THE ROLE OF CELL CYCLE PROTEINS IN ATHEROSCLEROSIS

BY

MARJORIE ELLEN ZETTLER

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

Department of Physiology
Faculty of Medicine
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Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA
FACULTY OF GRADUATE STUDIES
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Anybody who has ever been seriously engaged in scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: **"Ye must have faith."**

- Maxwell Planck

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aFGF	acidic fibroblast growth factor (FGF-1)
AP-1	activator protein-1
ADP	adenosine diphosphate
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor (FGF-2)
BHT	butylated hydroxytoluene
CDK	cyclin dependent kinase
Ci	Curie
CKI	Cdk inhibitor
CRP	C-reactive protein
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
DTT	dithiothreitol
EC	endothelial cell
EDTA	ethylenediamine tetra-acetic acid
EGTA	ethyleneglycotetra-acetic acid
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
Fe-ADP	ferrous adenine diphosphate
FGF-1	fibroblast growth factor-1 (acidic fibroblast growth factor)
FGF-2	fibroblast growth factor-2 (basic fibroblast growth factor)

FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage colony-stimulating factor
GST	glutathione-S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2O_2	hydrogen peroxide
IkappaB	inhibitor kappaB
IL	interleukin
IFNγ	interferon-γ
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride
IP_3	D-myo-inositol 1,4,5-triphosphate
JAK	janus activated kinase
LacCer	lactosylceramide
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LPC	lysophosphatidylcholine
LPDS	lipoprotein-depleted serum
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MDA	malondialdehyde
MEK1/2	MAP-ERK kinase 1/2
$MgCl_2$	magnesium chloride
mRNA	messenger ribonucleic acid

NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NCDC	2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate
NER	nuclear extraction reagent
NF-κB	nuclear factor kappaB
NP-40	nonidet P-40
OxLDL	oxidized low density lipoprotein
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PI 3-kinase	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMS	phenazine methosulphate
PMSF	phenylmethyl sulphonyl fluoride
PPAR-γ	peroxisome proliferator activator receptor-γ
Rb	retinoblastoma gene product
RIPA	radioimmunoprecipitation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM	standard error of the mean
SM α-actin	smooth muscle α-actin
STATs	signal transducers and activators of transcription
TBARS	thiobarbituric acid reactive substances
TBq	terabecquerel
TGF-β1	transforming growth factor-β1
TNF-α	tumor necrosis factor -α
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell

ABSTRACT

Oxidized low density lipoprotein (oxLDL) is a causative agent in the development and progression of atherosclerosis. It is believed to act in part by stimulating proliferation of cells in the vessel wall. The purpose of the first study was to test the hypothesis that oxLDL induces proliferation through changes in the expression, distribution, and activation of cell cycle proteins. Quiescent human fibroblasts and rabbit smooth muscle cells were treated with 0, 10 or 50 μg/ml oxLDL for 24-48 hours. This resulted in significant increases in total cell numbers at both concentrations of oxLDL, at both time points, for both types of cells. Western blot analysis revealed that oxLDL-stimulated cell proliferation was associated with significant increases in the expression of proteins that regulate entry into and progression through the cell cycle (Cdc 2, Cdk 2, Cdk 4, Cyclin B1, Cyclin D1 and PCNA). Surprisingly, the expression of cell cycle inhibitors ($p21^{cip1}$ and $p27^{kip1}$) was stimulated by oxLDL as well but this was to a lesser extent than the effects on cell cycle activating proteins. OxLDL also induced nuclear localization of all cell cycle proteins examined. The similar effects of oxLDL on the translocation and expression of both cell cycle activating and inhibitor proteins may explain the controlled proliferative phenomenon observed in atherosclerosis as opposed to the more rapid proliferative events characteristic of cancer.

Our next study tested the hypothesis that this mitogenic effect may be further enhanced by the presence of cytokines and growth factors known to be present in the atherosclerotic environment. Quiescent fibroblasts and smooth muscle cells were treated with 10 or 50 μg/ml oxLDL in combination with serum for 24 or 48 hours. Surprisingly,

these cells showed inhibited release from growth arrest and a significant reduction in the number of cells completing the cell cycle (as compared to cells treated with serum alone). This was not due to an induction of apoptosis. The anti-proliferative effects were not closely associated with changes in the expression of cell cycle proteins. Instead, oxLDL inhibited the translocation of cell cycle proteins Cdc 2, Cdk 2, Cdk 4, Cyclin A, Cyclin B1, Cyclin D1 and PCNA into the nucleus, as compared to separate treatments with serum alone. Kinase activation associated with specific cell cycle proteins was also inhibited by oxLDL. These data demonstrate that oxLDL has a surprising inhibitory effect on cell proliferation in the presence of serum that occurs by a mechanism involving an inhibited import of cell cycle proteins into the cell nucleus.

The preceding studies were carried out under *in vitro* cell culture conditions. The alterations in cell cycle proteins that may accompany atherosclerosis under *in vivo* conditions, however, remain unclear. Therefore, our final study examined the presence of cell proliferation within a primary atherosclerotic plaque. Identification of a change in the expression of several cell cycle proteins and the activities of their related kinases would provide valuable supportive evidence of mitotic activity in the atherosclerotic lesion. Atherosclerotic aortic vessels from cholesterol-fed rabbits were analyzed for expression of Cyclin A, Cdk 4 and PCNA. Activities of Cdk 4, Cdk 2 and Cdc 2 were also assessed. Whole aortic extracts were generated from rabbits fed a cholesterol-supplemented diet for 8 weeks to induce modest plaque development, or 16 weeks to induce severe plaque progression. At both time points, expression of Cyclin A, Cdk 4 and PCNA was significantly elevated. All three cyclin dependent kinase activities were also increased. There were no significant differences between early and late stage atherosclerosis. When

the plaques were selectively extracted from the 8 week aortic tissue and the non-plaque tissue removed, a significantly higher expression level for Cyclin A, Cdk 4 and PCNA was detected within the plaque. The tissue that contained no visible plaque had lower but significantly elevated expression levels for the three cell cycle proteins. In summary, the primary atherosclerotic plaque exhibits elevated expression levels and activities of several cell cycle proteins. This would confirm earlier studies that the primary plaque is in a mitotic state. Furthermore, cell proliferative activity appears to be similar in moderate and severe atherosclerotic plaques and is even detected in the neighboring non-atherosclerotic vascular tissue.

CHAPTER 1: INTRODUCTION

1.1 Cell cycle proteins

1.1.1 Inducers

The eukaryotic cell division cycle is controlled by periodic changes in the activity of cyclin dependent kinases (Cdks). Progression through the cell cycle depends on the coordinated synthesis, activation, and degradation of a family of cyclins that act as catalytic subunits of Cdks. Different cyclin/Cdk holoenzymes are activated at specific phases of the cell cycle and exert their regulatory control by phosphorylating key proteins involved in cell cycle progression (Figure 1). Passage through the first gap phase (G1) requires both Cyclin D-dependent Cdk 4 and Cdk 6 and Cyclin E/Cdk 2 holoenzymes. DNA synthesis (S phase) requires functional Cyclin A/Cdk 2 complexes. Following this, Cyclin A/Cdc 2 and Cyclin B/Cdc 2 pairs are assembled and activated during the second gap phase (G2) and mitosis (M phase) (71, 92, 96, 119, 165, 173, 181, 204). A complete list of all identified cyclins and Cdks and their sites of action within the cell cycle is shown in Table 1. An example of a change in Cyclin D1 nuclear fluorescence as a function of the cell cycle in fibroblasts is shown in Figure 2.

1.1.2 Suppressors

The activity of these kinases is directed in part by inhibitors of Cdks. Two classes of Cdk inhibitors (CKIs) exist. The first is the Cip/Kip family, which includes $p21^{cip1}$, $p27^{kip1}$ and p57. These CKIs regulate cell proliferation throughout the cycle. The second, sometimes referred to as the INK4 proteins (inhibitors of Cdk 4/6) are active only at G1 and include p15, p16, p18 and p19 (83, 182, 221). A complete list of all identified CKIs

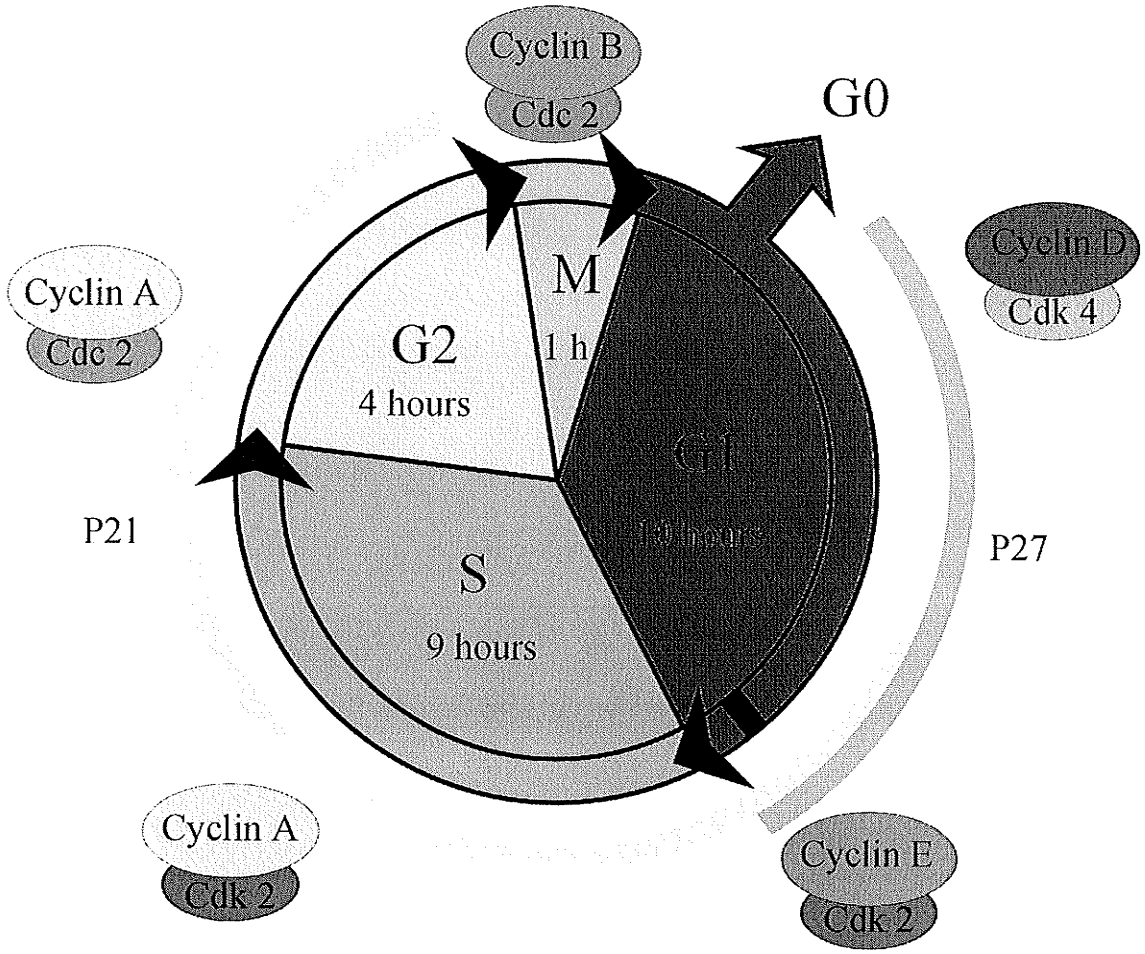

Figure 1. The involvement of specific cell cycle proteins during different phases of the cell cycle. Cyclin/Cdk complexes are shown adjacent to their points of action in the cell cycle. Periodicity of CKIs is indicated by bars outside cycle.

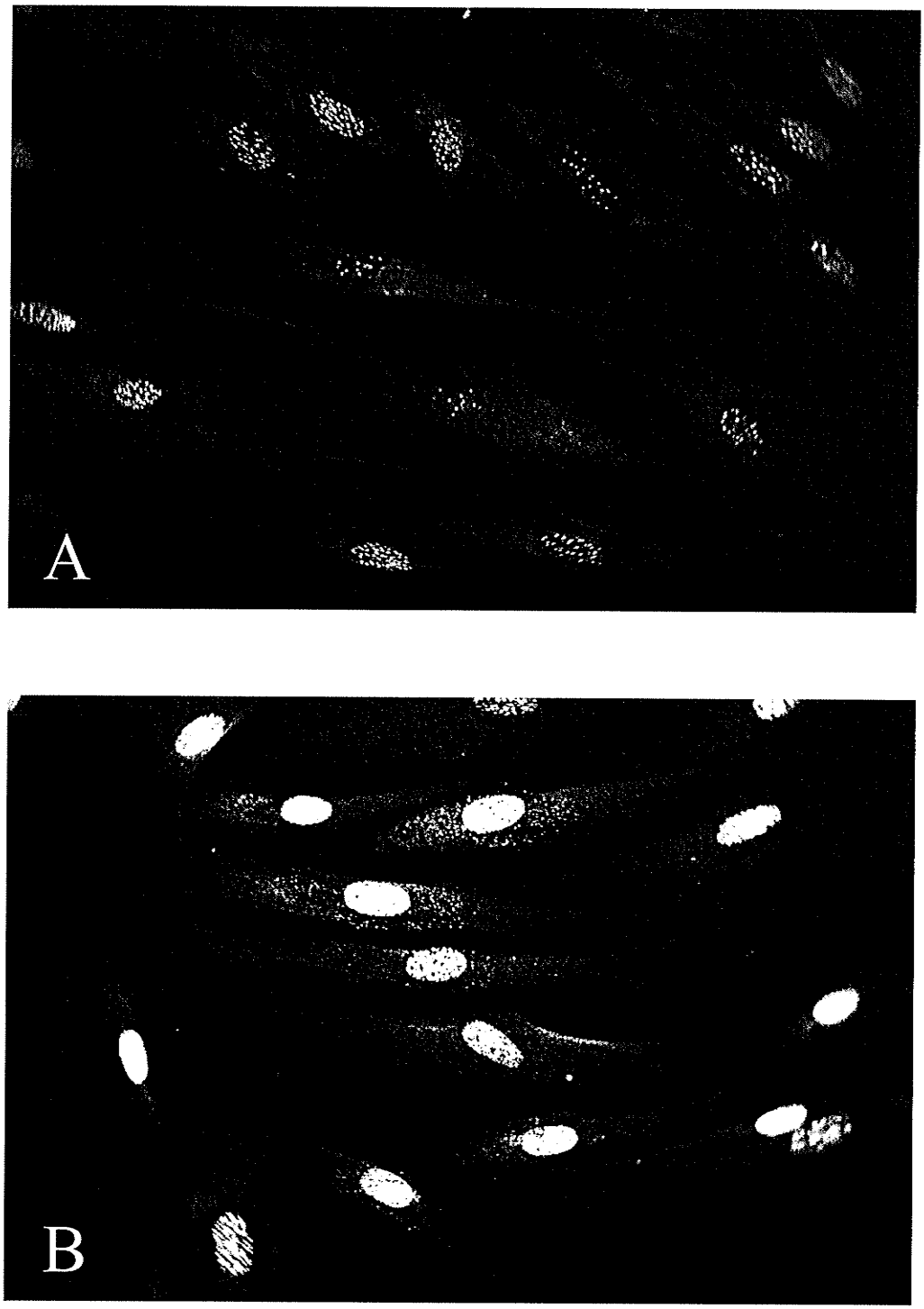

Figure 2. Confocal microscopic images illustrating nuclear fluorescence of Cyclin D1 in quiescent and proliferating fibroblasts. Human fibroblasts were stained with Cyclin D1 antibody and fluorescein isothiocyanate following A) 8 days in serum-free medium and B) 6 days in serum-free medium followed by 1 day in 5% FBS.

Table 1. Cell cycle regulatory proteins and sites of action within the cell cycle

Proteins	**Cell cycle**				
	G0-G1	G1-S	S	G2	M
Cyclins	C, D	A, E, H, X	A, E, B	B, F, A, H	A, B
Cdks	4, 5, 6	1, 2, 5, 7	1, 2, 5	1, 7	1
CKIs	p15, p16, p18, p19, $p21^{cip1}$, $p27^{kip1}$, p57	$p21^{cip1}$, $p27^{kip1}$, p57	$p21^{cip1}$, $p27^{kip1}$, p57	$p21^{cip1}$	$p21^{cip1}$
Others	p53, pRb, p107, p130	pRb, E2F, p107, p130	E2F, PCNA	p53	PCNA

Adapted from Li et al, Eur Heart J 1999:20;406-20.

.eferences: (31, 66, 77, 99, 108, 114, 138, 157, 162, 178, 180, 194, 197, 200, 225, 239, 258, 262, 263)

and their sites of action within the cell cycle is shown in Table 1.

The retinoblastoma gene product (Rb) is also an important negative regulator of proliferation (Figure 3). In the resting G0 state, Rb is present in the unphosphorylated form and is a potent inhibitor of cell cycle progression. Hypophosphorylated Rb binds to the cell cycle regulatory transcription factor E2F, inhibiting its activity and so preventing transcription of genes involved in the transition of cells from G1 to S phase, including Cdc 2 and proliferating cell nuclear antigen (PCNA) (43, 257). Rb is phosphorylated by Cyclin D/Cdk in response to growth factor stimulation and in this state permits cells to traverse the G1/S checkpoint of the cell cycle and proliferate (50, 54). The Rb-related proteins p107 and p130 contain a motif similar to one found in the $p21^{cip1}$ family which enables binding and inhibition of Cdks, and are also able to repress E2F activity (246, 262, 270). Ultimately, all upstream cellular mitogenic signaling cascades are dependent on G1 events, including the synthesis of D-type cyclins and their assembly with Cdk 4/6, to progress through the cell cycle (258). Therefore, the cell cycle becomes a point of convergence for all pathways affecting proliferation, relying on the Rb pathway to coordinate information from both extracellular and intracellular sources (137).

$p21^{cip1}$, the 21kD protein product of the Waf1/Cip1 gene, acts as a universal inhibitor, able to bind to and inhibit Cdks at all stages of the cell cycle (264) (Figures 1 and 3). $p21^{cip1}$ monomers normally associate with active Cyclin D/Cdk complexes in proliferating fibroblasts. Overexpression of $p21^{cip1}$ potently inhibits Cyclin D/Cdk kinase activity and arrests cells in G1 (274). Therefore, one mechanism by which $p21^{cip1}$ inhibits cell cycle progression may be by negatively regulating the Cyclin D/Cdk dependent phosphorylation of Rb. A second means by which $p21^{cip1}$ effects negative control of the

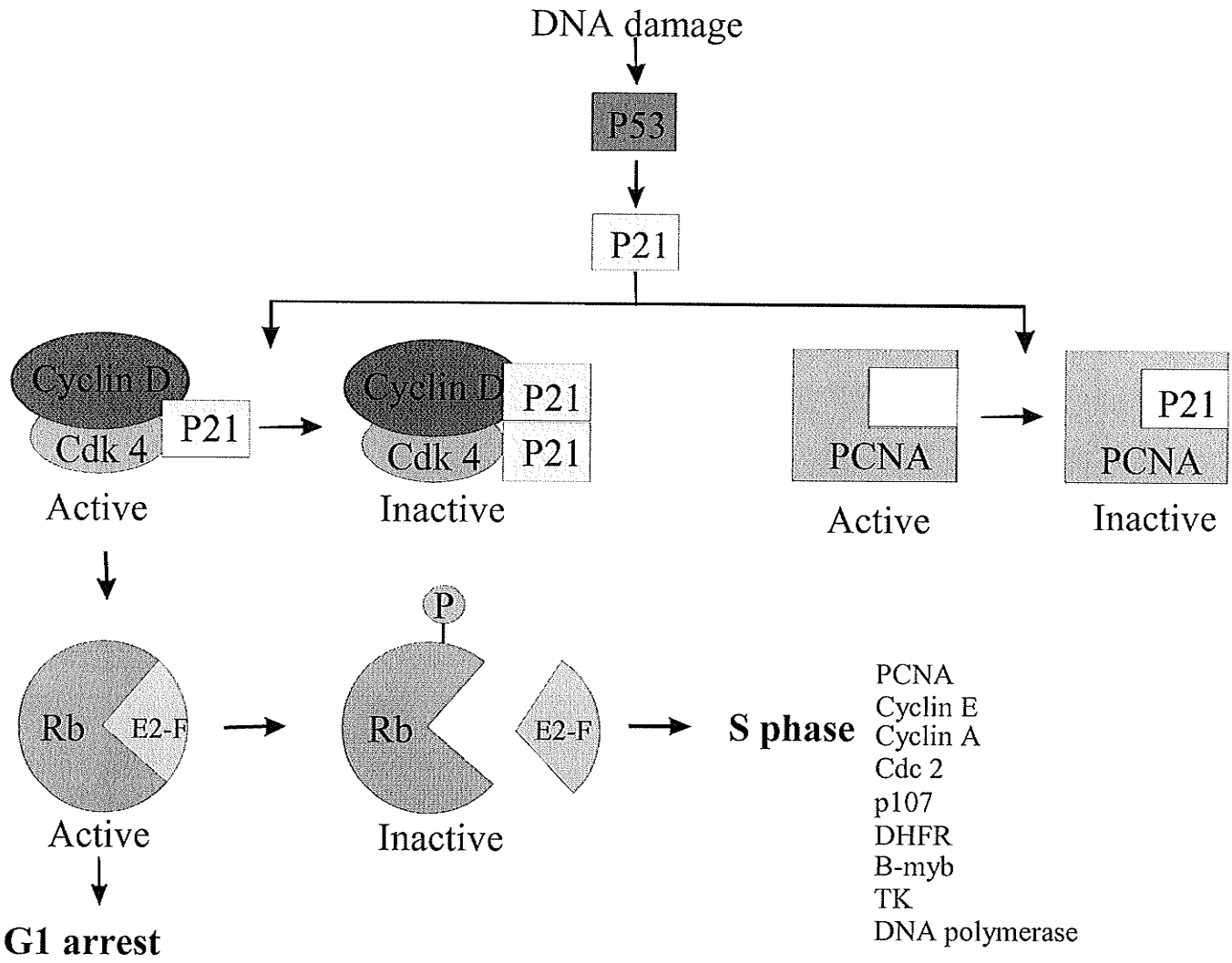

Figure 3. Schematic illustration of the mechanisms by which $p21^{cip1}$ effects cell cycle arrest. $p21^{cip1}$ is induced by p53. Binding of $p21^{cip1}$ to PCNA renders it inactive and prevents DNA replication. Binding of $p21^{cip1}$ to Cyclin D/Cdk 4/6 prevents phosphorylation of Rb. Hypophosphorylated Rb remains bound to transcription factor E2-F, prohibiting entry into the cell cycle.

cell cycle may be by binding to and inhibiting the DNA polymerase cofactor PCNA, preventing DNA replication (128). It is believed that different regions of the $p21^{cip1}$ protein are active in inhibiting Cdks and PCNA, so the two mechanisms for halting the cell cycle are independent (139). Consistent with this hypothesis, recent evidence indicates that adenovirus mediated overexpression of human $p21^{cip1}$ inhibits proliferation of rat vascular smooth muscle cells (VSMCs) *in vitro* by inhibiting phosphorylation of Rb and by formation of complexes between $p21^{cip1}$ and PCNA (35).

