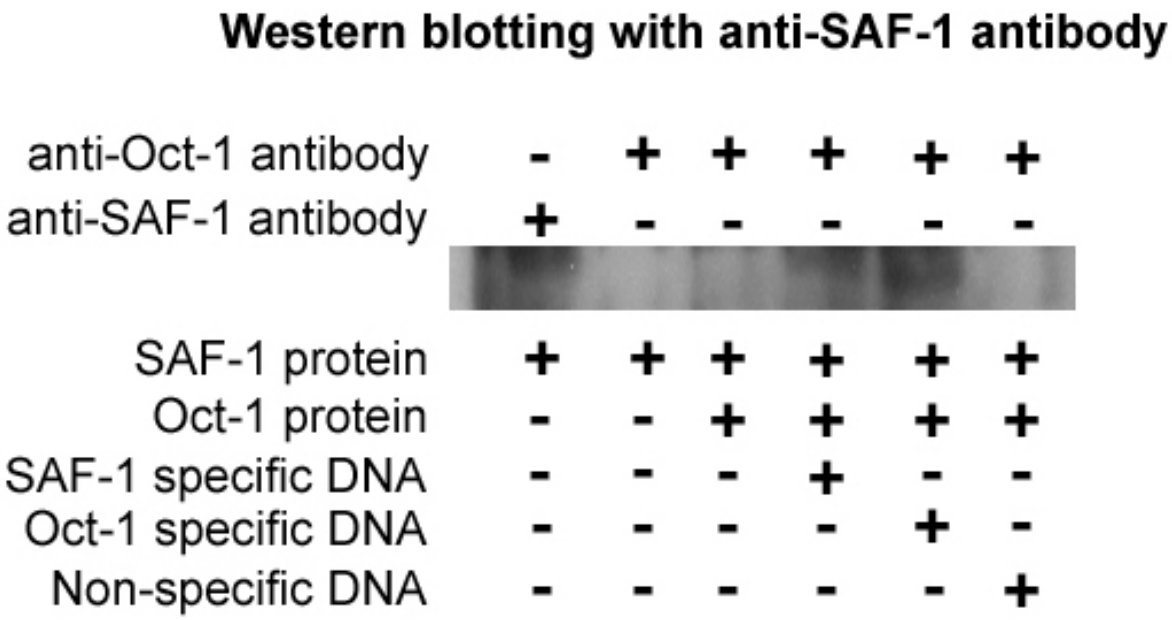

Figure 25: SAF-1 antibody immunoprecipitates Oct-1 *in vitro*. Bacterially expressed and purified SAF-1 and Oct-1 proteins were used for the co-immunoprecipitation assay to determine the interaction between these transcription factors. The results show that the interaction between SAF-1 and Oct-1 can occur only if either SAF-1 or Oct-1 proteins are bound to their respective binding sites. Absence of DNA or presence of unrelated DNA failed to facilitate the interaction between these two transcription factors.

Oct-1 inhibits the activation domain of SAF-1

The chimeric proteins with the DNA binding domain (DBD) of the GAL4 expression plasmid were made containing the activation domain of SAF-1, to test the possibility that Oct-1 may influence the SAF-1 activity by causing changes in the activation domain of SAF-1. The plasmids were transiently transfected in the THP-1 cells and the CAT activity was measured (Figure 25).

The results indicate that Oct-1 does not increase the expression of the reporter gene, whereas the expression of the SAF-1GAL4DBD chimeric protein increased the expression of CAT reporter five folds. Interestingly, the expression of the Oct-1 decreased the SAF-1 mediated expression of the reporter gene. These results suggest that Oct-1 brings about certain changes in the activation domain of SAF-1, such that it looses its ability to increase the transcription of the target gene.