$p21^{cip1}$ expression is also regulated by the tumor suppressor gene p53 (65) (Figure 3). p53 is induced in response to DNA damage (249). Phosphorylation of p53 by the S and G2/M cyclin/Cdk complexes induces a conformational change, increasing its ability to bind a target site within the promoter of the $p21^{cip1}$ gene (255). Transcription of $p21^{cip1}$ is activated and accumulation of $p21^{cip1}$ protein results in a G1 block of the cell cycle. In this way, $p21^{cip1}$ may carry out p53's growth suppressive function (63, 140, 247).

Recent evidence suggests that $p21^{cip1}$ may work to inhibit migration and adhesion as well as proliferation. Cultured rabbit VSMCs transfected with $p21^{cip1}$ exhibited decreased spreading, attachment and migration in response to platelet derived growth factor (PDGF). $p21^{cip1}$ effectively inhibited migration by preventing assembly and disassembly of actin filaments. $p21^{cip1}$ has also been shown to alter the expression of genes involved in cellular adhesion and translocation of adhesion molecules (73). Adhesion to the extracellular matrix is required for Cyclin D1 and Cyclin A expression, Cyclin E/Cdk 2 kinase activity, and Rb and p107 phosphorylation (9, 275). Thus, suppression of vascular cell growth by $p21^{cip1}$ may result from an altered interaction with the extracellular matrix.

$p27^{kip1}$, which shares a region of homology with $p21^{cip1}$, complexes with Cyclin E/Cdk 2 in cells growth arrested with either transforming growth factor β (TGF-β) or contact inhibition (196, 218). The increase in $p27^{kip1}$ levels is due in part to translational upregulation and in part to decreased degradation (56). Unlike $p21^{cip1}$, $p27^{kip1}$ does not associate with cyclin/Cdk complexes in proliferating cells. Ectopic overexpression of $p27^{kip1}$ in cultured VSMCs downregulated Cdk 2 activity and repressed transcription from the Cyclin A promoter (42). Therefore, $p27^{kip1}$ may be a mediator of VSMC growth arrest *in vitro*. Cellular changes normally associated with migration (such as alignment of actin filaments and focal adhesions) were also inhibited in rat VSMC infected with a retrovirus that overexpressed $p27^{kip1}$ (57). $p27^{kip1}$-dependent migration blockade required inactivation of Cdk/cyclin complexes (57). This effect may be mediated through the accumulation of hypophosphorylated Rb and repression of E2F target genes (57).

1.1.3 Cell cycle protein involvement in the pathology of disease

Eukaryotic cells respond to pathologic stimuli in different ways at the molecular level. Injury and stress may induce some cell types, including VSMCs, fibroblasts, and lymphocytes, to proliferate. The same cells may undergo cell cycle arrest and apoptotic death in response to more severe types of damage. While these responses serve an adaptive function, dysregulation may become pathological. A well-characterized example is cancer. One or more cell cycle regulatory components, either protooncogenes (cyclins, Cdks) or tumor suppressors (Cdk inhibitors) are defective in nearly every type of tumor. Mutations in protooncogenes or tumor suppressors tend to uncouple cells from environmental signals and so the cell cycle continues to be pushed forward. Regardless of

extracellular cues, G1 cyclins are induced, overriding Cdk inhibitors and preventing cell cycle arrest. The result is defective or abolished restriction point control. Deregulation by a number of molecular mechanisms has been documented, including gene amplification (Cyclin D1, D2, E, Cdk 4, Cdk 6), chromosomal translocation or inversion (Cyclin D1), mutations which cause activation (Cdk 4) or inactivation (Rb, p16, p57), inactivation by protein-protein sequestration (Rb), aberrantly enhanced ($p27^{kip1}$) or diminished (D cyclins) protein degradation, or activation by proviral integration (D cyclins) (14, 220).

In the case of VSMCs, mature cells which are post-mitotic and express markers of the differentiated phenotype at homeostasis can undergo phenotypic modulation in response to several environmental stimuli (189). This change from "contractile" to "synthetic" phenotype is necessary for both cell migration and proliferation to occur. Changes at the level of gene expression cause cells to increase their production of components of the extracellular matrix and synthetic organelles (such as rough endoplasmic reticulum and free ribosomes). At the same time, expression of α-actin is decreased. As a result of these changes, cells can no longer contract, but are capable of division. Dysregulated cell proliferation in response to arterial injury plays a key role in the development of atherosclerosis and restenosis (198, 215, 242). For this reason, an understanding of the fundamental basis of vascular cell proliferation and migration is crucial to an understanding of the pathogenesis of the disease.

1.2 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease (206). It is a systemic disorder, but the complications of the disease occur at focal sites in the circulatory system.

Atherosclerosis primarily affects the large and medium arteries, including the coronary, carotid, basilar and vertebral arteries, and can also involve the aorta, iliac and femoral arteries (129). It can lead to ischemia of the heart, brain, or extremities, resulting in infarction (129). Ischemic heart disease, the leading cause of morbidity and mortality in North America, Europe and Japan, is a direct consequence of atherosclerosis (1, 27, 28).

Cardiovascular disease is the underlying cause of death for 1 in 3 Canadians (1). Risk conditions for cardiovascular disease include age, sex, and family history of the disease. Modifiable risk factors include tobacco smoking, physical inactivity, obesity, less than the recommended consumption of fruits and vegetables, high blood pressure, and diabetes. Recent statistics indicate that 8 out of 10 Canadians have a least one of these risk factors, and 1 out of 10 have three or more (1). As our population ages, the incidence of cardiovascular disease is expected to grow.

Interventional procedures to treat cardiovascular disease, such as coronary artery bypass grafting, angioplasty, valve surgery, pacemaker implantation and heart transplantation, are on the rise (1). Similarly, the number of prescriptions for medication to manage cardiovascular disease is also increasing (1). The total cost of cardiovascular disease on the health care sector in Canada is estimated to be $18,472.9 million (1).

The process of atherogenesis begins in childhood, when "fatty streaks", the first clinical manifestation of the disease, are detectable in arteries (230) (Figure 4). These early lesions can progress slowly over a period of decades in an asymptomatic manner, but may also eventually cause stable symptoms such as angina pectoris or intermittent claudification (129) (Figure 5). As the lesion develops, it may produce thrombotic complications, leading to unstable coronary syndromes, thrombotic stroke, or critical

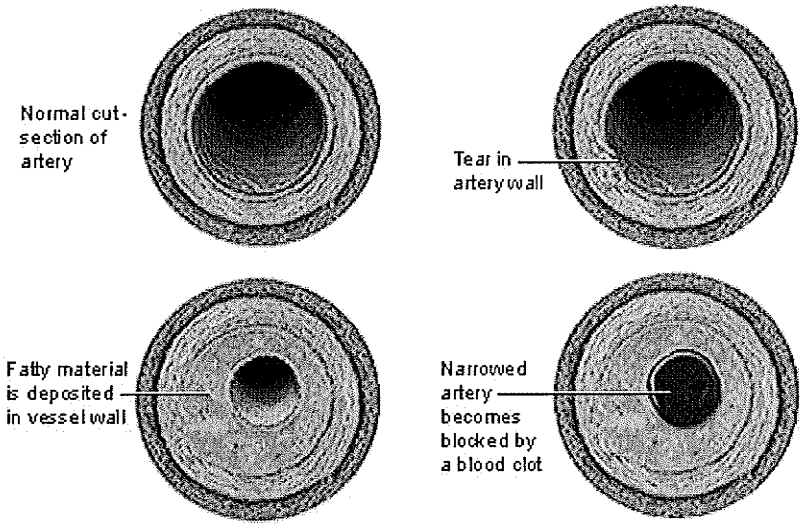

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Figure 4. Schematic illustration of the progression of an atherosclerotic plaque. From top, left to right: normal healthy artery, injury to endothelium resulting in beginning of "fatty streak", advanced lesion, and thrombosis.

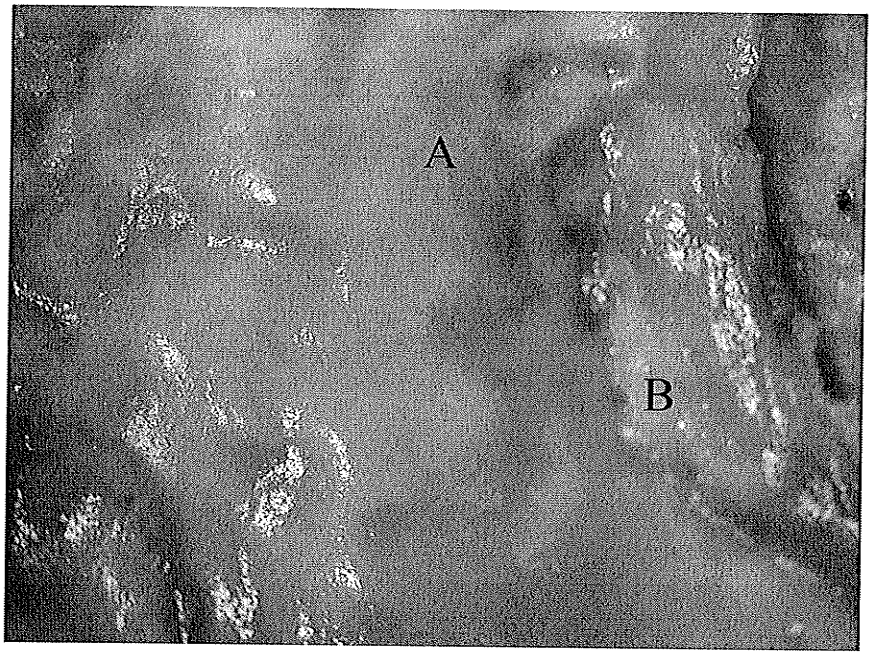


Figure 5. Gross pathological specimen of human carotid plaque, examined under 40x magnification. This advanced lesion was asymptomatic. **A** indicates an area of lipid deposition. **B** indicates an area of calcification.

limb ischemia (129).

Atherosclerotic lesion development is a complex process, involving cell growth, migration, inflammation, growth factor and cytokine secretion and extracellular matrix production. Atherosclerosis has traditionally been considered to be the result of a "response to injury", where the initial insult is any damage to the endothelium which eventually results in the proliferation of VSMCs (94, 207). The initial injury to the endothelium may be caused by shear stress, mechanical injury, inflammatory cells, or biological agents in the bloodstream. A critical step in this process may be the oxidation of low density lipoproteins (LDL) within the vessel wall and the subsequent participation of oxidized LDL (oxLDL) in the progression of the disease (231, 261).

1.3 Oxidized low density lipoprotein

Elevated levels of plasma LDL cholesterol lead to an increased movement of LDL into the vessel wall, where it becomes entrapped in the subendothelial space (Figure 6). Here it is known to become oxidatively modified in response to reactive oxygen intermediates and various cellular enzymes (190). LDL which has undergone modifications such as oxidation has a predilection for uptake through the scavenger receptor (232). OxLDL is taken up by scavenger receptors on VSMC, fibroblasts, and macrophages in the intima of the blood vessels in an unregulated process leading to the formation of lipid-engorged foam cells (Figure 7). This is believed to be a critical event in the atherosclerotic process (17, 191). The accumulation of oxLDL has been documented in atherosclerotic plaques, both in humans and animal models (268). A number of studies have demonstrated that

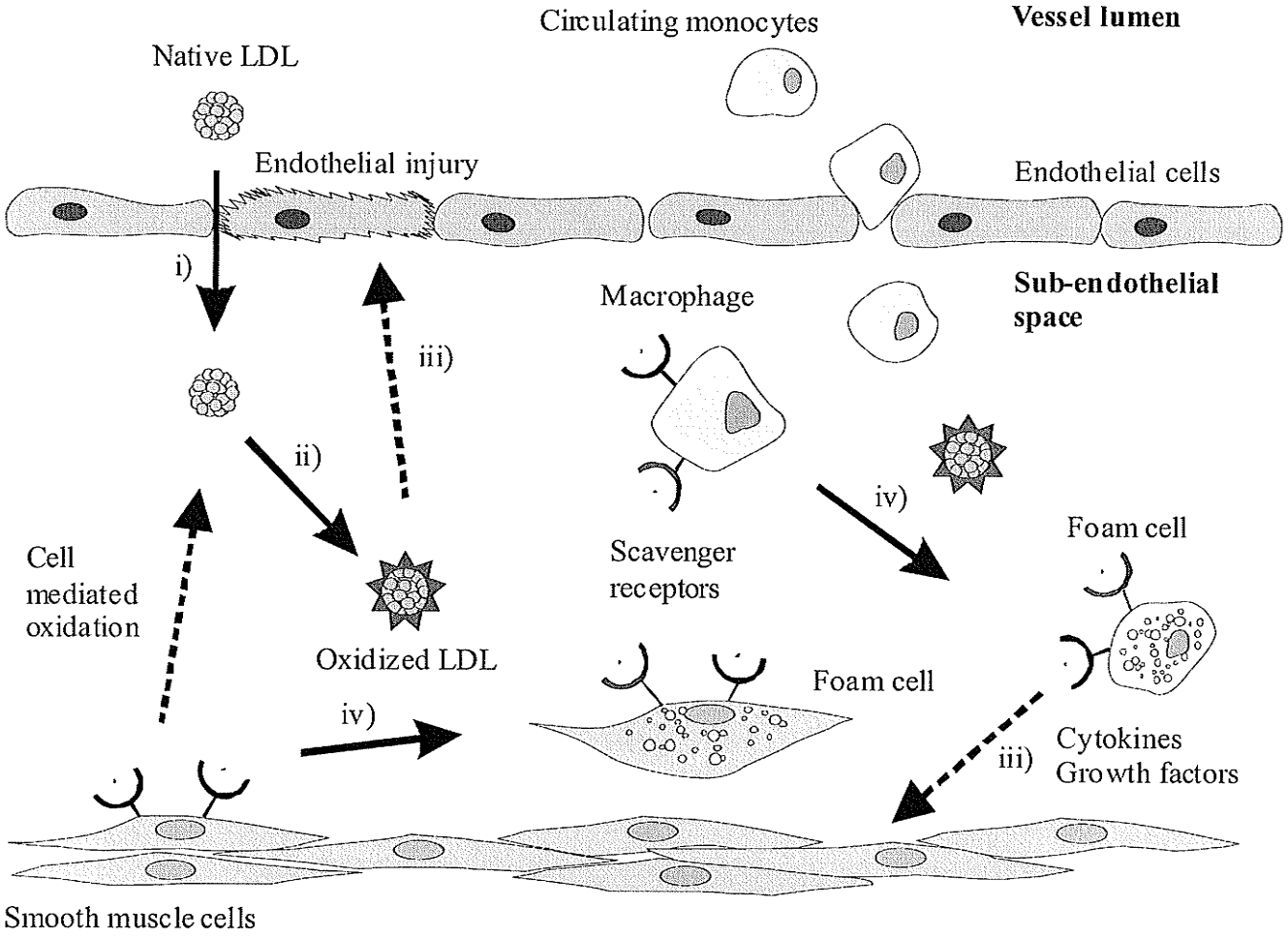


Figure 6. Schematic illustration of the proposed mechanism by which LDL becomes oxidized and contributes to the formation of foam cells.

i. Infiltration of LDL into the subendothelial space and entrapment.
ii. Oxidative modification of LDL.
iii. Pro-atherogenic actions of oxLDL (endothelial injury, release of cytokines and growth factors).
iv. Uptake of oxLDL via scavenger receptors to create foam cells.

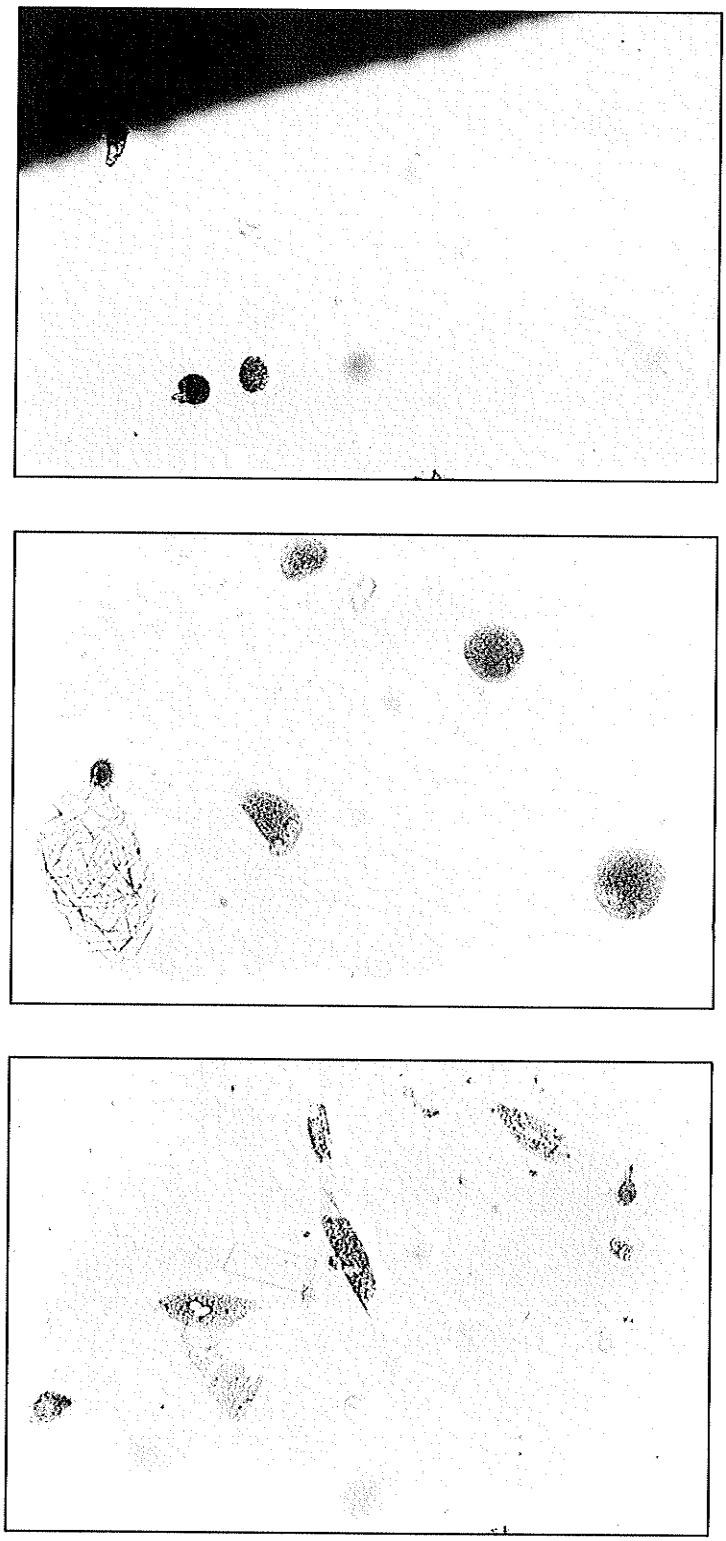


Figure 7. Fluorescent microscopic images illustrating foam cells migrating from an atherosclerotic plaque. Top panel: initial migration of lipid-laden cells from tissue. Middle panel: Macrophage foam cells. Lower panel: VSMC foam cells. Cholesterol crystals are also evident in the middle and lower panels.

circulating oxLDL in the plasma is associated with the clinical manifestations of atherosclerosis, such as coronary artery disease (101-103, 134, 245). More recently, this relationship has also been demonstrated for subclinical atherosclerotic changes in the carotid and femoral arteries (107). The amount of oxLDL and degree of oxidation is likely to be low during early stages of atherosclerosis. Under these conditions, effects are mainly proinflammatory (18, 72, 159). As the disease progresses, increased accumulation of oxLDL causes cytotoxic effects leading to plaque destabilization and rupture (116, 192). Growing evidence suggests that oxLDL is a likely candidate for involvement in the pathological cell growth associated with atherosclerosis. However, to date the effect of oxLDL on cell cycle protein expression has not been examined.

1.3.1 Proliferation

The ability of oxLDL to induce proliferation in a variety of cells is well established. Mildly oxidized LDL exerts a concentration dependent stimulation of proliferation in cultured human SMCs (233), rabbit VSMCs (39, 121), bovine VSMCs (11), murine macrophages (22, 89, 148, 210), human fibroblasts (22) and human endothelial cells (EC) (141). The mitogenic effect of oxLDL on these cells is several times that of native LDL, indicating the specificity of the response to oxLDL. As further evidence to support this observation, the antioxidant butylated hydroxytoluene (BHT) inhibits LDL oxidation and its proliferative effects on SMC (11). In humans, circulating oxLDL is associated with intima-media thickness and plaque occurrence (107). This suggests that the proliferation of vascular cells observed in response to oxLDL *in vitro* is pathologically relevant.

Cellular proliferation is dependent upon the induction of cell cycle proteins, including cyclins and cyclin-dependent kinases (96). OxLDL has not yet been linked to changes in cell cycle protein expression or distribution. However, preliminary experiments from our laboratory indicate that exposure of serum-starved rabbit VSMC and human fibroblasts to mildly oxidized LDL results in movement into the cell cycle, associated with a significant increase in the level of Cyclin B1. This increase is accompanied by a redistribution of Cyclin B1 from the cytoplasm to the nucleus (Figure 8), further implicating the involvement of oxLDL in mitogenesis. Expression of cell cycle proteins occurs in response to intracellular signals that activate transcription factors.

1.3.2 Transcription factors

Changes in gene expression must occur in order for the cell to enter the cell cycle and begin the process of proliferation. Signals received by cell surface receptors are relayed to the nucleus, where transcription factors integrate these signals and mediate responses. OxLDL can affect proliferation in the vasculature through an action on various transcription factors, resulting in an alteration of gene expression.

Activator protein-1 (AP1), composed of members of the Jun and Fos families, is induced by growth factors and cytokines (reviewed in (8)). Binding sites for AP1 are important in transcriptional regulation of tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) (8). AP1 binding activity is controlled by the redox state of the

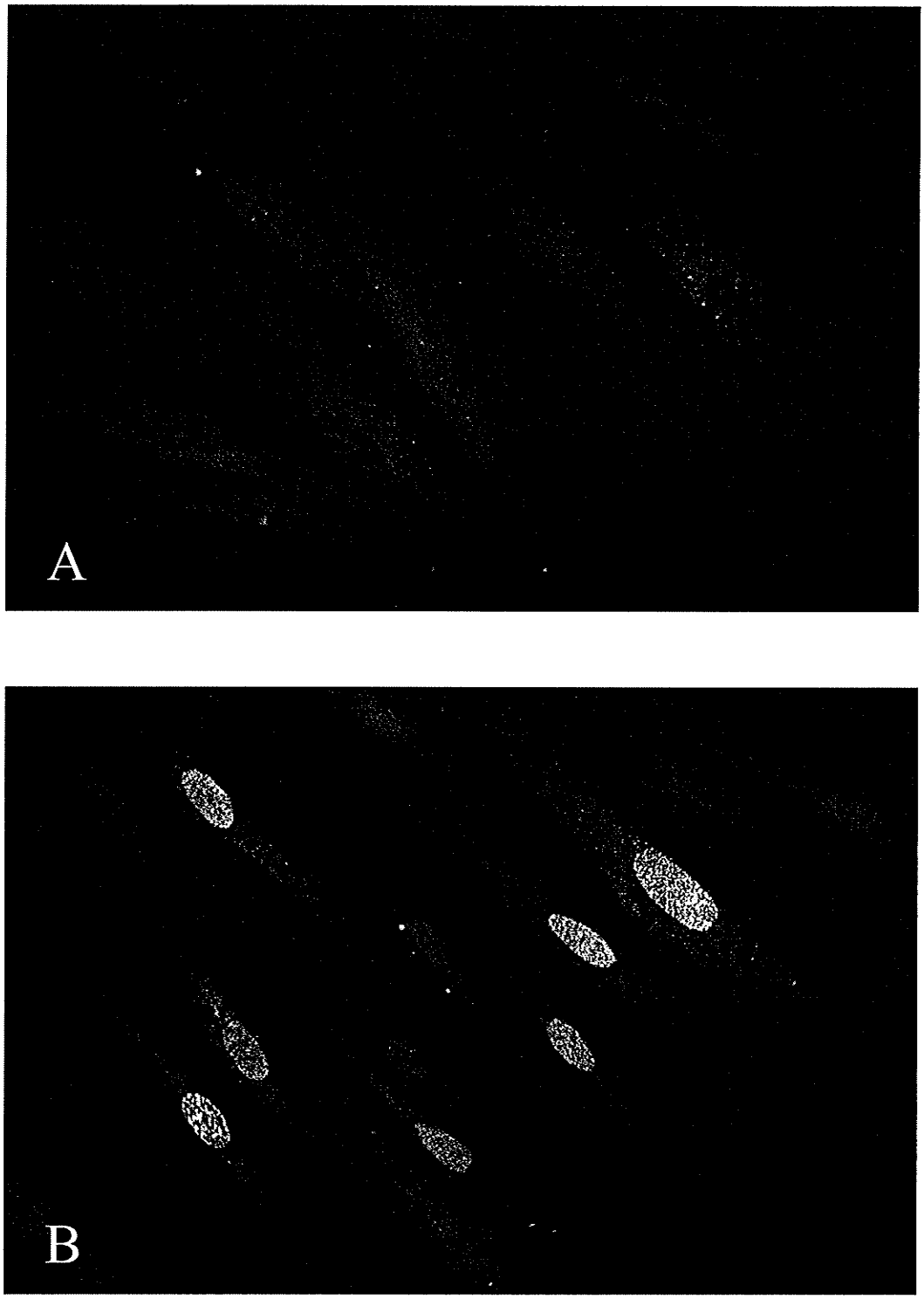


Figure 8. Increased nuclear fluorescence of Cyclin B1 in fibroblasts treated with oxLDL. Cyclin B1 is believed to be required for completion of the M phase of the cell cycle. (A) Control (no oxLDL). (B) 10 μg/ml oxLDL for 24 hours. All cells were maintained in serum-free media for 5 days preceding treatment.

cell and is stimulated by oxidative stress and oxygen radicals (16, 195). OxLDL induces AP1 activation in fibroblasts, SMC (155) and human vascular EC (45). This effect, due in part to lipid peroxidation products, does not require de novo protein synthesis and is partially inhibited by the antioxidants α-tocopherol and N-acetylcysteine. OxLDL alters AP1's activity through a post-translational modification of both c-Fos and c-Jun subunits (155).

The STATs (signal transducers and activators of transcription) are a family of transcription factors activated by both janus activated kinase (JAK) and tyrosine phosphorylation in response to engagement of various cytokine receptors. OxLDL and its lipid extracts enhances STAT1 and STAT3 binding in human EC. The increase in binding parallels an increase in the intracellular level of lipid peroxidation and the level of reactive oxygen species, suggesting that STAT binding activity is also dependent on the redox state of the cell. Consistent with this hypothesis, STAT binding is inhibited by vitamin E and partially inhibited by N-acetylcysteine (153).

OxLDL and its lipid extracts also induces activation of the oxidative stress transcription factor nuclear factor kappaB (NF-κB) in fibroblasts, SMC (154), human THP-1 monocytic cells (24) and human coronary artery EC (104, 126). NF-κB plays a critical role in proliferation, migration and inflammation (reviewed in (163)). Activated NF-κB is also found in human atherosclerotic lesions (24). Activation in monocytic cells is inhibited by the antioxidant/H_2O_2 scavenger pyrrolidine dithiocarbamate (24), while activation in fibroblasts and SMC is partially inhibited by vitamin E (154). OxLDL also induces degradation of inhibitor kappaB (IkappaB) protein in human coronary artery EC, which is attenuated with γ-tocopherol (126).

The response of NF-κB to oxLDL is, however, specific to cell type. OxLDL decreases NF-κB binding to DNA in *in vitro*-activated T lymphocytes, inhibiting proliferation. Levels of the active form of IkappaBα are unchanged in these cells (32). Alterations in IkappaBα and β degradation result in delayed or reduced kappaB binding activity in macrophages pretreated with oxLDL; consequently, cytokine gene expression is inhibited or delayed (91). OxLDL also suppresses lipopolysaccharide (LPS) -induced binding of macrophage extracts to a NF-κB sequence oligonucleotide (104, 219).

1.3.3 Inflammatory response/cytokines

The immune/inflammatory response has recently been suggested to be a key component of the atherogenic process. A positive correlation between circulating oxLDL levels and C-reactive protein (CRP, a sensitive marker for inflammation and infection) has been observed in humans (103). Similarly, plasma oxLDL levels were positively associated with TNFα levels in humans (107). OxLDL itself is immunogenic and stimulates antibody production (212). OxLDL-containing immune complexes taken up by receptors on human macrophages lead to transformation into foam cells and activation and release of cytokines (106). Cytokines are thought to play a crucial role in atherogenesis. For example, IL-1β induces SMC proliferation (130), and mRNA levels for IL-1β (174), TNFα (243) and IL-6 (217) are elevated in atherosclerotic plaques. OxLDL stimulates the release of IL-8 by human THP-1 monocytic cells (240) and a protein component of the oxLDL particle stimulates the production of IL-1α by rabbit arterial macrophage-derived foam cells (133). Multiple components of oxLDL have been reported to induce IL-1β release by mononuclear cells (241), however, the concentrations

of oxLDL used in these experiments were in the cytotoxic range, raising the possibility that the observed IL-1β release may have been due to apoptosis of the cells. Treatment of murine macrophages with low doses of oxLDL stimulates the proliferative actions of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF), although proliferation was not dependent on GM-CSF or M-CSF production (89). OxLDL also enhances mRNA levels and protein release of GM-CSF in murine macrophages (21). This effect is inhibited by the protein kinase C (PKC) inhibitor calphostin C (21). OxLDL-induced murine macrophage growth was found to require specific uptake of the oxLDL component lysophosphatidylcholine (LPC) by scavenger receptors by Biwa et al. (21), although Martens et al. report that the proliferative effect of oxLDL could be mimicked using an oxidatively modified protein (148). Biwa et al. report that the oxLDL-induced growth of macrophages is significantly inhibited by anti-GM-CSF antibodies (21). Growth is also inhibited by replacing the medium at 24 hours. Since GM-CSF levels are basal at this time, another cytokine may be involved at later phases. Therefore release of GM-CSF may have a priming role in conjunction with other cytokines in oxLDL-induced murine macrophage growth (21).

Cytokine release in response to oxLDL can influence cellular function at several levels. Interferon-γ (IFNγ) (176), IL-4 (105), GM-CSF and TNFα are all reported to influence expression of the classical scavenger receptors and CD36 (53). The lectin-like oxLDL receptor can also be upregulated by TNFα in macrophages (171). IL-4 (105) and M-CSF both stimulate expression of peroxisome proliferator activator receptor γ, a nuclear receptor transcriptionally activated by oxLDL and its lipid components, in macrophages and monocytic cell lines (203). This suggests a feedback loop: cytokine

release in response to oxLDL leads to upregulation of oxLDL receptors and hence increased uptake of oxLDL.

Immune responses to oxLDL appear to be specific for different cell types. For example, oxLDL inhibits IL-2 production and IL-2 receptor expression in *in vitro*-activated T lymphocytes (32). OxLDL also inhibits release of TNFα (90), IL-1β and IL-6 by macrophages (69), and TNFα, IFNγ and IL-12 by mitogen-stimulated peripheral blood mononuclear cells (144).

1.3.4 Growth factors/second messengers

Growth factors mediate intercellular communication by activating cell surface receptors linked to second messenger pathways. Complex cell functions such as proliferation are generally stimulated by combinations of these signals. OxLDL can influence this process both by stimulating the production and release of growth factors and by causing an increased sensitivity to the effects of these growth factors.

Platelet-derived growth factor (PDGF)-A (13) and PDGFβ surface receptors (208) are expressed by SMC from human plaques. Preincubation of SMC with either native or oxLDL can enhance the responsiveness of cells to exogenous PDGF. Mitogenic effects disappear if cells are treated with superoxide dismutase (233), again suggesting a role for oxygen derivatives as second messengers. OxLDL can also increase the production of PDGF, PDGF A-chain mRNA and expression of PDGFα and β-receptors in SMC (233) but it inhibits the generation of PDGF B chain by macrophages (143) and EC (70).

OxLDL, 4-hydroxynonel and LPC can upregulate mRNA expression and protein secretion of the EC specific mitogen and angiogenic factor VEGF (vascular endothelial

growth factor) in a monocytic cell line. This effect is time and dose dependent. VEGF immunoreactivity has been detected in subendothelial macrophage-rich regions of human early atherosclerotic lesions (199).

OxLDL induces the release of fibroblast growth factor-1 (FGF-1) from FGF-1 transfected mouse NIH-3T3 cells and rabbit SMC in a concentration dependent manner. FGF-1 released in response to oxLDL does not require de novo synthesis and is suggested to be mediated by sublethal and transient changes in membrane permeability (7). Similarly, LPC transiently influences membrane permeability, which may be related to FGF-2 release. Neutralizing antibody against FGF-2 significantly inhibits oxLDL and LPC-induced DNA synthesis in SMC (33).

Incubation of human macrophages (120) and rabbit SMC (10) with oxLDL leads to an increase in ceramide, a lipid mediator involved in stress-induced signaling. Incubation of SMC with oxLDL increases activities of both acidic and alkaline ceramidases and sphingosine kinase. Inhibitors of these enzymes abrogate the mitogenic effect of oxLDL (10). OxLDL also stimulates biosynthesis of lactosylceramide (LacCer). LacCer stimulates proliferation of VSMC *in vitro* and mediates TNFα-induced NF-κB expression in EC via a redox-dependent transcription pathway. LacCer also activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which produces superoxide (37).

Increases in intracellular Ca^{2+} have been associated with the initiation of transcription, translation and post-translational regulation (19). Addition of oxLDL to rat (259) or rabbit VSMC (151) or murine macrophages (152) induces an increase in intracellular free Ca^{2+}. This Ca^{2+} is likely released from internal stores (151, 152). It has

been suggested that cell cycle Ca^{2+} transients are mediated by increases in intracellular inositol triphosphate (IP_3) (46). OxLDL activated phosphoinositide turnover in rabbit VSMC which was blocked by phospholipase C inhibitors (151). In macrophages, oxLDL-induced phosphoinositide turnover was blocked by inhibitors of receptor-mediated endocytotic processes (202). These actions on intracellular Ca^{2+} may be associated with the proliferative effects of oxLDL. Therefore, in addition to the direct effects of the growth factors released in response to oxLDL, oxLDL enhances the responsiveness of cells to other growth factors and cytokines.

1.3.5 Enzymes involved in mitogenesis

Mitogen-activated protein kinases (MAPK) are stimulated by a wide range of stresses and extracellular signals, which may in turn activate receptor tyrosine kinases or G-protein coupled receptors, initiating phosphorylation cascades. OxLDL strongly evokes phosphorylation and activation of p38 MAPK in rat VSMC in a time and dose dependent manner (115). Activated MAPK is translocated to the nucleus within 15 minutes after exposure to oxLDL (115). The activation of MAPK is not mediated by classical scavenger receptors and is not affected by tyrosine kinase inhibitors (115). OxLDL appears to activate MAPK through a phospholipase C and G-protein dependent action (115).

OxLDL also affects other components of the MAPK pathway. OxLDL stimulated p42/p44 (extracellular signal-regulated kinase ERK2/ERK1) MAPK activity in baboon (123) and rat VSMC (44). These effects were time and dose dependent, and the active component was a lipid-based moiety. The stimulation appears to involve PKC, since

pretreatment with phorbol ester blocked MAPK activation (123). OxLDL immune complexes also induce ERK2 MAPK phosphorylation in THP-1 macrophage-like cells in a time and dose dependent manner. Nuclear translocation of the phosphorylated ERK2 markedly increases after stimulation and cholesterol loading results in longer phosphorylation times (106). However, ERK2 MAPK activation in an oxLDL-treated human monocytic line requires scavenger receptor uptake but is independent of PKC or G-protein activation (55).

Sustained activation of PKC is essential for long term cellular responses such as proliferation and differentiation. OxLDL induces activation of membrane PKC in murine macrophages. Pertussis toxin reduces oxLDL-induced growth in these cells by 50% (33, 152). OxLDL also differentially affects levels of individual PKC isoenzymes (74). Polyinosinic acid exhibited a concentration-dependent inhibition of the oxLDL-induced PKC activation, suggesting scavenger receptor interactions are critical to PKC activation (48).

OxLDL and its lipid peroxidation product 4-hydroxynonenal can increase tyrosine phosphorylation and consequent activation of several receptors (including the epidermal growth factor receptor) and their signaling pathways (235). Increased protein tyrosine phosphorylation is accompanied by a two-fold increase in phosphatidylinositol 3-kinase (PI 3-kinase) activity in phorbol ester pretreated THP-1 human monocytic-like cells. PI 3-kinase phosphorylates the inositol ring of IP_3 and is thought to be involved in cell proliferation. Two different PI 3-kinase inhibitors can reduce oxLDL stimulated growth of murine macrophages by 40-50% (149). PI 3-kinase and its downstream effector, Akt, also play a key role in cell survival (60). OxLDL has been shown to activate the PI 3-

kinase/Akt signaling cascade in rat cultured VSMC (44). Inhibition of PI 3-kinase or Akt partially blocked activation of p42/p44 MAPK in response to oxLDL as well. This suggests that induction of MAPK by oxLDL is mediated in part through the activation of the PI 3-kinase/Akt pathway (44).

Activation of phospholipase D results in the generation of phosphatidic acid, which can elicit a wide variety of biological responses, including DNA synthesis. OxLDL can enhance phospholipase D activation in both rabbit SMC (177) and murine macrophages (80). In both cell types, exposure to the phosphatase inhibitor sodium orthovanadate enhances oxLDL-mediated phospholipase D activation, but pretreatment with the tyrosine kinase inhibitor genistein attenuates the effect (177). OxLDL-induced phospholipase D activation is independent of PKC and Ca^{2+} in both cell types. In murine macrophages, pertussis toxin decreases phospholipase D activation in response to oxLDL, while PAF receptor antagonists cause inhibition (80).

1.3.6 Future directions

While ample evidence exists to support the contention that oxLDL promotes a proliferative response in a variety of cell types, several issues have yet to be clarified. The first issue concerns whether oxLDL is a mitogen itself, or whether it acts by increasing sensitivity to other mitogens. The presence of other mitogens in the media can be eliminated by using serum-deprived cells and changing the media every 24 hours in order to prevent a response to oxLDL-induced growth factor or cytokine release. Experiments from our own laboratory under these conditions resulted in a significant

increase in the number of VSMC, suggesting that oxLDL has mitogenic properties in and of itself.

A second issue concerns the component(s) of oxLDL responsible for the mitogenic effects. Several studies have isolated components of oxLDL associated with cell growth. For example, Heery et al. (93) found that PAF-like oxidized phospholipids were responsible for oxLDL-induced growth in bovine and rat VSMC. However, Martens et al. (148) identified modified apoB100 as the active mitogenic component of oxLDL in their experiments using murine macrophages. Other studies point to lipid peroxidation products and other components of the oxLDL molecule as capable of inducing specific effects, such as release of VEGF (199) or activation of MAPK (123). The results of these experiments are difficult to interpret given the diversity in experimental conditions (cell type, method and extent of LDL oxidation, concentration of oxLDL in the media, duration of exposure, etc.).

The final issue to be addressed concerns the signal transduction pathway employed by oxLDL in generating the proliferative response. It would appear that the effects of oxLDL are mediated both by interaction with cell surface receptors and signaling mechanisms initiated by components of oxLDL following internalization. A number of studies have investigated the participation of specific enzymes (e.g. MAPK, PKC, PI 3-kinase), second messengers (e.g. Ca^{2+}, reactive oxygen intermediates), growth factors (e.g. GM-CSF) and receptors (e.g. scavenger receptor, FGF receptor). Again, it is difficult to draw conclusions given the wide variety of models used. While no enzymatic pathway emerges as the clear route by which oxLDL exerts its proliferative effects, activation of any of these enzymes may result in the formation or activation of factors

that feed into other pathways. OxLDL appears to induce a generalized cellular activation involving multiple receptors and pathways. Cell type may determine the predominant route by which the mitogenic response is mediated. Clearly, the growth-promoting effects of oxLDL are consistent with its proposed role in atherosclerosis and further implicate this lipoprotein as a critical component in atherosclerotic disease of the coronary and carotid circulations.

1.4 The involvement of cell cycle proteins in atherosclerotic and restenotic cell proliferation

To date there has been only limited investigation into the role of cell cycle proteins in the pathology of vascular disease (23, 127, 175). The majority of this work has been focused on models of restenosis. Balloon injury to arteries elicits a well-characterized response (185). Tissue remodeling in injured arteries involves migration of medial SMCs to the intima, followed by intimal cell proliferation and extracellular matrix synthesis (111, 131). Of particular importance is the arrest of cell growth seen after the initial proliferative response. The inhibition of VSMC replication may be the result of multiple mechanisms, including p53 activation, induction of Cdk inhibitors, or the binding of Rb to transcription factors.

1.4.1 Preclinical models

Diez-Juan et al. found that $p27^{kip1}$-deficient mice fed a high cholesterol diet remained normocholesterolemic and did not develop atherosclerotic lesions (58). However, inactivation of one or two $p27^{kip1}$ alleles accelerated atherosclerosis in

apolipoprotein E-null mice (compared with apolipoprotein E-null mice with intact alleles) (58). This suggests that $p27^{kip1}$ may provide some protection against diet-induced atherogenesis (58).

Endogenous $p27^{kip1}$ was downregulated immediately following balloon injury in a porcine femoral model (238). Expression was upregulated in later phases of arterial remodeling associated with a decline in cell proliferation and concomitant with an increase in procollagen and TGF-β synthesis. Expression of p16 was observed shortly after injury but was not at later time points. Constitutive expression of $p27^{kip1}$, but not p16, was observed in control arteries (238).

In a rat model of arterial injury, an upregulation and activation of Cdk 2 and Cyclins E and A was observed between 1 and 2 days post-injury (256). This was correlated with PCNA expression up to 10 days post-injury, declining after 18 days. Cdk 2 and Cyclin E expression was seen in medial VSMCs 36 hours after injury, then became undetectable in the media and confined to the luminal surface of the intima. Basal $p21^{cip1}$ expression was not observed in uninjured vessels (256).

Yang et al. (265) found that termination of intimal cell growth after angioplasty in porcine arteries was associated with the induction of endogenous $p21^{cip1}$. This increase in expression was associated with a decline in intimal cell proliferation, from one day post-injury when less than 5% of intimal VSMCs expressed $p21^{cip1}$, to three weeks post-injury when $p21^{cip1}$ expression was present in the majority of VSMCs. In normal, uninjured porcine arteries, $p21^{cip1}$ expression was undetectable by immunostaining or Western blot analysis (265).

A summary of therapeutic manipulation of cell cycle proteins in animal models is

Table 2. Therapeutic manipulation of cell cycle proteins in animal models

Oligodeoxynucleotide (ODN) therapy

Gene	ODN	Species	Vessel	Neointimal inhibition
Cdc 2 + PCNA	antisense	rabbit	jugular vein graft	>90%
Cdc 2 + PCNA	antisense	rat	carotid artery	>50%
PCNA	antisense	rat	carotid artery	80%
Cdk 2	antisense	rat	carotid artery	60%
Cdc 2	antisense	rat	carotid artery	40%
Cdk 2 + Cdc 2	antisense	rat	carotid artery	85%
Cdk 2	antisense	rat	carotid artery	55%
Cdc 2	antisense	rat	carotid artery	47%
Cyclin B1+ Cdc 2	antisense	rat	carotid artery	78%
Cdk 2	antisense	mouse	coronary artery	54%
E2F	decoy	rat	carotid artery	74%

Viral therapy

Gene	Vector	Species	Vessel	Neointimal inhibition
$p21^{cip1}$	adenoviral	porcine	ileofemoral artery	37%
$p27^{kip1}$	adenoviral	rat	carotid artery	49%
$p21^{cip1}$	adenoviral	rat	carotid artery	46%
pRb	adenoviral	rat	carotid artery	50%
pRb	adenoviral	porcine	femoral artery	47%
$p27^{kip1}$-p16	adenoviral	rabbit	carotid artery	60%
p53	HVJ	rabbit	carotid artery	80%

References: (3, 34, 35, 42, 145, 156, 167-170, 222, 236, 265, 269)

outlined in Table 2. Overexpression of $p21^{cip1}$ by adenoviral gene transfer in the model employed by Yang et al. reduced the extent of intimal hyperplasia post-angioplasty by about 37%. The pattern of $p21^{cip1}$ expression was inversely related to the time course of cellular proliferation (265). Previous studies using adenoviral vectors for $p21^{cip1}$ protein have shown a 46% reduction of intimal thickening in rat arteries, with the human $p21^{cip1}$ protein expressed in >70% of medial SMC in the rat carotid artery (34).