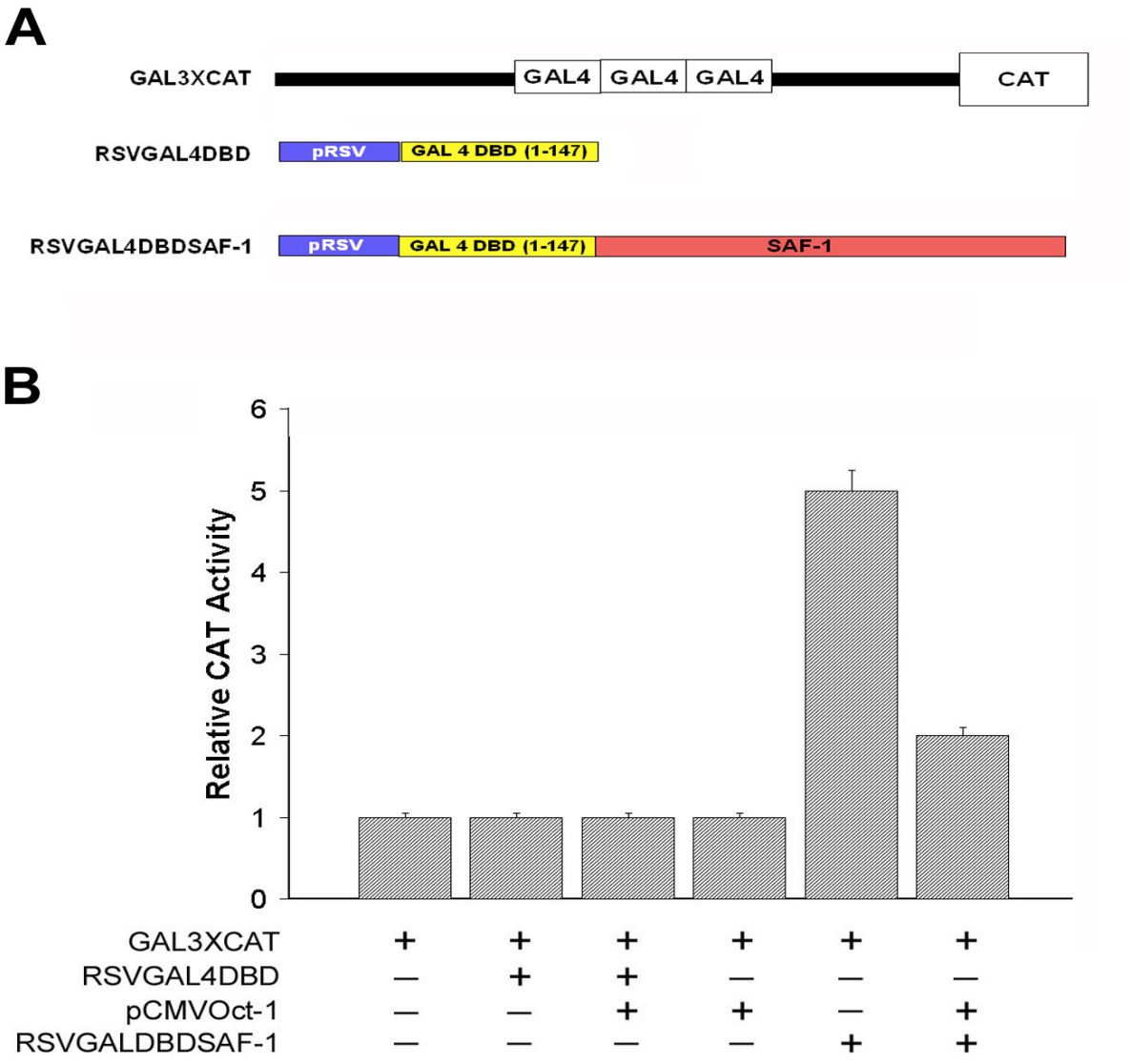

Figure 26: Oct-1 represses the SAF-1 activity by decreasing its transactivation poteintial. The GAL4CAT reporter construct was made with 3 copies of yeast GAL4 DNA binding site. We also constructed mammalian expression plasmids for GAL4 DNA binding domain, Oct-1 expression and a chimeric protein with DNA binding domain of yeast GAL4 and sctivation domain of SAF-1. Using this chimeric protein with DNA binding domain of GAL4 gene and activation domain of SAF-1, we here show that Oct-1 is able to inhibit the action of SAF-1 by altering its transactivation potential.

Discussion

In this study we have identified that the inflammation responsive transcription factor SAF-1 can interact with an ubiquitously expressed transcription factor Oct-1, both in presence and absence of DNA. By using different approaches we have shown that SAF-1 has high affinity for Oct-1 protein.

The close proximity of the SAF-1 and Oct-1 binding sites on the MMP-14 promoter suggested us that there may be some physical interaction between these two transcription factors. Our experiments have clearly suggested that SAF-1 and Oct-1 can physically interact with each other, both *in vivo* and *in vitro*. The *in vitro* co-immunoprecipitation experiments have shown that SAF-1 and Oct-1 can not interact with each other in the absence of DNA, which is not in agreement with our *in vivo* co-immunoprecipitation experiments, where we have shown that SAF-1 can interact with Oct-1 even in the absence of DNA. This phenomenon can be explained by the fact that there is no post-translational modification of the proteins expressed in bacterial cells which were used in the *in vitro* experiments, which may be required for the interaction to occur under *in vivo* conditions.

Our experiments involving protein-protein interaction between SAF-1 and Oct-1 have suggested that both SAF-1 and Oct-1 can physically interact with each other without inhibiting their respective DNA binding activity. We have also shown by transient transfection assays that Oct-1 is not fully able to inhibit the action of SAF-1. Thus, we hypothesize that under normal physiological conditions when the expression of SAF-1 is

at the basal level, Oct-1 can inhibit the binding of SAF-1 to the MMP-14 promoter, as suggested by chromatin immunoprecipitation experiments. But under inflammatory conditions, when the expression of SAF-1 increase several folds, with relatively very less increase in the nuclear localization of Oct-1, Oct-1 fails to inhibit the SAF-1 binding to its binding site on MMP-14 promoter.

By EMSAs, we have seen that there is slight increase in the DNA-binding activity of SAF-1 under the conditions of atherosclerosis and the complex formed by interaction of SAF-1 and Oct-1 can bind to the MMP-14 promoter. This raised the possibility whether Oct-1 can still inhibit the SAF-1 mediated transcription of MMP-14, as these results can open up the potential for the development of new therapeutic measures directed towards Oct-1, for the control of MMP-14 gene expression. We here also show that Oct-1 is able to inhibit the expression of the reporter gene to almost basal level by inhibiting the transactivation potential of SAF-1. This phenomenon may be an additional level of control of MMP-14 gene expression by Oct-1.

Taken together, these findings suggest the multiple level of control exerted by Oct-1 on the SAF-1 mediated transcription of MMP-14 gene. On one hand SAF-1 and Oct-1 can physically interact with each other forming an inhibitory complex which can reduce the ability of SAF-1 to interact with MMP-14 promoter. Moreover, some degree of inhibition is also provided by Oct-1 inhibiting the transactivation potential of SAF-1 when the complex is able to bind with the MMP-14 promoter.

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