Chen et al. (42) observed induction of endogenous $p27^{kip1}$ protein in the injured arterial wall of rat at late time points after angioplasty. Using the same model, overexpression of $p27^{kip1}$ by adenovirus gene transfer reduced neointima formation by 49% (42). Therefore, termination of intimal cell growth following angioplasty may involve more than one Cdk inhibitor.

A unique strategy was employed by McArthur et al., who created a novel fusion protein of the CKIs $p27^{kip1}$ and p16 (156). Introduced into the balloon-injured carotid arteries of cholesterol-fed rabbits by adenovirus, this $p27^{kip1}$-p16 chimera inhibited neointimal hyperplasia by 60% vs. a control virus (156). The chimera virus was more effective than either of the parental genes in reducing intimal thickening (156).

Upstream regulators have also proven to be effective targets. Localized infection of the arterial wall with an adenovirally encoded constitutively active non-phosphorylatable form of Rb significantly reduced medial VSMC proliferation and restenosis in both rat carotid and porcine iliac models of balloon angioplasty (35). Transfection with a phosphorylation-competent Rb also inhibited neointimal thickening (223).

Several studies indicate that low dose irradiation reduced neointimal formation after balloon injury or stenting in both rabbits and pigs, presumably by activating p53 (95, 251, 252). p53 also suppresses transcription of protooncogenes such as c-fos and c-jun, which are frequently present at high levels during the early stages following vascular injury (79). p53 overexpression via the hemagglutinating virus of Japan/liposome-mediated gene transfer method reduced neointimal thickening in balloon injured rabbit carotid arteries by 80%, growth arresting VSMCs at G1 or G2/M (269).

The expression of several genes involved in cell cycle regulation has been successfully targeted with antisense oligonucleotides *in vivo*. Suppression of neointimal thickening using this approach has been demonstrated in the rat carotid artery balloon injury model using antisense oligonucleotides to Cyclin B1, Cdc 2, Cdk 2, and PCNA, as well as combinations, such as Cdc 2 kinase with PCNA or Cdk 2 kinase (3, 168-170, 222, 228). Transfection into injured arteries resulted in a reduction of neointimal formation by up to 85% (see Table 2) (168).

1.4.2 Human specimens

Expression of various cell cycle proteins in primary and restenotic human atherectomy specimens has been assessed by immunostaining. Numerous studies indicate little or no evidence of proliferation using PCNA as an indicator (26, 82, 183). However, Wei et al. (256) report Cdk 2 and Cyclin E expression in PCNA-positive VSMCs in restenotic but not primary lesions. Similarly, O'Sullivan et al. (188) found that VSMCs within in-stent restenotic tissue expressed significantly higher levels of both Cyclins A and E, and significantly lower levels of $p27^{kip1}$, relative to normal coronary arteries.

Another study using in-stent restenotic tissue found levels of Cyclin D1 to be correlated with neointimal hyperplasia (in the early stages following stenting), and levels of $p27^{kip1}$ to be associated with later stages, when proliferation is reduced (211).

It has been shown by several groups that p53 is present in the nuclei of SMCs, ECs, and macrophages in human atherosclerotic tissue. Ihling et al. (109) demonstrated that p53 often colocalized with $p21^{cip1}$, suggesting a coordinated response of both proteins. These same cells were negative for the proliferation marker Ki-67, providing further evidence for the role of p53 and $p21^{cip1}$ in inhibiting cellular proliferation. Control tissue had very low levels of p53 and $p21^{cip1}$ and no positive staining for Ki-67 (109). A second study by Ihling et al. found that, in plaques from patients undergoing carotid endarterectomy, cells expressing Cyclin E were simultaneously positive for $p27^{kip1}$ (110). Quantitative analysis revealed that upregulation of these cell cycle proteins was associated with an upregulation of TGFβ receptor II, suggesting a link between inflammation and cell cycle control (110).

Tanner et al. (238) observed $p27^{kip1}$ expression in non-replicating cells of both control and atherosclerotic vessels. The degree of $p21^{cip1}$ expression was correlated with the severity of the disease in the specimen. p16 was not observed in control or diseased tissue (238). Braun-Dullaeus et al. (26) found that $p27^{kip1}$ levels were significantly decreased in both primary and in-stent restenotic lesions, relative to control aorta, internal mammary artery and carotid artery thrombendarterectomy specimens. In this study, levels of PCNA and Cdk 2 were low in both types of specimens, but $p21^{cip1}$ levels were significantly higher in restenotic lesions compared with primary lesions (26).

1.5 Targeting the cell cycle for the treatment of vascular proliferative disease

As our understanding of the cell cycle and its role in vascular disease develops, new treatment strategies targeting the cell cycle are emerging (20, 64, 67, 112). Because atherosclerosis has such a long incubation period, and because the precise time of the initial injury to the vessel is unknown, most of the treatments currently in development are proposed for other vasculoproliferative therapeutic indications (such as in-stent restenosis or bypass graft failure). Regardless, information gained from the study of cell cycle inhibitors for these conditions may provide valuable insight into potential application of this therapeutic strategy to atherosclerosis. Cell cycle inhibition may be achieved through pharmacological agents, irradiation, or gene therapy.

1.5.1 Pharmacological agents

The development of pharmacological cell cycle inhibitors is focused on agents that will inhibit the cell cycle (i.e. a "cytostatic" mechanism) as compared to killing the cell (i.e. a cytotoxic mechanism). Several drugs currently on the market meet this criterion. For example, a number of pharmacological agents already in use to treat cardiovascular disease (such as non-steroidal anti-inflammatory drugs and statins) have recently been found to also have anti-proliferative activity via specific effects on cell cycle proteins (30, 184).

Another drug already in clinical use is rapamycin, a macrocyclic triene antibiotic. It has been used as an immunosuppressant to prevent transplant rejection, but it has also been shown to prevent proliferation of VSMC (186). Rapamycin's antiproliferative effect was found to be due to an increase in levels of $p27^{kip1}$, resulting in a block in cell cycle

progression at G1 (150). The use of rapamycin was observed to be effective in preventing neointimal hyperplasia and reducing arteriopathy in both a porcine balloon angioplasty model (75) and a rat femoral artery allograft model (85). A rapamycin-eluting stent was developed and first tested in a porcine angioplasty model (237). This stent (BX Velocity™) has since been used in several clinical trials (201, 226), including the SIRIUS (SIRolImUS-eluting stent in de novo native coronary lesions) (172) and RAVEL (RAndomized study with the sirolimus-eluting Velocity balloon-Expandable stent in the treatment of patients with de novo coronary artery Lesions) (166) trials (Table 3). Thus far the stents have proven successful in preventing restenosis at one year (100, 227).

A second cell cycle-inhibiting drug that has been used for the prevention of restenosis is paclitaxel (Taxol). Paclitaxel promotes polymerization of microtubules, preventing disassembly of the mitotic spindle and effectively inhibiting cell division (214). Cells are arrested at G0/G1 and G2/M (59). This drug, which has primarily been used in the treatment of cancer, is cytotoxic (254). *In vivo* local administration of paclitaxel has been effective in preventing neointimal hyperplasia in a rat model of balloon angioplasty (224), as well as both rabbit (61, 98) and pig (97) stent models. Several clinical trials have demonstrated the efficacy of paclitaxel-coated stents in humans, including the EvaLUation of pacliTaxel-Eluting Stents (ELUTES) and TAXUS™ trials (117) (Table 3). However, it appears that there is a late "rebound" phenomenon in these patients, after the drug in the stent has been exhausted (132, 248). Similar to paclitaxel, docetaxel is another microtubule polymerizing agent capable of cell cycle inhibition (76). This drug has been tested and found effective in a rabbit model of balloon angioplasty (266).

Losartan is an angiotensin II receptor antagonist that is administered orally for the treatment of hypertension. Preliminary evidence suggests that it may also be useful in preventing restenosis, however doses required to achieve this result cannot be given orally (260). To circumvent this problem, losartan was mixed with surgical fibrin glue and applied directly to the adventitial surface of balloon-injured porcine saphenous arteries (164). This strategy proved effective, with neointimal area decreased by 82% (164).

Recent drug development has focused on specific inhibitors of Cdks. Flavopiridol, which targets the ATP-binding sites on Cdks, is one example (84). This drug is taken orally, and has been shown to reduce neointimal formation in a rat carotid model of balloon angioplasty (209). CVT-313 is a purine analog that inhibits Cdk 2 (29). Local administration of this drug also reduced neointimal thickening in a rat carotid model of balloon angioplasty (29).

1.5.2 Irradiation

The use of β or γ radiation on newly-stented arteries is another strategy to prevent restenosis. Also called "brachytherapy", this treatment causes DNA damage and subsequent upregulation of p53 (25, 216). P53 then promotes $p21^{cip1}$-induced growth arrest at G1 (25, 216). Evidence from clinical trials suggests that this therapeutic strategy is effective for in-stent restenosis (86, 250). However, regions proximal and distal to the stent occasionally restenose (the "candy-wrapper effect") (4). Rates of late stage thrombosis are also higher in these patients (51).

Table 3. Clinical trials utilizing anti-proliferative therapy with cell cycle targets for restenosis

Trial	Intervention	Therapy	# of Patients	Primary endpoint	Angiographic restenosis (%)
RAVEL	Stent	Rapamycin	238	in-stent late luminal loss by quantitative angiography at 12 months	0
SIRIUS	Stent	Rapamycin	1100	cardiac death, myocardial infarction, or target vessel failure at 9 months	9
TAXUS I	Stent	Paclitaxel	61	diameter stenosis by angiography at 6 months	0
ASPECT	Stent	Paclitaxel	177	diameter stenosis by angiography at 6 months	4
ELUTES	Stent	Paclitaxel	192	binary restenosis rate	3
TAXUS II	Stent	Paclitaxel	536	in-stent lesion volume by intravascular ultrasound	6
TAXUS IV	Stent	Paclitaxel	1314	target vessel revascularization at 9 months	8
PREVENT	CABG	E2F decoy	41	>75% stenosis by ultrasonography at 12 months	29
AVAIL	Balloon catheter	c-myc antisense ODN	57	diameter stenosis by angiography at 6 months	8

Adapted from Ferguson et al, Cell Cycle 2003;2(3):211-9.

References: (2, 117, 146, 166, 172)

1.5.3 Gene therapy

As outlined in section 1.4.1, many different gene therapy strategies have been employed to target the cell cycle in preclinical models of vasculoproliferative disease. To date, however, only two have reached clinical trials in humans.

In the PREVENT study (PRoject of Ex-vivo Vein graft ENgineering via Transfection), saphenous vein grafts were pre-treated in a pressurized solution of E2F decoy nucleotides before implantation (146) (Table 3). These grafts showed increased patency at one year relative to untreated grafts (146).

In the AVAIL trial, c-myc antisense oligonucleotides (Resten-NG®) were delivered by balloon catheter to the site of angioplasty and stent placement (2). Preliminary results indicate that low doses of c-myc antisense oligonucleotides were effective in preventing restenosis in these patients (2) (Table 3).

As our understanding of the involvement of cell cycle proteins in cardiovascular disease grows, potential new targets for gene therapy may be identified. For example, a recent study by Gonzalez et al. (81) identified a single nucleotide polymorphism in the human $p27^{kip1}$ gene (-838C>A), which was associated with a significantly increased risk of myocardial infarction. This study, which compared the genotypes of 180 patients who had suffered an episode of myocardial infarction to 250 healthy controls, found that carriers of the -838A polymorphism had an almost twofold risk of suffering an episode of myocardial infarction compared to controls (81). This finding may have important implications for future gene therapies.

1.5.4 Future directions

Much has been learned in the last decade about the role of cell cycle proteins in pathological cell growth. However, their participation in proliferative atherosclerotic disease remains poorly understood. Little is known of the expression of the entire family of cell cycle proteins in normal as well as atherosclerotic arteries, what factors regulate their expression under normal and disease conditions, and most importantly, which proteins change during the critical initial proliferative phase of atherosclerosis. This knowledge will be crucial to direct therapy efficiently for restenotic and atherosclerotic disease in the future.

To date, drug and treatment development efforts have focused on vasculoproliferative diseases with well-characterized, local, rapid growth responses (such as restenosis or coronary artery bypass graft failure). In contrast, atherosclerosis is a chronic, systemic disease, and so will prove more difficult to treat effectively by any of the strategies described. However, understanding patterns of cell cycle protein expression and activation in atherosclerosis is a critical first step towards this goal.

CHAPTER 2: HYPOTHESES AND OBJECTIVES

Three separate hypotheses concerning the role of cell cycle proteins in atherosclerosis are tested in this thesis:

1. OxLDL has mitogenic properties and is capable of inducing changes in the expression, activation, and distribution of cell cycle proteins. The objectives of this study are:
   1.1. To characterize changes in the cell cycle of cultured vascular cells in response to oxLDL, serum and growth factors. This will be accomplished using both flow cytometry (to track movement through the cycle) and hemacytometer counts (to mark completion of the cycle).
   1.2. To determine if exposure of cultured vascular cells to varying levels of oxLDL induces changes in expression, activation, or distribution of cell cycle proteins. This will be achieved using Western blotting, kinase assays, and immunocytochemical methods.
   1.3. To delineate pathways by which oxLDL mediates its effects within the cell by investigating the role of MEK 1/2, PLC, PI 3-kinase and the scavenger receptor. This will be accomplished using pharmacological inhibitors of these pathways, as well as a receptor blocker.
2. The response to oxLDL is dependent upon the metabolic state of the cell, and is influenced by the presence of other mitogens or growth factors in the media. The objectives of this study are:

2.1. To characterize changes in the cell cycle of cultured vascular cells in response to oxLDL and serum in combination. This will be accomplished using both flow cytometry (to track movement through the cycle) and hemacytometer counts (to mark completion of the cycle).

2.2. To determine if exposure of cultured vascular cells to varying levels of oxLDL in combination with serum induces changes in expression, activation, or distribution of cell cycle proteins. This will be achieved using Western blot analysis, kinase assays, and immunocytochemical methods.

2.3. To delineate pathways by which oxLDL in combination with serum mediates its effects within the cell by investigating the role of PLC/A_2, PKC, PI 3-kinase and MAPK. This will be accomplished using pharmacological inhibitors of these pathways.

3. The stimulation of vascular cell proliferation that occurs during primary atherosclerosis is mediated by changes in the expression and activation of specific cell cycle proteins. The objectives of this study are:

3.1. To compare patterns of cell cycle protein expression and activation in vascular tissue from an animal model of atherosclerosis, using Western blot analysis and kinase assays.

3.2. To relate the progression of atherosclerosis (as measured by plaque formation) to changes in cell cycle protein expression and activity in aortas from an animal model of atherosclerosis.

3.3. To determine if non-atherosclerotic aortic tissue in an atherogenic environment exhibits changes in cell cycle protein expression and activity, using Western blot analysis and kinase assays.

CHAPTER 3: METHODS

3.1 Animal protocol

Male albino New Zealand white rabbits were given free access to food and water in the St. Boniface Research Centre animal care facility. Anaesthesia was induced by administration of 5% halothane in 2 L/min oxygen, followed by 3% halothane in 2 L/min oxygen by face mask. The animals were sacrificed with a dose of 1 ml/kg body weight of 10:1 (vol:vol) ratio of ketamine (100 mg/ml) to xylazine (100 mg/ml). In order to prevent blood coagulation, 0.2 ml of sodium heparin stock (1,000 i.u./ml) was added to the euthanasia cocktail. The rabbits received 3 ml of the euthanasia cocktail through the marginal ear vein. Aortae from animals fed the control diet were removed immediately. Cardiac puncture was performed on cholesterol-fed animals to obtain blood for lipoprotein isolation. Aortae from these animals were removed after blood collection.

3.2 Cell culture

An established fibroblast cell line from human neonatal foreskin was a kind gift from the laboratory of Dr. Michelle Alfa.

Primary cultures of VSMCs were generated by explant techniques (213). The thoracic aorta from a male New Zealand White rabbit (2.5 to 3 kg body weight) was isolated and cleaned of connective tissue, excess fat and adherent blood cells. The endothelial layer was gently scraped off. The aorta was then cut into 2- to 3-mm sections and transferred to a culture dish with growth medium. This medium consists of 20% FBS in DMEM and 5% antibiotic-antimycotic (Gibco-BRL). The explants were incubated in a

humidified incubator equilibrated with 5% CO_2 and maintained at 37°C. After initial migration, the aortic sections were allowed to proliferate for another 7 days before they were transferred to a new culture dish. VSMCs from the second phase of migration were used in our experiments. For all experiments, VSMCs from the first or second passage were used.

Confluent cultures of human neonatal fibroblasts and rabbit VSMC were trypsinized with trypsin-EDTA (Gibco BRL) and seeded at 500,000 cells per 100 mm x 20 mm dish. Following 24 hours in DMEM supplemented with 5% FBS, cells were washed twice with phosphate buffered saline (PBS). The media was replaced with serum-free DMEM supplemented with transferrin (5 μM), selenium (1 nM), ascorbate (200 μM) and insulin (10 nM) for 6 days in order to induce growth arrest. Cells were then incubated with this medium and 10 or 50 μg cholesterol/ml LDL or oxLDL for various time points for up to 48 hours (for experiments involving starved cells). For experiments involving fed cells, cells were incubated with 10 or 50 μg cholesterol/ml LDL or oxLDL in combination with FBS (5% for fibroblasts, 10% for VSMC) for 24 or 48 hours. LDL was oxidized with a Fe-ADP free radical generating system. The method and the characteristics of the minimally modified oxLDL are reported in detail elsewhere (88, 151). Typically, this preparation of oxLDL exhibits a modest increase in electrophoretic mobility, a ~20% depletion of vitamin E, and a ~30% increase in malondialdehyde (MDA) content (151). Cholesterol concentrations were assessed prior to oxidation and these concentrations were used for both native LDL and oxLDL-treated groups. Protein concentrations were unchanged throughout the course of the experiments. Cultures were maintained at 37°C in humidified 5% CO_2 and medium was replaced every 24 hours.

Freshly prepared oxLDL was also replaced on a daily basis. Control cells were maintained in an identical media (in the absence of oxLDL) for the same period of time.

3.2.1 Use of enzyme inhibitors and receptor blockers

For experiments involving inhibitors, cells were pretreated for 15 minutes with either 25 μM polyinosinic acid (Sigma) (121), 20 μM LY294002 (Sigma) (149), 50 μM NCDC (Sigma) (151), 4 μM PD98059 (Calbiochem) (118), 200 nM calphostin C (Sigma) (152), or 3 μM U73122 (Sigma) (118) before exposure to oxLDL. These concentrations were maintained in the media for the duration of the experiments.

3.2.2 Measurement of lactate dehydrogenase (LDH) activity

To demonstrate that the concentrations of oxLDL utilized in these experiments were not toxic, LDH release into the culture medium was assayed as an indicator of cell damage according to the method of Bergmeyer (87). Briefly, cells treated as described were maintained in phenol-free DMEM (Gibco BRL). Aliquots of media were collected daily and frozen at –20°C for the assay. Frozen aliquots were thawed and 50 μl of each sample was mixed with 100 μl of Tris buffer (1 M, pH 8.5) and 25 μl 0.1 M L-lactate solution. After a 5 minute incubation in a 37°C water bath, 100 μl of color reagent (7.5 mM nicotinamide adenine dinucleotide (NAD), 4 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), and 1.6 mM phenazine methosulphate (PMS) solution) were added. The test tubes were mixed and incubated for another 5 minutes in a 37°C water bath, before 2.5 ml of 0.1 N hydrochloric acid was mixed into each tube. Optical density was measured at 500 nm within 20 minutes. A blank

containing all components of the substrate solution (replacing 11 mM oxalate and 5.4 mM EDTA for the media) was used against the samples. No significant differences were observed in levels of LDH release between oxLDL treated cells and untreated controls over 24 or 48 hours for either 10 or 50 μg/ml oxLDL (data not shown). Furthermore, we could detect no significant increases in the release of LDH after cells were incubated with any of the drugs, either alone or in combination with oxLDL.

3.2.3 Use of growth factors and cytokines

For experiments involving growth factors, aFGF (Sigma) and bFGF (Sigma) were used at concentrations of 50 nM and 10 nM, respectively. TGF-β1 (Sigma) was used at a concentration of 5 nM. These concentrations were maintained in the media for the duration of the experiments.

3.2.4 Preparation of lipoprotein-depleted serum

Lipoprotein-depleted serum (LPDS) was prepared as described by Auge et al. (11). Briefly, serum was warmed at 56°C for 2 hours. Sodium azide was added (1 g/100 ml serum) and density was equilibrated at 1.21 g/ml with potassium bromide. The serum was then placed into 37 ml tubes for the ultracentrifuge vertical rotor Vti50 (Beckman). Tubes were centifuged for 16 hours at 4°C, at 40,000 rpm. The supernatant (containing total lipoproteins) was removed, as well as half of the liquid between supernatant and bottom of the tube (which could be contaminated by free fatty acids). The lipoprotein-depleted serum was then dialyzed in 0.9% NaCl, 3 times with at least 40 times volume of LPDS. The LPDS was then tested for lipoprotein or free fatty acid content.

3.3 Plasma lipoprotein isolation and oxidation

3.3.1 Isolation

LDL (density 1.019-1.063 g/ml) was prepared by sequential ultracentrifugation of the plasma male albino New Zealand rabbits fed a 0.5% cholesterol supplemented diet, as described (135, 151). Blood was collected in 5 ml vacutainer tubes containing 7.5 mg EDTA. The plasma was separated from blood cells by centrifugation at 3,000 rpm for 20 minutes at 4°C. Dithiobisnitrobenzoic acid (1.5 mM), phenylmethylsulfonyl fluoride (PMSF) (2 mM), thimerosal (0.08 mg/ml) are added to the plasma after separation of the blood cells to inhibit lecithin-cholesterol acyl transferase, proteolysis and bactericides, respectively. Plasma lipoproteins were isolated by a serial ultracentrifugation technique. Plasma was centrifuged at 38,000 rpm in a Ti70 rotor (Beckman) at 4°C. After 24 hours of centrifugation, the top layer (consisting of chylomicrons (density < 0.996 g/ml), and very low density lipoprotein (VLDL; density < 1.0063 g/ml) was removed. The density of the remaining plasma was adjusted to 1.019 g/ml with the addition of NaCl, using the equation: NaCl g = plasma volume x 0.11698. Low density lipoprotein (LDL; density 1.019 – 1.063 g/ml) was then isolated by centrifugation at 43,000 rpm for another 24 hours. EDTA (0.1 mM) is added throughout the isolation to prevent oxidation of LDL. The LDL fraction is extensively dialyzed against 0.15 M NaCl, 0.1 mM EDTA (pH 7.4), sterile filtered (0.2 μm pore size) and stored at 4°C. The protein content of LDL was determined by Lowry's method (136) and cholesterol (free and esterified) was measured enzymatically as described. The absence of LDL oxidation during isolation or prior to its

use in experiments was determined by an absence of MDA reactive products (68) and oxidized cholesterol (124).

3.3.2 Oxidative modification

The EDTA content of the native LDL was reduced prior to the oxidative modification. LDL was oxidized with a Fe-ADP free radical generating system (88). The Fe-ADP system will generate a variety of free radicals including superoxide anions, hydrogen peroxide and hydroxyl radicals (88). In a typical experiment, 1 mg/ml LDL was incubated at 37°C for 3 hours with freshly prepared 0.05 mM Fe and 0.5 mM ADP in sterile filtered 150 mM NaCl, pH 7.4. The same concentrations of Fe and ADP added to control cells in the absence of LDL had no effect (data not shown).

3.3.2.1 Thiobarbituric acid reactive substances (TBARS) assay

The extent of oxidation was determined by measuring MDA content (68). Briefly, a 1 ml aliquot of 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N HCl was added to the LDL and heated for 15 minutes at 100°C. A pink chromogen developed, and its absorbance was measured at 535 nm. Freshly diluted malondialdehyde bis-dimethyl acetal 1,1,3,3-tetramethoxypropane was used as a reference standard, and thiobarbituric acid reactive substances were measured as nmol MDA/mg protein.

3.4 Cell cycle analysis by flow cytometry

Following exposure to 0, 10 or 50 μg/ml oxLDL for 2, 6, 12, 24 and 48 hours, cells were trypsinized, fixed in ice-cold 100% ethanol, and treated with RNase A (500

U/ml in 1.12% sodium citrate) for 15 minutes at 37°C. DNA was stained with propidium iodide (5% solution in 1.12% sodium citrate) for 30 minutes at room temperature in the dark. Samples were analyzed on a Becton Dickinson FacsCalibur flow cytometer. The percentage of cells in each phase of the cell cycle was estimated using CellQuest software.

3.5 Measurement of cell numbers

For quantification of the number of cells in culture following treatments, cells were trypsinized and counted in a hemacytometer. For each condition and time point, a minimum of 18 fields were counted.

3.6 Immunocytochemistry

Cells were seeded onto glass coverslips and maintained as described above. After oxLDL treatment, cells were fixed in 50% acetone/50% methanol for 3 minutes. A blocking solution of wash buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) plus 10% skim milk powder was used before antibody treatments. Cells were then immunostained with primary antibodies to Cdc 2, Cdk 2, Cdk 4, Cyclin A, Cyclin B, Cyclin D1, $p21^{cip1}$, $p27^{kip1}$, p53 and Rb (Transduction Laboratories), Cyclin E sc-481 (Santa Cruz) and PCNA (Sigma) according to the directions of the manufacturer. Following incubation with primary antibody for 1 hour at room temperature, coverslips were rinsed repeatedly with wash buffer before incubation with a secondary antibody conjugated to fluorescein isothiocyanate (FITC) (Sigma) for an additional hour at room

temperature in the dark. Nuclear staining was done using 50 mM Hoescht No. 33258 in PBS. Coverslips were mounted on slides using Fluorsave reagent (Calbiochem).

3.6.1 Confocal microscopy

Changes in the distribution of cell cycle proteins were observed using a Bio-Rad 600 UV-confocal microscope running COMOS 7.0a software, attached to a Nikon Diaphot 300 with a 40x oil immersion planapochromat Fluor objective (N.A. 1.3). To visualize the FITC fluorophore, the VHS filter block was used; the UVHS filter was used to visualize Hoescht staining. The FITC fluorescence was obtained by exciting the cells with a 488 nm laser line and the emission was collected at 520 nm. The Hoescht No. 33258 fluorescence was obtained by exciting the nuclei with a 351 nm laser line and the emission was collected at 450 nm. For each experiment, cells were viewed at 3% transmission, with black levels set at 5.2 and gain at 7.5. Images of cells were Kalman filtered 3 times. Images were processed using Confocal Assistant 4.02. Nuclear fluorescence was quantified using Molecular Dynamics Imagespace software, v.3.2.1. Background reactivity was checked in the presence of preimmune sera or in the absence of primary antibody. For each experiment, multiple fields of cells were visualized.

3.7 Apoptosis assay

Apoptotic cells were detected using an ApoDETECT Annexin V-FITC Kit (Zymed). Cells were visualized by confocal microscopy.

3.8 Protein assay

Protein content was determined using the modified Lowry assay (136).

3.9 Preparation of cell extracts and Western blot analysis

Cells treated as described above were washed twice with PBS and lysed with SDS lysis buffer (62.5 mM Tris-HCl [pH 7.6], 100 mM NaCl, 1% SDS, 1 mM PMSF, 21μM leupeptin). Protein concentrations of each sample were determined using the modified Lowry assay (136). Samples were diluted using 2x sample buffer (375 mM Tris-OH, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.04% bromophenol blue, pH 8.8), and denatured by heating to 100°C for 5 minutes. For each sample, 50 μg total protein was fractionated by SDS-PAGE (Bio-Rad Protean II system) in a gradient gel (3 - 15%) with a 4% stacking gel for 4 hours at 550 mV, 80 mA (constant current). Running buffer contained 0.025 mM Tris-HCl, 0.192 mM glycine, and 0.1% SDS. Gels were calibrated using prestained molecular weight markers (Gibco BRL). Following electrophoresis, gels were incubated in Towbin's buffer (25 mM Tris-OH, 192 mM glycine, 20% methanol, pH 8.3) for 15 minutes. Nitrocellulose membranes (Gibco BRL) were pre-soaked in the same buffer prior to transfer. Transfer onto nitrocellulose membrane was performed using a BioRad apparatus for 75 minutes at 50 V (constant voltage). Following completion of transfer, equal loading of lanes was confirmed by staining with Ponceau S stain (Sigma) for 5 minutes. After removing Ponceau stain with several washes using wash buffer, the membrane was then placed in blocking buffer for an hour at room temperature on a multi-mixer. Antibody treatments were performed according to the manufacturer's instructions. Primary antibodies were typically diluted in 1% blocking buffer and

incubated with the membranes on a multi-mixer for one hour at room temperature or overnight at 4°C. Membranes were then washed five times (15 minutes per wash) in wash buffer and antibody reactions were detected using horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's instructions. Secondary antibodies were typically diluted in 1% blocking buffer and incubated with the membranes on a multi-mixer for one hour at room temperature or overnight at 4°C. Reactions were detected with enhanced chemiluminescent detection reagents (Pierce). Densitometry was performed on a BioRad GS-670 Imaging Densitometer. For Western blots of rabbit tissue samples, antibodies PCNA (Sigma), Cyclin A (Abcam), Cdk 4 (BD Transduction Laboratories) and PARP (BD PharMingen) were used.

3.10 Assay of kinase activity

Immunoprecipitation of Cdk 4 was carried out by adding 1 μg Cdk 4 antibody (Transduction Laboratories) to 200 μg total cell lysate, 250 μl 2x immunoprecipitation buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris [pH 7.4], 2 mM EDTA, 2 mM EGTA [pH 8.0], 0.4 mM sodium orthovanadate, 0.4 mM PMSF, 1% NP-40), and H_2O to a final volume of 500 μl. The immunoprecipitation reaction was carried out overnight at 4°C with gentle rotation. The next day, 20 μl 50% protein G agarose beads (Calbiochem) were added, and the sample was incubated at 4°C with gentle rotation for 30 minutes. The beads were collected by centrifugation (1 minute at 7,000 rpm, 4°C) and the supernatant was removed. The bead pellet was washed with 1x immunoprecipitation buffer, centrifuged (4 minutes at 14,000 rpm, 4°C), and the supernatant discarded. The washing step was repeated twice more using 1x immunoprecipitation buffer, and a final wash was

done using kinase reaction buffer (40 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 1 mM DTT, 2 mM EDTA [pH 8.0]). Following the last wash, the bead pellet was resuspended in 30 μl of kinase reaction buffer plus 0.2 μCi/μl [γ-^{32}P]ATP (specific activity 3,000 Ci; 111 TBq/mM) and the kinase substrate (0.01 μg/μl GST-pRb (Santa Cruz)). The reaction was carried out for 30 minutes at 30°C and stopped by the addition of 4x SDS-PAGE loading buffer. Samples were then loaded onto a 10% gel and separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250 stain to confirm equal amounts of kinase substrate in each sample, and then destained and dried. Phosphorylated substrate was visualized by autoradiography and quantitated by densitometry. Immunoprecipitation reactions for Cdc 2 and Cdk 2 were carried out using an identical protocol, but with 0.2 μg/μl histone H1 (Gibco BRL) used as the kinase substrate in place of GST-pRb.

3.11 IP_3 assay

Cells treated as described were washed with PBS, scraped down and homogenized. The D-myo-inositol 1,4,5-triphosphate (IP_3) content of the homogenate was measured using a radioisotopic assay kit (Amersham) according to manufacturer's instructions.

3.12 Animal model of atherosclerosis

Male albino New Zealand white rabbits (2.5-3.0 kg) were used. These animals do not generate atherosclerotic lesions on a normal chow diet. In order to elevate the serum cholesterol levels and induce atherosclerotic lesions in these rabbits, a diet supplemented with 0.5% cholesterol was fed to these animals (Purina Test Diets). Rabbits in these

experiments were maintained on regular rabbit chow (control) or a 0.5% cholesterol diet (treated) for 8 or 16 weeks.

3.13 Quantification of plaque in aortas

Aortas were harvested, cleaned, cut open lengthwise and into sections, and photographed using a Nikon Coolpix 995 camera. Photographs were digitized and amount of plaque was quantified using Molecular Dynamics Imagespace software, v.3.2.1.

3.14 Preparation of tissue samples

Approximately 0.4 g wet weight of rabbit aortic tissue was chopped finely and added to a tube containing 1 ml of modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, aprotinin 1 μM, leupeptin 1 μM, pepstatin 1 μM]. Homogenization was performed with a Polytron Homogenizer for 1 minute. The homogenate was ultracentrifuged for 30 minutes at 100,000 xg and the supernatant was removed. The pellet was further subjected to nuclear protein extraction using NER (Nuclear Extraction Reagent), purchased from Pierce. 250 μl of NER was added to each tube. The tube was vortexed for 15 seconds to resuspend the pellet, then placed on ice for 10 minutes. The tube was vortexed again and returned to ice, repeating this process every 10 minutes for a total of 40 minutes. The tube was then centrifuged at 16,000 xg in a microfuge for 10 minutes. The supernatant was removed and added to the original supernatant. The combined supernatants were mixed thoroughly and assayed for

protein concentration using the modified Lowry assay (136). All samples were kept on ice throughout the experiments, and all centrifugations were performed at 4°C.

3.15 Data analysis

Values were calculated with standard errors of the mean (SEM). Results were analyzed by one-way ANOVA followed by a Student-Newman-Keuls or Dunnett's post-hoc test. The statistics were computed with the program SigmaStat. A value of $P < 0.05$ was considered significant.

CHAPTER 4: RESULTS

4.1 Experiments using quiescent cultured vascular cells

Though the cell cycle represents the final common pathway of all mitogenic signaling cascades, there has been no evidence to date linking oxLDL to the induction of cell cycle proteins. Furthermore, the pattern of expression of cell cycle proteins and the upstream signaling pathway by which they are induced in the progression of vascular disease has not been elucidated. The purpose of the present study, therefore, was to determine if oxLDL is capable of inducing proliferation in quiescent cells, to identify if oxLDL is capable of altering the expression and distribution of specific cell cycle proteins, and finally to identify the signaling pathways involved in the mitogenic response.

4.1.1 Proliferation of vascular cells following exposure to oxLDL

The ability of oxLDL to stimulate entry of cells into the cell cycle was first analyzed by flow cytometry (Figure 9). Cells maintained in starvation medium (no oxLDL) for 24 hours remained at approximately 92–95% G0/G1 arrested. In contrast, cells treated with 10 or 50 μg/ml oxLDL had substantial decreases in the proportion of cells in G0/G1 over time. For example, after treatment with 10 μg/ml oxLDL, 91, 93, 82 and 78% of cells were in G0/G1 at 2, 6, 12 and 24 hours. After treatment with 50 μg/ml oxLDL, 89, 91, 74 and 72% of the cells remained in G0/G1. Therefore, oxLDL released cells from growth arrest in a time and dose dependent manner.

The ability of oxLDL to stimulate proliferation was then assessed by total cell

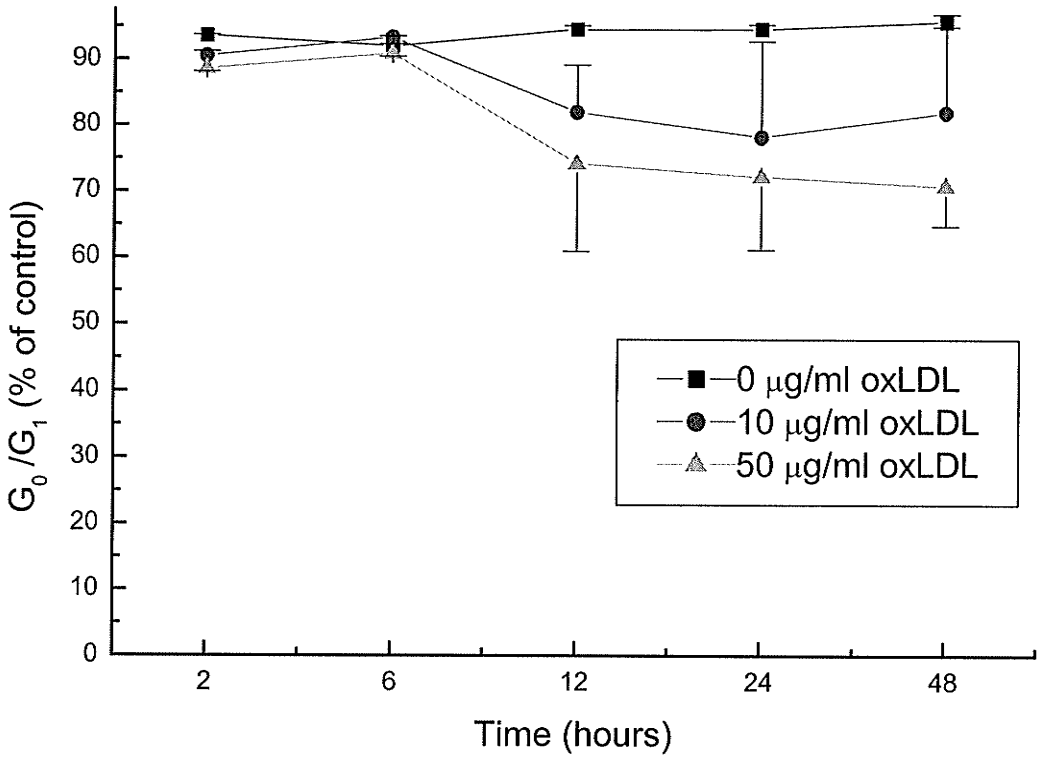


Figure 9. OxLDL stimulates cell cycle entry in quiescent fibroblasts. Cells were maintained in serum-free media for 6 days before oxLDL treatment. DNA synthesis was assessed by propidium iodide staining using a FACsCalibur flow cytometer. The proportion of cells in G0 /G1 following exposure to 0, 10 or 50 μg/ml oxLDL for 24 or 48 hours is expressed as a percentage of control, ± SEM.

numbers (Figure 10). Treatment of cells with 0, 10, and 50 μg/ml oxLDL for 24 or 48 hours resulted in significant increases in the numbers of both fibroblasts and smooth muscle cells. At least 995 cells were counted for each treatment and time point. Exposure to 10 μg/ml oxLDL resulted in increases of 39% at both 24 and 48 hours in fibroblasts. The same concentration of oxLDL increased VSMC numbers by 25% and 27% at 24 and 48 hours respectively. Exposure of fibroblasts to 50 μg/ml oxLDL increased cell numbers by 59% at 24 hours and 40% at 48 hours, while VSMC numbers increased by 55% (24 hours) and 33% (48 hours) under the same conditions. Treatment of both types of cells with native LDL did not result in the same magnitude of change in cell numbers at either concentration or time point.

For comparative purposes, we examined the effects of exposing quiescent fibroblasts to bFGF (data not shown). Over 24 hours of exposure, fibroblast cell numbers increased 39.4% to 10 μg/ml oxLDL and 33.6% to 10 ng/ml bFGF. Over 48 hours of exposure time, fibroblast cell numbers increased 38.7% to 10 μg/ml oxLDL and 86% to 10 ng/ml bFGF. Thus, oxLDL appears to possess a mitogenic activity similar to bFGF for 24 hours exposure times but does not induce as sustained a proliferative effect over 48 hours.

To evaluate the possible role of the scavenger receptor in the proliferative mechanism of oxLDL, the scavenger receptor blocker polyinosinic acid was used. At a concentration of 25 μg/ml, polyinosinic acid effectively inhibited the mitogenic action of oxLDL on serum-starved fibroblasts (Figure 11). The PI 3-kinase inhibitor LY294002 (at a concentration of 20 μg/ml) also prevented oxLDL-induced proliferation, as did the PLC inhibitor NCDC (used at a concentration of 50 μg/ml). The MEK 1/2 inhibitor PD98059

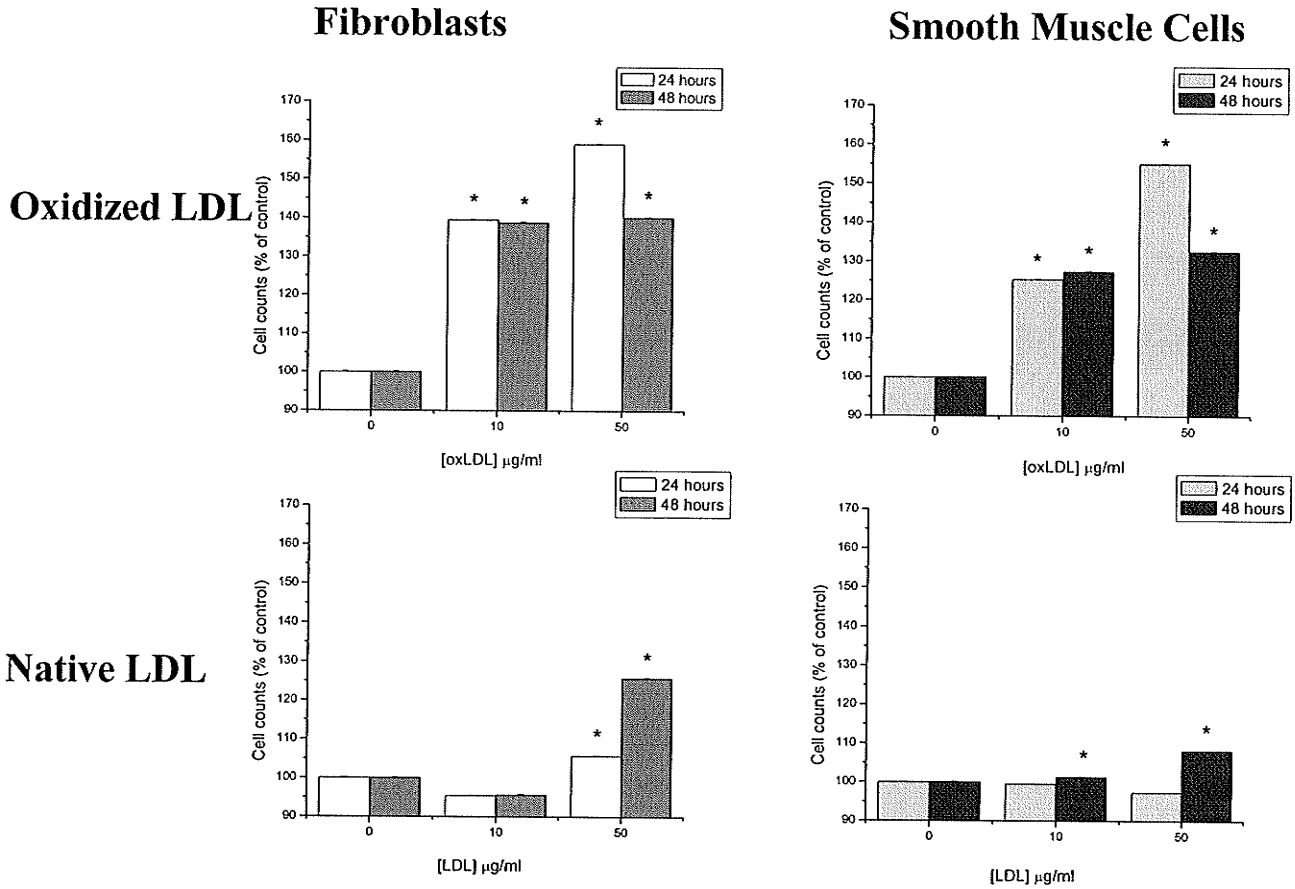


Figure 10. Increase in total number of cells following exposure of quiescent fibroblasts and VSMC to oxLDL. Mean number of cells per field, as counted using a hemacytometer, is expressed as a percentage of control, ± SEM (*$p<0.05$). Data represents at least 4 independent experiments. A minimum of 1046 cells were counted per treatment. In some cases, the SEM bars are too small to resolve on this figure.

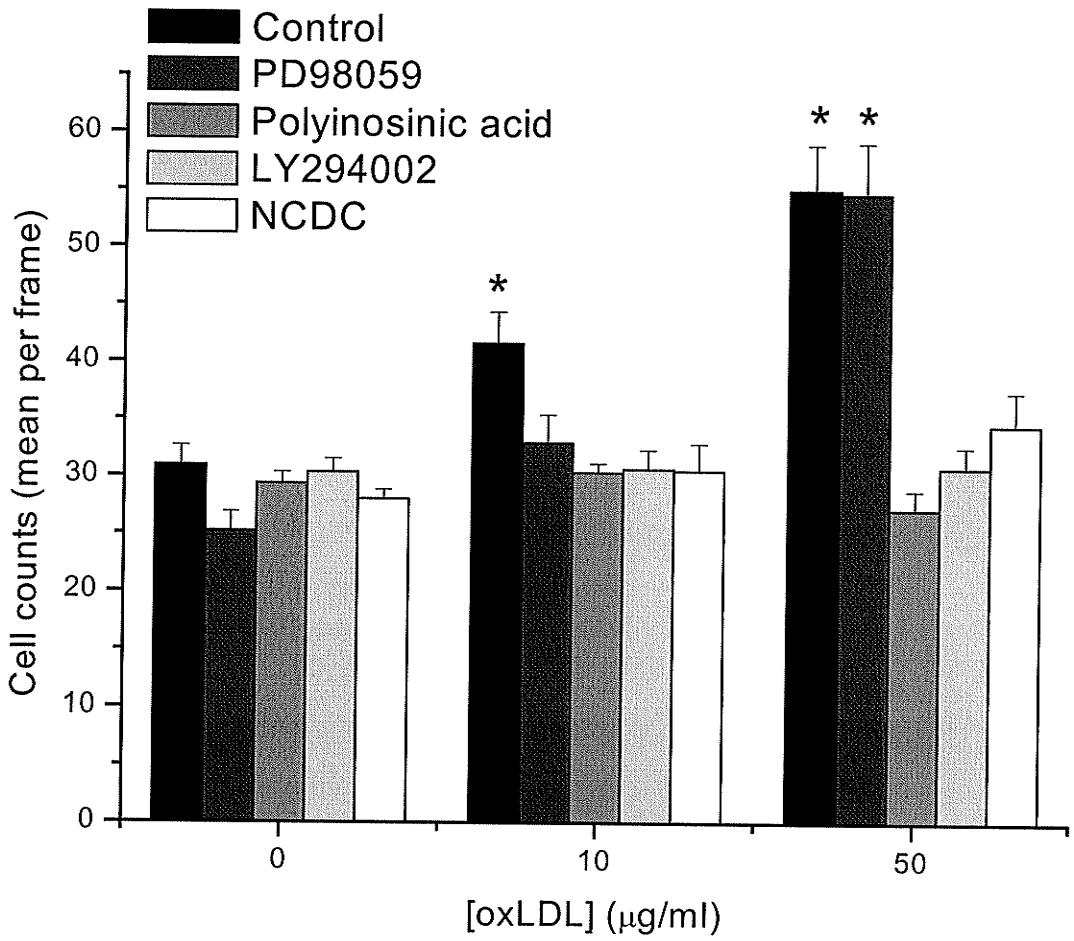


Figure 11. Effect of inhibitors on cell numbers in fibroblasts exposed to oxLDL. Cell numbers are expressed as a mean per field ± SEM (*$p<0.05$). Cells were pretreated with inhibitors alone for 15 minutes before exposure to oxLDL in combination with the inhibitors for 24 hours. Data represents at least 4 independent experiments. A minimum of 112 cells were counted per treatment.

(used at a concentration of 4 μg/ml), while effective in preventing growth in response to 10 μg/ml oxLDL, did not prevent growth in response to 50 μg/ml oxLDL. In each experiment, cells treated with the inhibitor in the absence of oxLDL showed no evidence of increased LDH release as compared to cells maintained in starvation medium (Figure 11).

Because the PLC signaling pathway appeared to be involved in the proliferative action of oxLDL, we speculated that the signaling molecule IP_3 might also play a role. Fibroblasts were treated with 0 or 50 μg/ml oxLDL in the presence or absence of 50 μg/ml NCDC for 24 hours. Treatment of fibroblasts with 50 μg/ml oxLDL resulted in a significant increase in IP_3 levels (Figure 12). This increase was prevented by NCDC treatment.

4.1.2 Cell cycle protein expression following exposure to oxLDL

Western blot analysis was used in order to determine if oxLDL could induce changes in total cellular levels of cell cycle proteins. Expression of the cell cycle proteins was examined in whole cell extracts of fibroblasts exposed to 10 and 50 μg/ml oxLDL for 24 and 48 hours. Total cellular levels of PCNA were significantly increased at both concentrations and time points with respect to controls (Figure 13a). Exposure to 10 μg/ml oxLDL resulted in an increase of 39% over control at 24 hours and 34% over control at 48 hours. Higher concentrations of oxLDL caused similar effects. Surprisingly, the expression of a cell cycle inhibitor, $p27^{kip1}$, was also induced by oxLDL treatment (Figure 13b). Exposure of cells for 24 hours to 10 and 50 μg/ml oxLDL resulted in

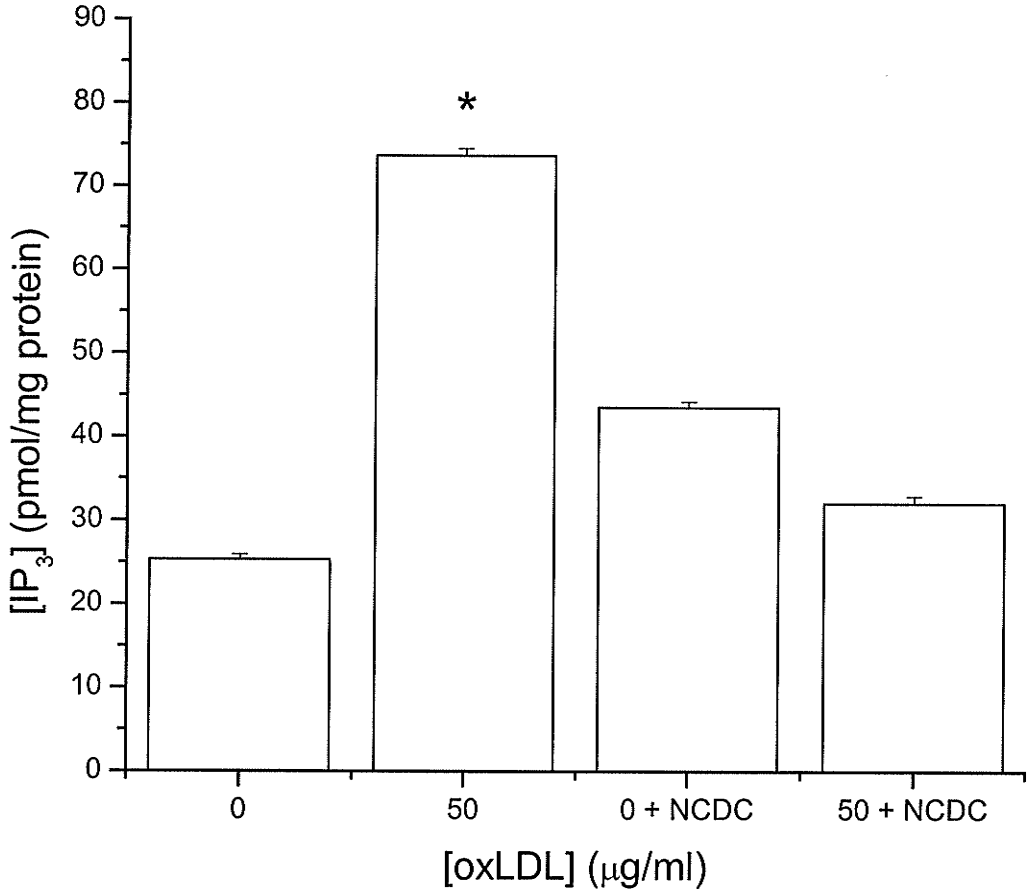


Figure 12. IP_3 content in oxLDL-treated cells. Fibroblasts were treated with 0 or 50 μg/ml oxLDL in the presence or absence of 50 μg/ml NCDC for 24 hours. Data represents 4 independent experiments. IP_3 content is expressed as pmol/mg protein, ± SEM (*$p<0.05$ vs. all other treatments).

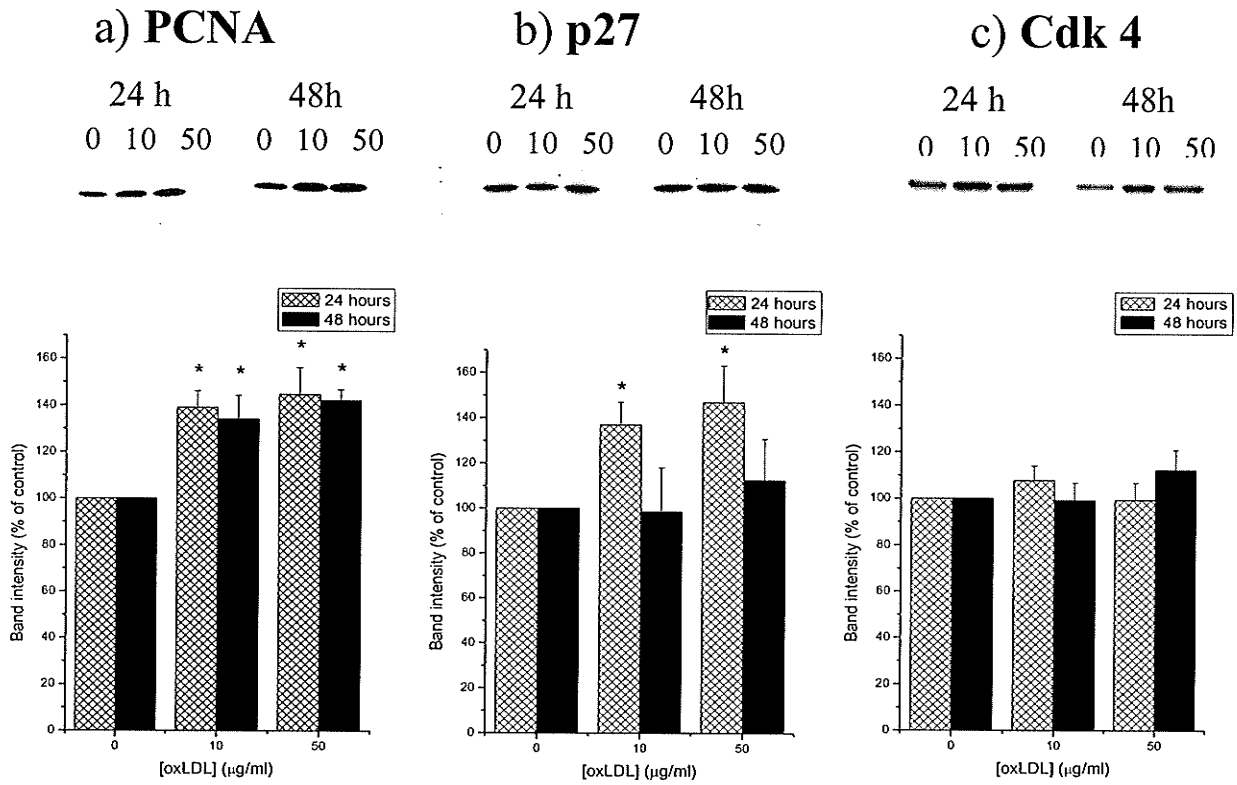


Figure 13. Expression of (a) PCNA, (b) $p27^{kip1}$ and (c) Cdk 4 in fibroblasts following exposure to oxLDL. Representative bands from Western blots of whole cell extracts from oxLDL-treated cells. 50 µg of protein was loaded in each well. Densitometric comparisons of expression in cells exposed to 0, 10 or 50 µg/ml oxLDL for 24 or 48 hours, expressed as a percentage of control, ± SEM (*$p<0.05$). Data represents at least 3 independent experiments for each protein examined.

increases in expression of 36% and 47% over control. Longer exposure times did not change expression. Not all cell cycle proteins were affected by oxLDL treatment. No significant changes in the expression of Cdk 4 were observed (Figure 13c).

The effects of oxLDL on both cell cycle activators and inhibitors in Figure 13 prompted us to examine other representative proteins in greater depth. We examined Cyclin D1 and $p21^{cip1}$ expression at earlier time points (6-48 hours) after exposure of cells to oxLDL (Figure 14). These targets were chosen because of their importance in regulating the cell cycle. Cyclin D1 is the first cyclin necessary for movement of the cells from a growth-arrested state into the cell cycle, and $p21^{cip1}$ is a potent inhibitor of cell proliferation throughout the entire cycle (83). Expression of Cyclin D1 increased over control as early as 6 hours after exposure to 10 μg/ml oxLDL. Maximal effects were observed at 24 hours followed by a sharp decline in expression at 48 hours. Although the effects of oxLDL on $p21^{cip1}$ expression followed a similar pattern, the induction in expression was delayed and less pronounced. Following exposure to a higher [oxLDL] (50 μg/ml) for 24 hours, total cellular levels of $p21^{cip1}$ were also significantly increased by 20% over control (data not shown).

Cdc 2, Cdk 2 and Cyclin B1 were difficult to detect in control cells (Figure 15). However, by 24 and 48 hours following exposure to oxLDL, the levels of these proteins clearly increased. Both 10 and 50 μg/ml oxLDL induced significant changes in expression of all of these proteins. However, because of the low levels of expression in the control cells, it was not possible for us to quantitate this increase. The results depicted in Figure 15 are representative of several experiments (n=4).

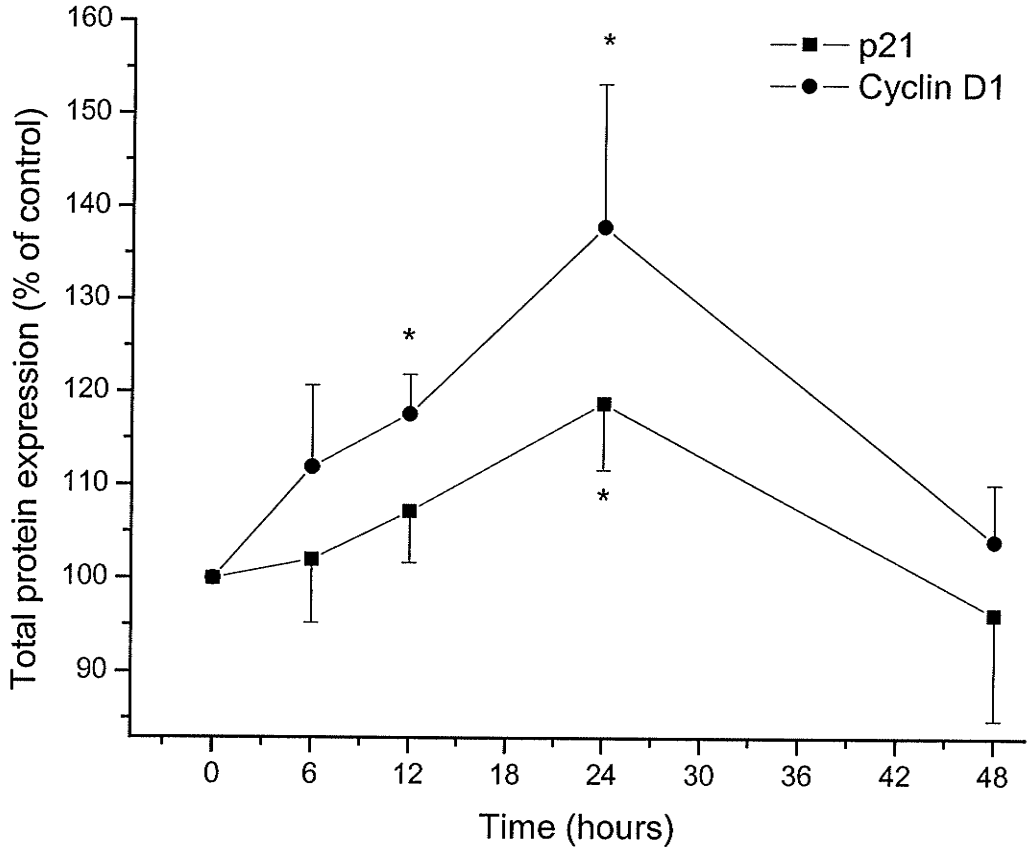


Figure 14. Expression of Cyclin D1 and $p21^{cip1}$ in quiescent fibroblasts following exposure to oxLDL. Data is representative of at least 3 independent experiments for each protein and time point. Densitometric comparisons of expression in cells exposed to 50 μg/ml oxLDL for 6, 12, 24 and 48 hours are expressed as a percentage of control, ± SEM *$p<0.05$ indicates significant difference from respective untreated values.

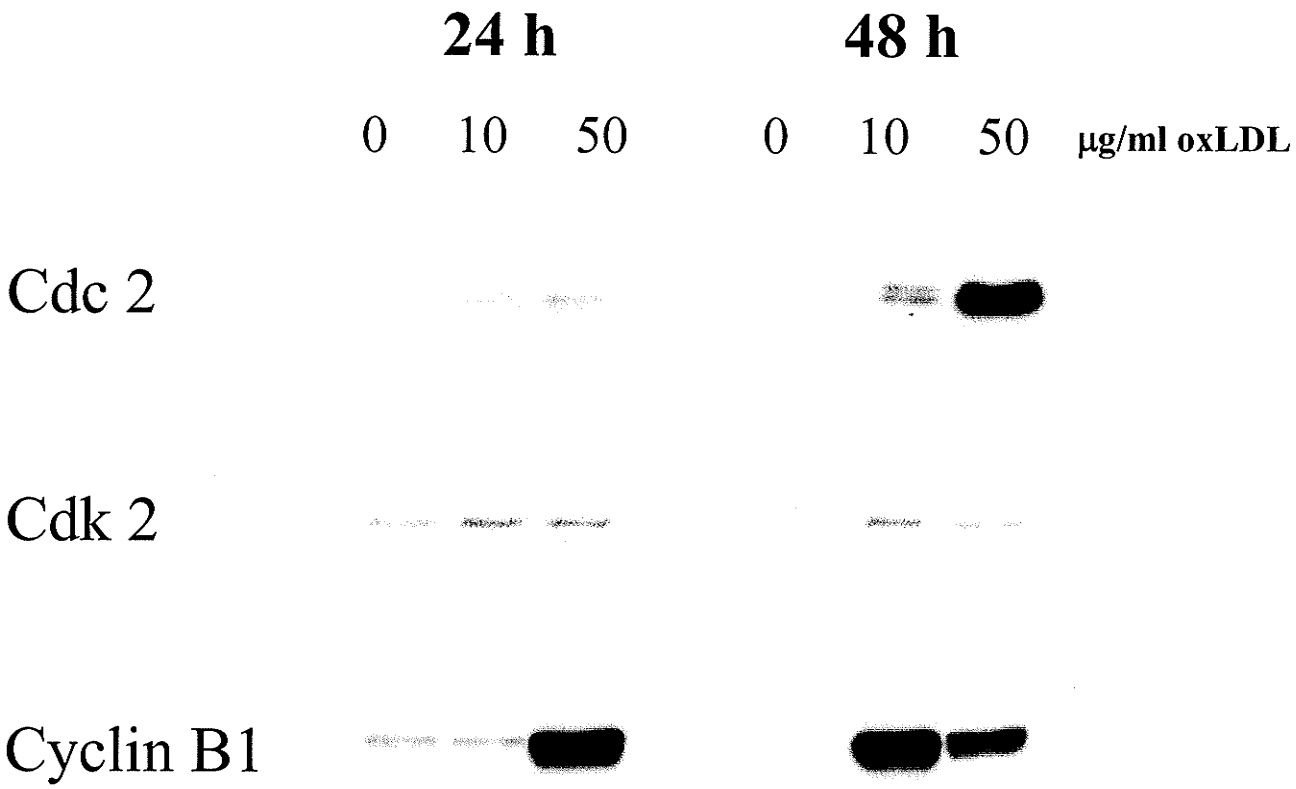


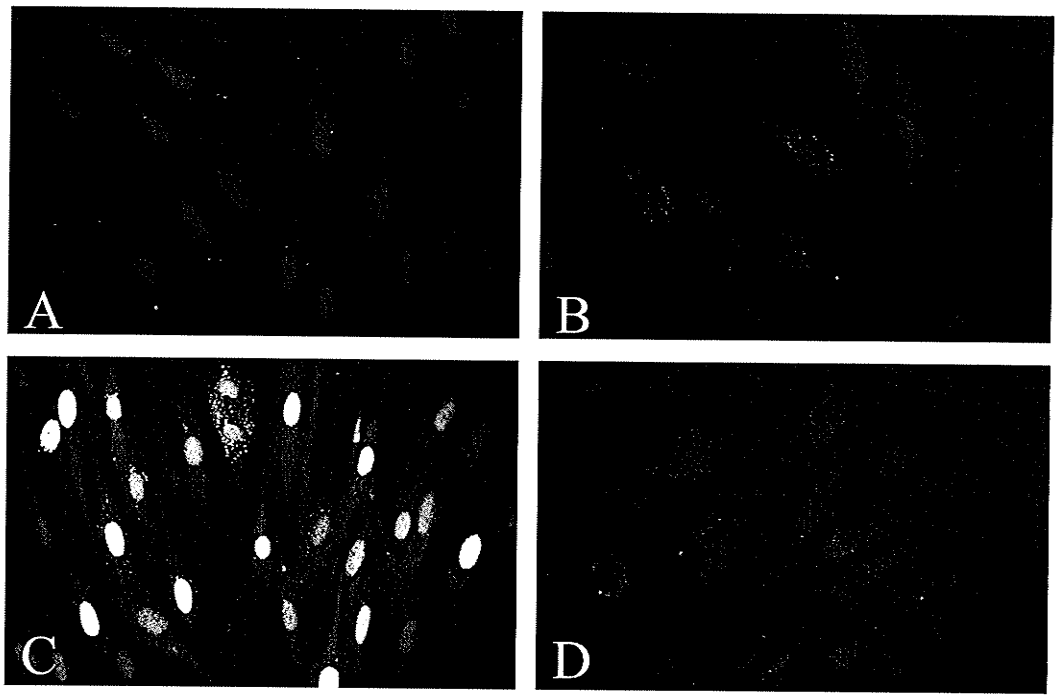
Figure 15. Increased expression of Cdc 2, Cdk 2 and Cyclin B1 in fibroblasts following exposure to oxLDL. 50 µg of protein was loaded in each well. Bands from Western blots are representative of 3 independent experiments. Because control expression was minimal, quantification via densitometric comparisons could not be completed in a reliable fashion.

4.1.3 Cell cycle protein distribution following exposure to oxLDL

Cellular distribution of cell cycle proteins was then studied in cells exposed to 10 and 50 μg/ml oxLDL for 24 and 48 hours. Translocation into the nucleus is a key step in the activation of cyclin/cyclin-dependent kinase complexes (83). Nuclear levels of PCNA were significantly higher than controls in fibroblasts treated with 50 μg/ml oxLDL for 24 hours (38% over control) (Figure 16). Forty-eight hours of 10 and 50 μg/ml oxLDL treatment elevated nuclear levels of PCNA by 92% and 124% over control, respectively ($p < 0.05$).

At the 24 hour time point, nuclear levels of Cyclin D1 were significantly increased at both 10 and 50 μg/ml oxLDL, by 49% and 45% respectively as compared to control (Figure 17). By 48 hours, levels of nuclear Cyclin D1 had risen by 119% (10 μg/ml oxLDL) and 221% (50 μg/ml oxLDL) versus control.

Similar comparisons were made for the cell cycle proteins Cdc 2, Cdk 2, Cdk 4, Cyclin A, Cyclin B1, Cyclin E, $p21^{cip1}$, $p27^{kip1}$, p53 and Rb (Table 4). Following 24 hours of exposure to 10 μg/ml oxLDL, significant increases in nuclear levels of Cdc 2 and Cdk 4 were noted. After 48 hours of exposure to 10 μg/ml oxLDL, significant increases were observed in nuclear levels of all cell cycle proteins but $p27^{kip1}$. Twenty-four hours of exposure to 50 μg/ml oxLDL induced significant increases in nuclear levels of every cell cycle protein examined but Cyclin B1. Exposure to 50 μg/ml oxLDL for 48 hours resulted in significant increases in nuclear levels of all cell cycle proteins but Cyclin B1 and Cyclin E. Therefore, these data suggest that exposure of fibroblasts to oxLDL induces increases in nuclear levels of Cdc 2, Cdk 2, Cdk 4, Cyclin A, Cyclin B1, Cyclin D1, Cyclin E, $p21^{cip1}$, $p27^{kip1}$, p53, PCNA and Rb.



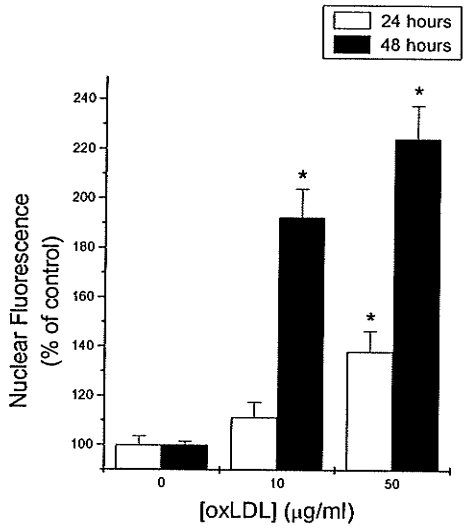


Figure 16. Increased nuclear fluorescence of PCNA in fibroblasts following 48 hours of 50 μg/ml oxLDL treatment. A) Cells treated with 0 μg/ml oxLDL stained with primary antibody to PCNA and secondary antibody to FITC. B) Cells treated with 0 μg/ml oxLDL stained with secondary antibody to FITC (no primary antibody). C) Cells treated with 50 μg/ml oxLDL stained with primary antibody to PCNA and secondary antibody to FITC. D) Cells treated with 50 μg/ml oxLDL stained with secondary antibody to FITC (no primary antibody). Magnification = 400x. A comparison of nuclear fluorescence in cells exposed to 0, 10 or 50 μg/ml oxLDL for 24 or 48 hours, expressed as a percentage of control, ± SEM (*$p<0.05$). N=3 for each condition and time point.

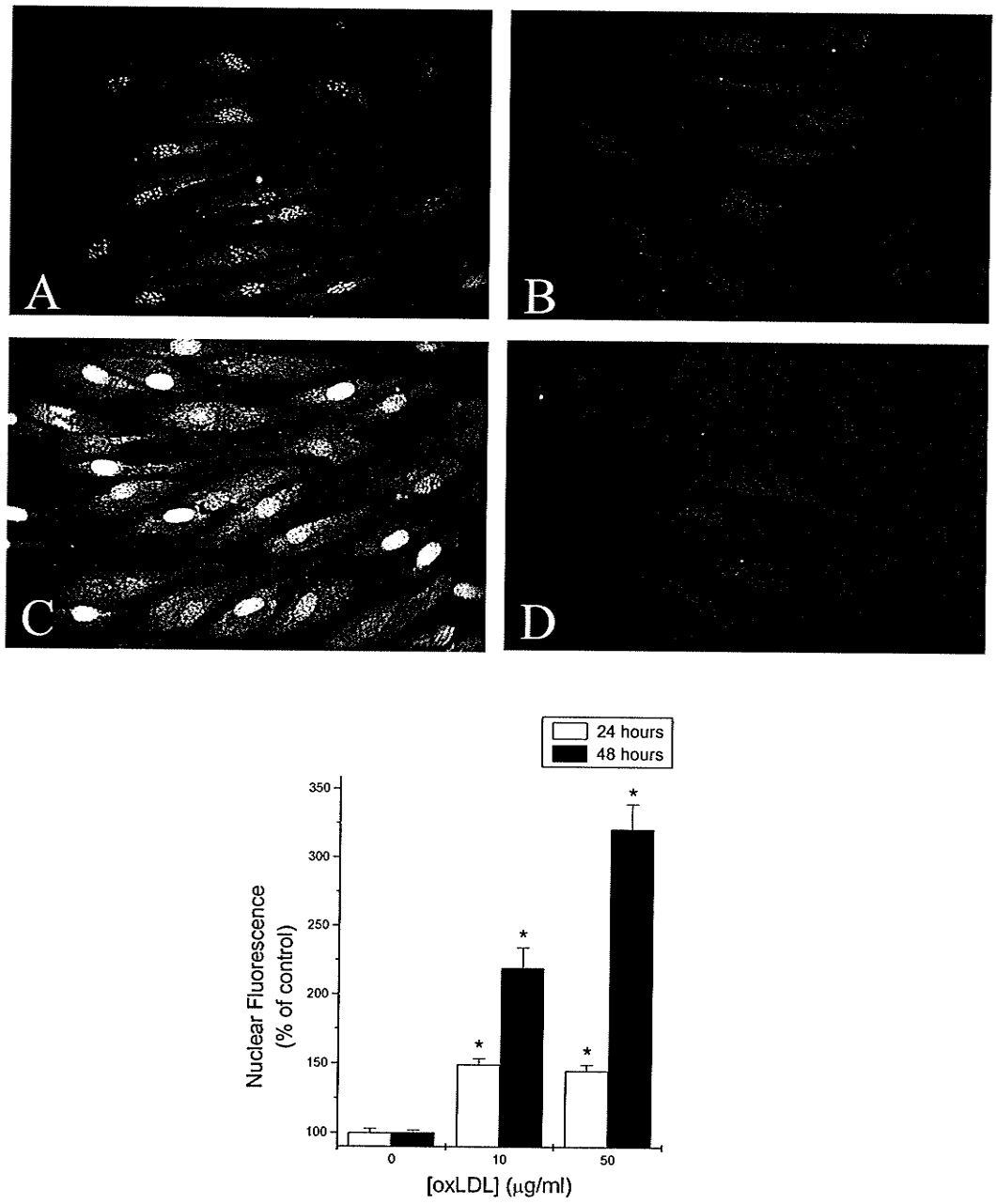


Figure 17. Increased nuclear fluorescence of Cyclin D1 in fibroblasts following 48 hours of 50 μg/ml oxLDL treatment. A) Cells treated with 0 μg/ml oxLDL stained with primary antibody to Cyclin D1 and secondary antibody to FITC. B) Cells treated with 0 μg/ml oxLDL stained with secondary antibody to FITC (no primary antibody). C) Cells treated with 50 μg/ml oxLDL stained with primary antibody to Cyclin D1 and secondary antibody to FITC. D) Cells treated with 50 μg/ml oxLDL stained with secondary antibody to FITC (no primary antibody). Magnification = 400x. A comparison of nuclear fluorescence in cells exposed to 0, 10 or 50 μg/ml oxLDL for 24 or 48 hours, expressed as a percentage of control, ± SEM (*$p<0.05$). N=4 for each condition and time point.

Table 4. Increased nuclear fluorescence of cell cycle proteins following exposure of quiescent fibroblasts to oxLDL.

	10 μg/ml oxLDL		50 μg/ml oxLDL	
	24 h	**48 h**	**24 h**	**48 h**
Cdc 2	112 ± 2*	131 ± 2*	127 ± 2*	122 ± 3*
Cdk 2	101 ± 4	163 ± 8*	139 ± 10*	206 ± 8*
Cdk 4	110 ± 2*	175 ± 7*	110 ± 2*	146 ± 3*
Cyclin A	115 ± 5	201 ± 15*	174 ± 9*	192 ± 11*
Cyclin B1	98 ± 3	273 ± 17*	96 ± 1	118 ± 2
Cyclin E	100 ± 2	107 ± 2*	123 ± 2*	98 ± 1
$p21^{cip1}$	103 ± 2	248 ± 16*	128 ± 4*	131 ± 4*
$p27^{kip1}$	99 ± 2	96 ± 2	108 ± 2*	108 ± 3*
p53	95 ± 3	124 ± 4*	174 ± 6*	111 ± 4*
Rb	93 ± 2	113 ± 4*	112 ± 3*	118 ± 3*

Mean increases in nuclear fluorescence of cell cycle proteins, expressed as a percentage of control ± SEM (*$p<0.05$). Results are from at least 3 independent experiments. In each experiment, a minimum of 95 cells were counted.

4.1.4 Kinase activation following exposure to oxLDL

Although translocation of cell cycle proteins into the nucleus suggests the activation of cyclin/cyclin-dependent kinase complexes, it is not direct proof of such activation. We examined the Cdk 4 kinase activity following oxLDL exposure as a representative marker of kinase activation under our experimental conditions. Exposure of cells to 50 μg/ml oxLDL for 24 hours resulted in a significant increase of 20% in Cdk activity in comparison to control cells (Figure 18).

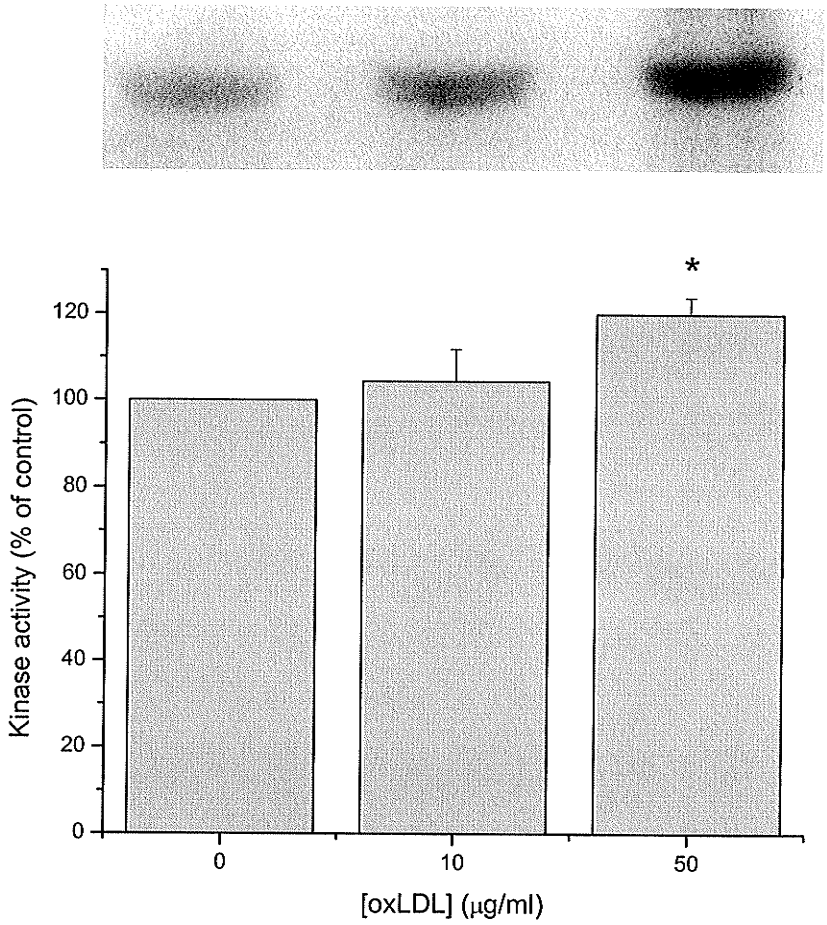


Figure 18. Increased kinase activity of Cdk 4 in fibroblasts following exposure to oxLDL. Upper figure: Representative autoradiograph showing Cdk 4 activity (with GST-pRb as a substrate) in whole cell extracts from fibroblasts treated with 0, 10 or 50 μg/ml oxLDL for 24 hours. Lower graph: Densitometric comparison of Cdk 4 activity, expressed as a percentage of control, SEM (*$p<0.05$). N=4 for each condition and time point.

4.2 Experiments using proliferating cultured vascular cells

Changes in the expression of a number of cell cycle proteins in the vessel wall during atherosclerosis or restenosis have been identified and are thought to represent critical cellular events that determine the proliferative potential of the cells during these pathological states (42, 109, 238, 256, 265, 272). Recently, however, this hypothesis has been challenged (78, 82, 183). Some studies report minimal evidence in favor of an accelerated cell proliferation in plaques, despite the presence of an atherogenic environment that would be expected to stimulate cell proliferation (82, 183). Therefore, instead of a stimulation of cell proliferation during atherosclerosis, other plausible mechanisms have been proposed (i.e. inhibited apoptosis) (78). It is also possible, however, that mitogenic factors like oxLDL may not have been studied optimally in past studies to define their proliferative potential. Previous investigations have studied the effects of oxLDL in isolation, however, in an *in vivo* atherosclerotic environment, vascular cells are exposed to oxLDL in the presence of a multitude of cytokines and growth factors. The proliferative action of oxLDL under these circumstances has not been defined. The purpose of the present study, therefore, was to elucidate the effects of oxLDL on cell proliferation in the presence of a variety of growth factors and cytokines found in serum. To obtain mechanistic insights, the effects of oxLDL on cell cycle proteins in this atherosclerotic environment were a focus for our study.

4.2.1 Proliferation of vascular cells following exposure to oxLDL in combination with serum

The effect of oxLDL on entry of cells into the cell cycle was analyzed by flow cytometry (Figure 19). Cells kept in serum-free medium for 5-6 days remained in a growth-arrested state (90-95% in G0/G1). The addition of serum to the medium caused these cells to move out of G0/G1 and progress through the cell cycle. Only 31% of cells maintained in 5% FBS (no oxLDL) for 24 hours remained in G0/G1. In contrast, cells treated with 5% FBS and 10 or 50 μg/ml oxLDL had significantly higher proportions of cells remaining in G0/G1 over time. For example, at 24 hours, cells treated with 10 μg/ml oxLDL were 66% arrested and cells treated with 50 μg/ml oxLDL were 78% arrested. Therefore, oxLDL inhibited the release of cells from growth arrest in a time and dose dependent manner.

Total cell numbers were assessed to demonstrate that the effects of oxLDL resulted in inhibited movement through the complete cell cycle. Exposure to 10 or 50 μg/ml oxLDL in combination with serum for 24 or 48 hours resulted in significant decreases in the numbers of fibroblasts (Figure 20). To determine if this effect was cell type-specific, VSMC were exposed to an identical experimental protocol. The same qualitative effect was observed. In order to determine if this was specific to oxLDL, native LDL was also tested. Treatment of both fibroblasts and VSMCs with native LDL resulted in an increase rather than a decrease in cell number at both concentrations and time points (Figure 20).

In order to determine if the decrease in cell numbers was due to cell death via apoptosis, annexin V staining was evaluated in cells treated with serum and oxLDL. No

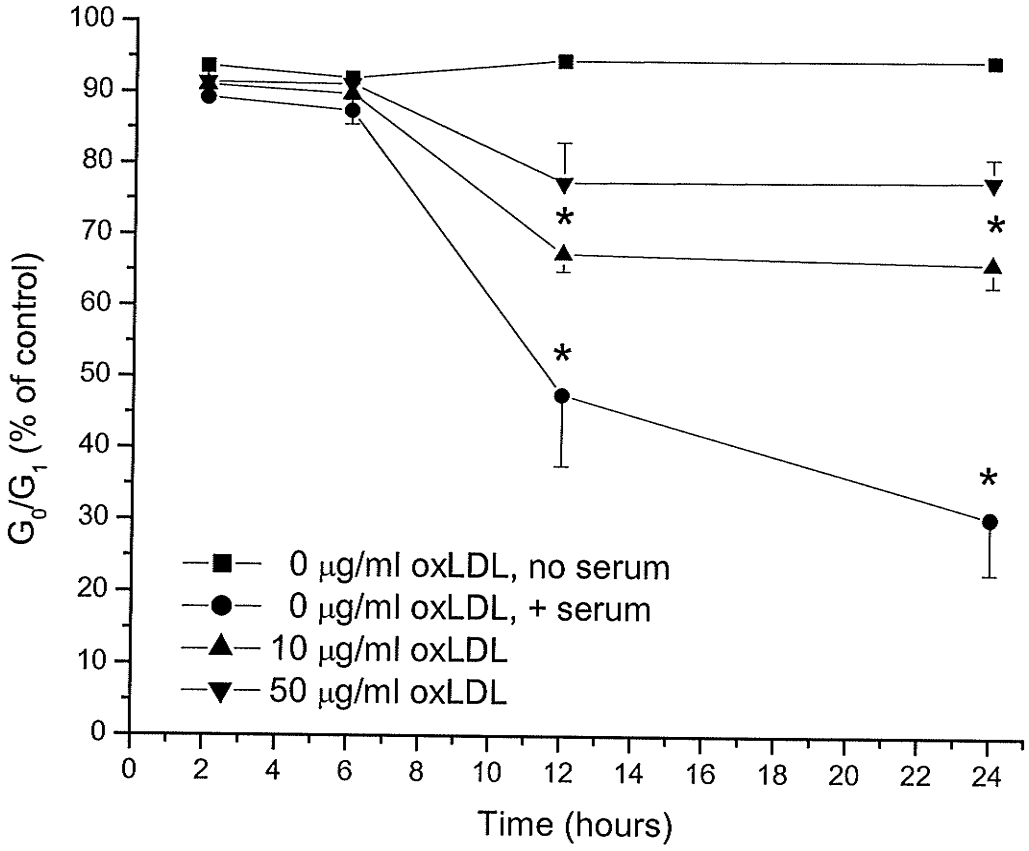


Figure 19. Cell cycle entry in fibroblasts treated with serum and oxLDL. In cells maintained as described, DNA synthesis was assessed by propidium iodide staining using a FACsCalibur flow cytometer. The proportion of cells in G0/G1 following exposure to 0, 10 or 50 μg/ml oxLDL is expressed as a percentage of serum-starved control ± SEM (*$p < 0.05$).

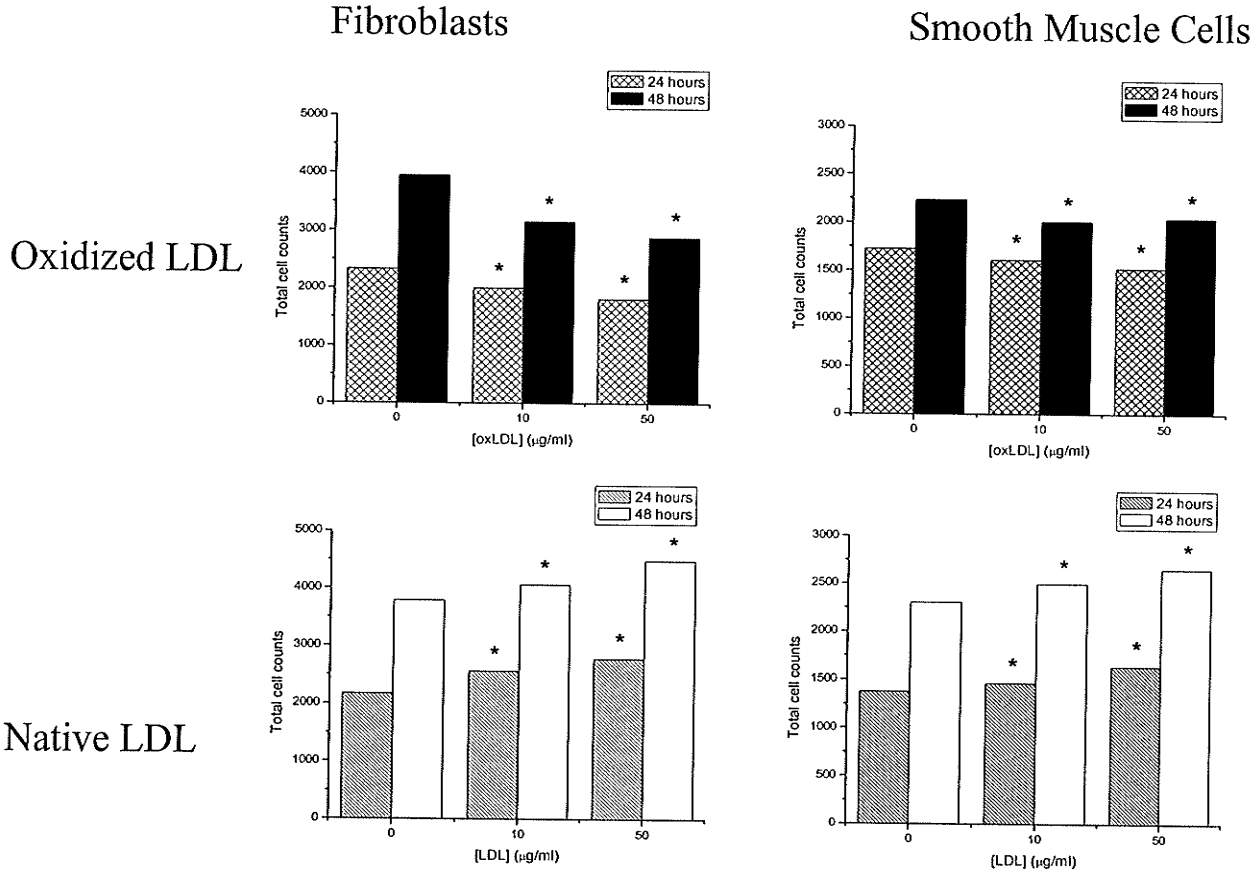


Figure 20. Decrease in total number of cells following exposure of fibroblasts and VSMC to oxLDL or native LDL in combination with serum. Cells were maintained in serum-free media for 6 days before oxLDL or native LDL and serum treatment. Data represents total number of cells as counted using a hemacytometer, ± SEM (*$p < 0.05$) Error bars are too small to resolve given the large N.

apoptotic cells were observed in either the serum-treated group in the presence or absence of oxLDL over 48 hours, as assessed by annexin V staining (Figure 21). Conversely, cells treated with H_2O_2 displayed clear evidence of apoptosis, including positive staining for annexin V.

For comparative purposes, we examined the effects of exposing quiescent fibroblasts to growth factors (aFGF, bFGF and TGF-β1) or lipoprotein-depleted serum in combination with oxLDL. Treatment with either bFGF or TGF-β1 in combination with oxLDL produced a significant increase in cell number, while aFGF plus serum diminished cell growth to an extent similar to serum (see Table 5). Lipoprotein-depleted serum in combination with oxLDL had no effect (cell numbers increased by 6% in combination with 10 μg/ml oxLDL, and 4% in combination with 50 μg/ml oxLDL. These increases were not significant.)

In order to ascertain the mechanism(s) involved in oxLDL's inhibitory effect on cell growth, a number of pharmacological inhibitors of selected signaling pathways were employed. The PI 3-kinase inhibitor LY294002 (used at a concentration of 20 μg/ml), and the PKC inhibitor calphostin C (at a concentration of 200 ng/ml) both failed to prevent the reduction in cell proliferation in response to oxLDL (Figure 22). However, treatment with the PLC/A_2 inhibitor U73122 (used at a concentration of 3 μg/ml) effectively reversed the oxLDL-induced inhibition of proliferation (Figure 20). Treatment with U73122 also blocked growth in response to native LDL plus serum (cell numbers were increased by 8% and 3% over control with 10 and 50 μg/ml native LDL, respectively. These increases in cell number were not significant.) In the experiments using LY294002, calphostin C and U73122, cells treated with the inhibitor in the absence

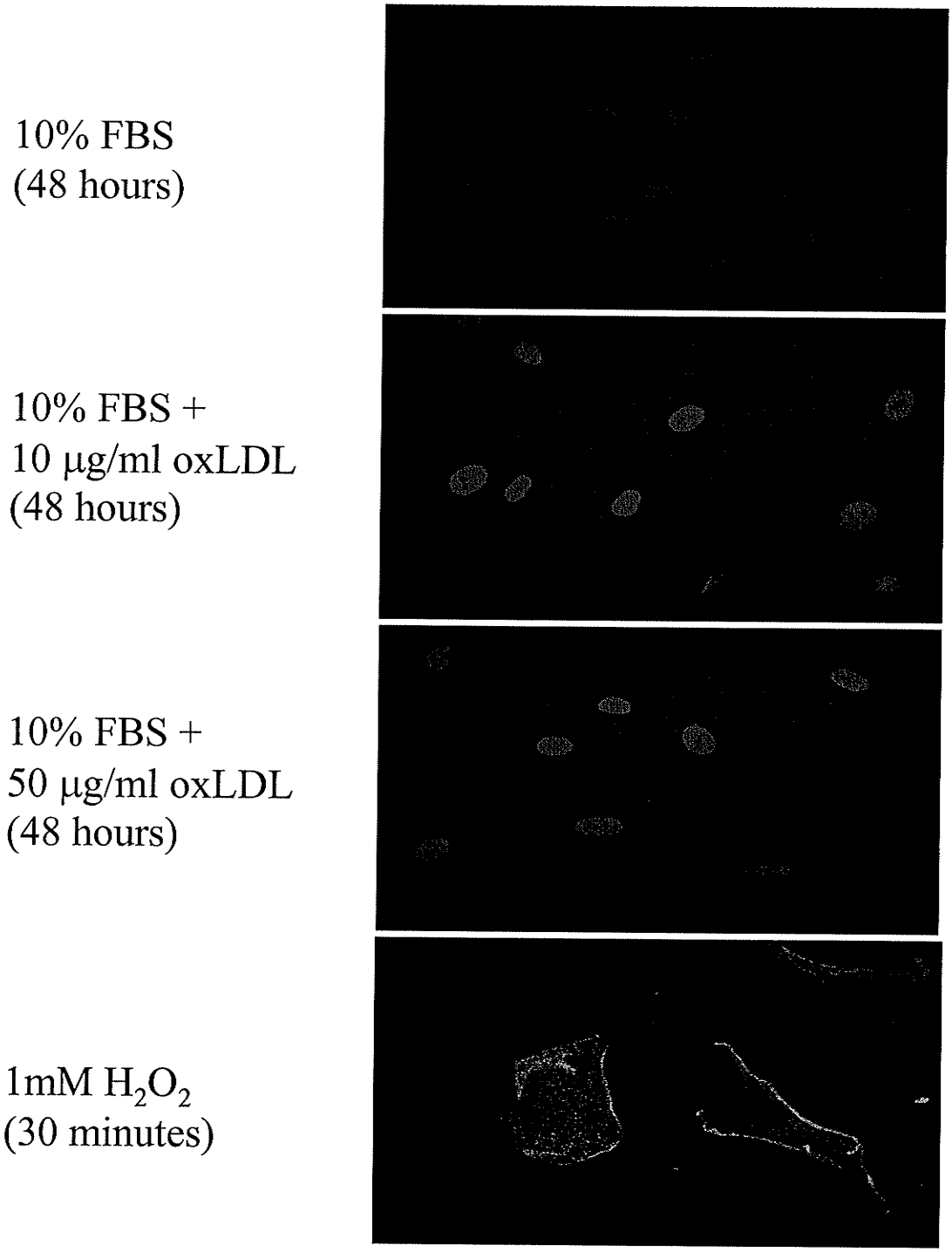


Figure 21. Representative confocal images of annexin V staining in VSMC treated with: 10% serum and no oxLDL for 48 hours, 10% serum and 10 μg/ml oxLDL for 48 hours, 10% serum and 50 μg/ml oxLDL for 48 hours, and 1 mM hydrogen peroxide for 30 minutes. Annexin V staining is indicated in green; Hoescht staining is indicated in red. Cells were maintained in serum-free media for 6 days preceding treatment.

Table 5. Change in cell number following exposure of quiescent fibroblasts to oxLDL in the presence of growth factors.

	10 μg/ml oxLDL	50 μg/ml oxLDL
bFGF	110 ± 6	162 ± 15*
TGFβ1	107 ± 5	145 ± 5*
aFGF	93 ± 4	80 ± 2*

Mean changes in cell number, expressed as a percentage of control (serum-starved cells) ± SEM (*$p<0.05$). Results are from at least 6 independent experiments. In each experiment, a minimum of 60 cells were counted.

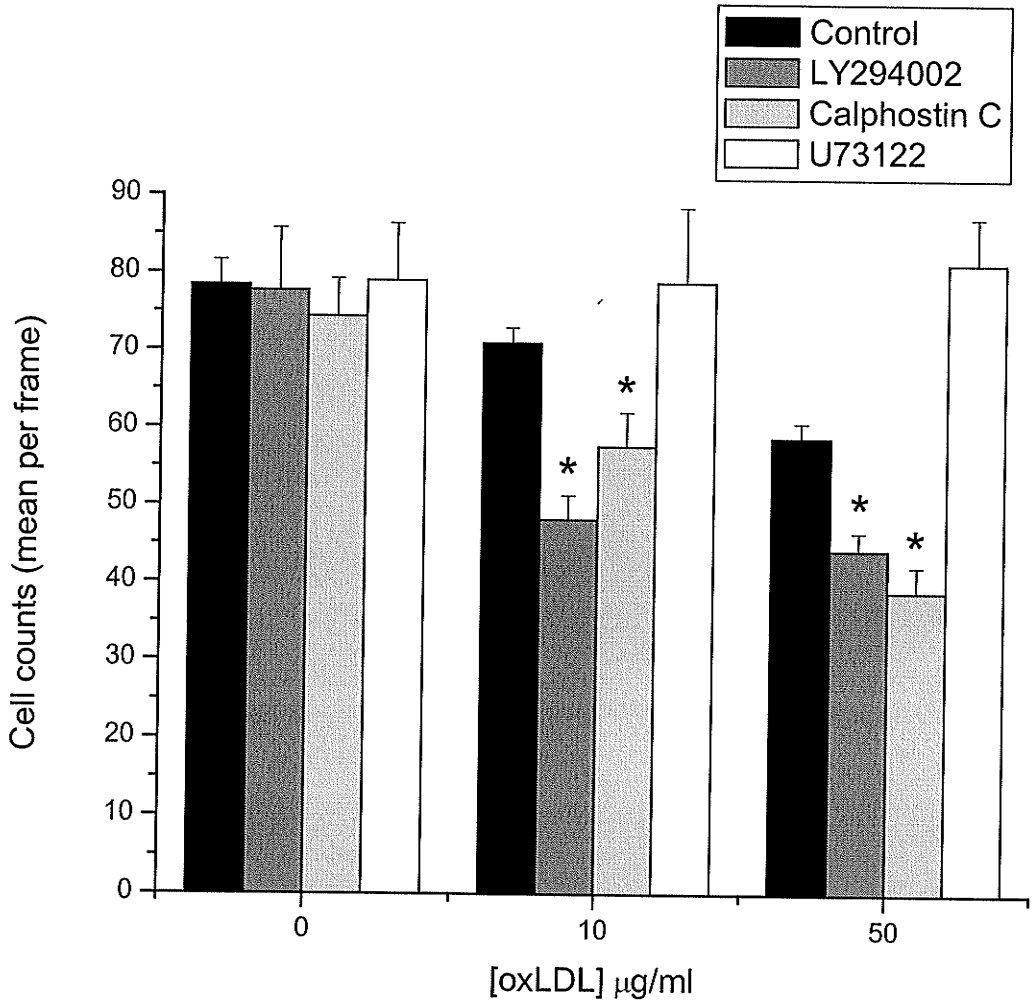


Figure 22. Effect of inhibitors on cell numbers in fibroblasts exposed to oxLDL in the presence of serum. Cell numbers are expressed as mean per field, ± SEM (*p < 0.05). Cells were pretreated with inhibitors alone for 15 minutes, before exposure to oxLDL in combination with serum and the inhibitors for 24 hours.

of oxLDL showed no evidence of increased LDH release as compared to cells maintained in starvation medium. Because U73122 has also been reported to inhibit MAP kinase, the activation of ERK1 and ERK2 was evaluated in U73122-treated cells. Western blots of extracts from cells treated with serum and oxLDL in combination with U73122 showed that ERK1/ERK2 activation (as detected using a monoclonal antibody to phospho-p44/p42 MAP kinase) was completely abolished in U73122-treated cells relative to controls (Figure 23).

4.2.2 Cell cycle protein expression following exposure to oxLDL in combination with serum

Cell cycle proteins regulate the cell's progression through the cell cycle. Therefore, oxLDL may be inhibiting serum-induced proliferation through a specific effect on the expression or distribution of these proteins in the cell. Expression of cell cycle proteins was examined by Western blot analysis. At the 24 hour time point in fibroblasts treated with serum + 10 μg/ml oxLDL (when cell numbers were reduced by 16% and the percentage of cells leaving G0/G1 was decreased by 35%), only the expression of Cdk 4 was significantly decreased relative to control (Figure 24). In the presence of 50 μg/ml oxLDL, when cell numbers were reduced by 22% and the percentage of cells leaving G0/G1 was decreased by 47%, total cellular levels of Cdc 2, Cdk 4, Cyclin B1 and PCNA were significantly decreased relative to controls, while levels of Cyclin D1 were unchanged (Figure 24). By 48 hours, when cell numbers were reduced by 20%, serum + 10 μg/ml oxLDL had no effect on the expression of any cell cycle protein (Figure 24). After treatment with serum + 50 μg/ml oxLDL, when cell

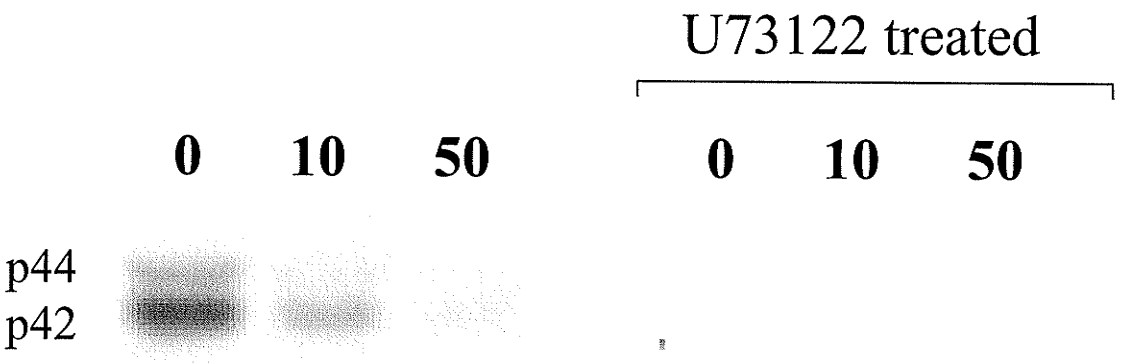


Figure 23. Densitometric comparison of expression of phospho-p44/p42 in serum-treated fibroblasts following exposure to oxLDL for 24 hours, in the presence or absence of U73122. Signal was not detectable in U73122-treated cells.

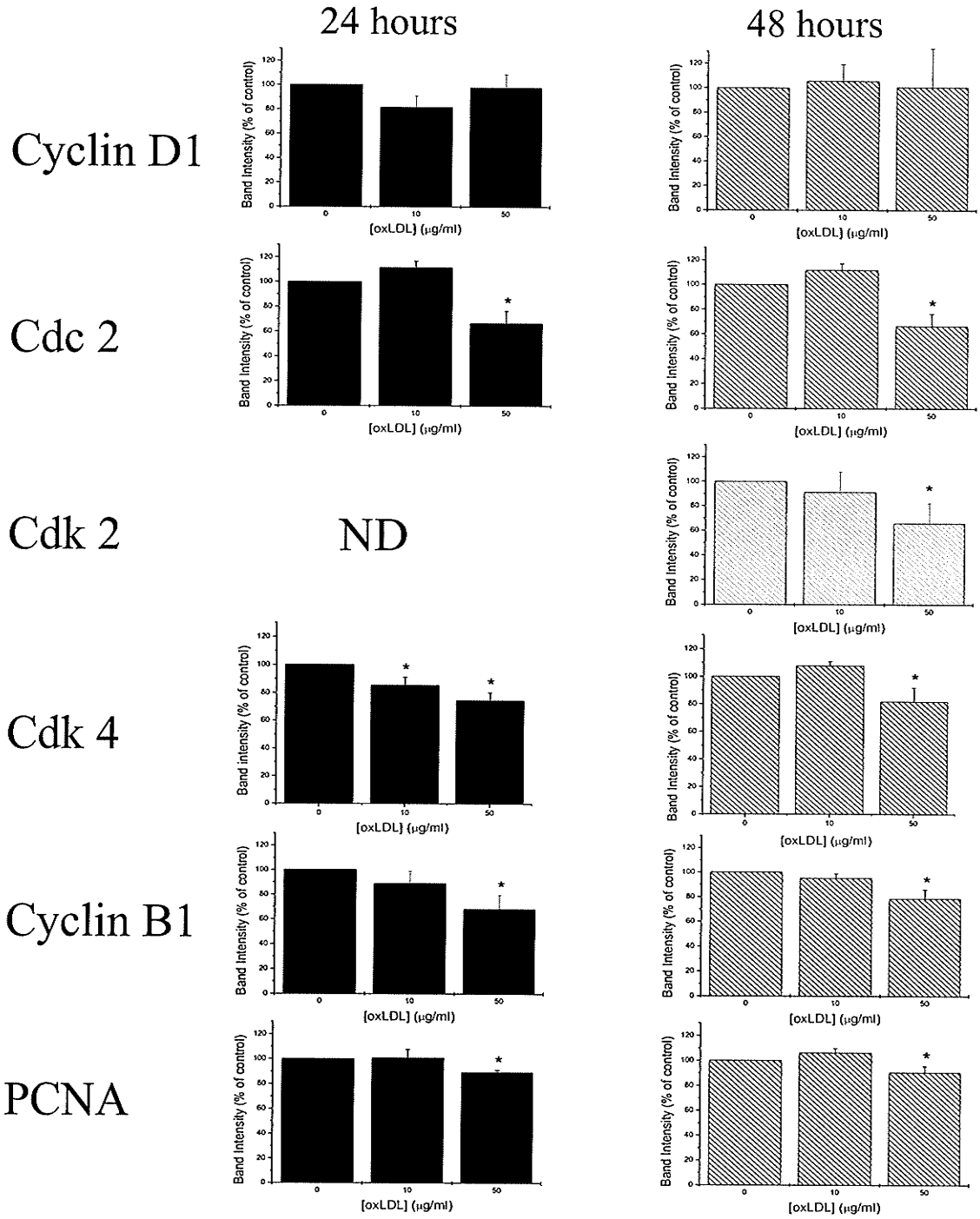


Figure 24. Densitometric comparisons of expression of Cyclin D1, Cdc 2, Cdk 2, Cyclin B1 and PCNA in cells exposed to 0, 10 or 50 µg/ml oxLDL with serum for 24 or 48 hours, expressed as a percentage of control, ± SEM (*$p < 0.05$). 50 µg of protein was loaded in each well. Data represents at least 3 independent experiments for each protein. ND indicates not detectable signal.

numbers were reduced by 27%, the levels of Cdc 2, Cdk 2, Cdk 4, Cyclin B1 and PCNA were significantly reduced relative to controls, while levels of Cyclin D1 were again unchanged (Figure 24).

4.2.3 Cell cycle protein distribution following exposure to oxLDL in combination with serum

Due to the seeming discrepancy between the expression of the cell cycle proteins (Figure 24), the movement of the cells into the cycle (Figure 19) and the decrease in cell number (Figure 20), a change in the cellular localization of these proteins was investigated as another potential mechanism for the observed effects of oxLDL. Representative results are shown for Cyclin D1 distribution in Figure 25. After 48 hours, nuclear levels of Cyclin D1 were increased after serum treatment. However, this cange in localization was inhibited by both 10 and 50 μg/ml oxLDL plus serum as compared to serum alone (Figure 25). This effect was in stark contrast to that seen with oxLDL treatment of cells in the absence of serum (Figure 25). Nuclear fluorescence was quantitated over a number of experiments to obtain an objective measurement of the redistribution of Cyclin D1. These measurements were also made for other cell cycle proteins including Cdc 2, Cdk 2, Cdk 4, Cyclin A, Cyclin B1 and PCNA (Figure 26). Following 24 hours of exposure to 10 μg/ml oxLDL, significant decreases in nuclear levels of Cdc 2, Cyclin A, Cyclin D1 and PCNA were noted. After 48 hours of exposure to 10 μg/ml oxLDL, significant decreases were observed in nuclear levels of Cdk 4, Cyclin A, Cyclin D1 and PCNA. Twenty-four hours of exposure to 50 μg/ml oxLDL induced significant decreases in nuclear levels of Cdc 2, Cyclin D1 and Cyclin A.

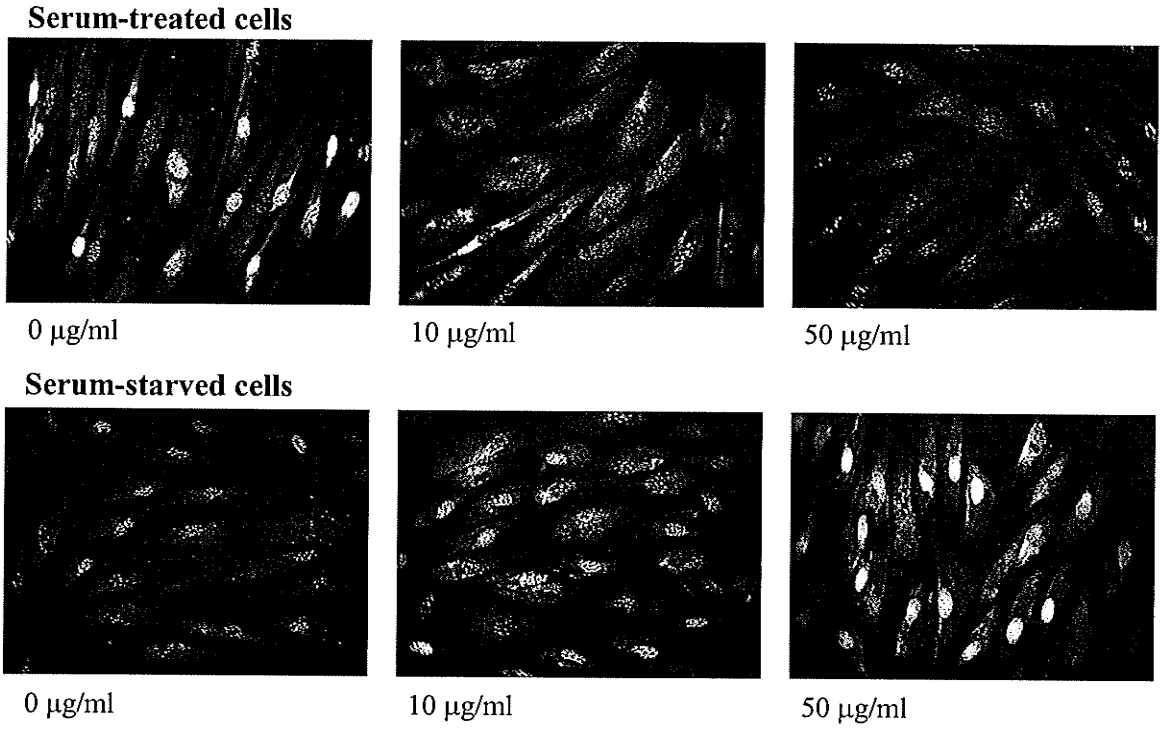


Figure 25. Representative confocal micrographs showing nuclear fluorescence of Cyclin D1 in fibroblasts following 48 hours of: (upper panel) no oxLDL in the presence of 5% serum, 10 μg/ml oxLDL in the presence of 5% serum, and 50 μg/ml oxLDL in the presence of 5% serum, (lower panel) no oxLDL under starvation conditions, 10 μg/ml oxLDL under starvation conditions, 50 μg/ml oxLDL under starvation conditions.

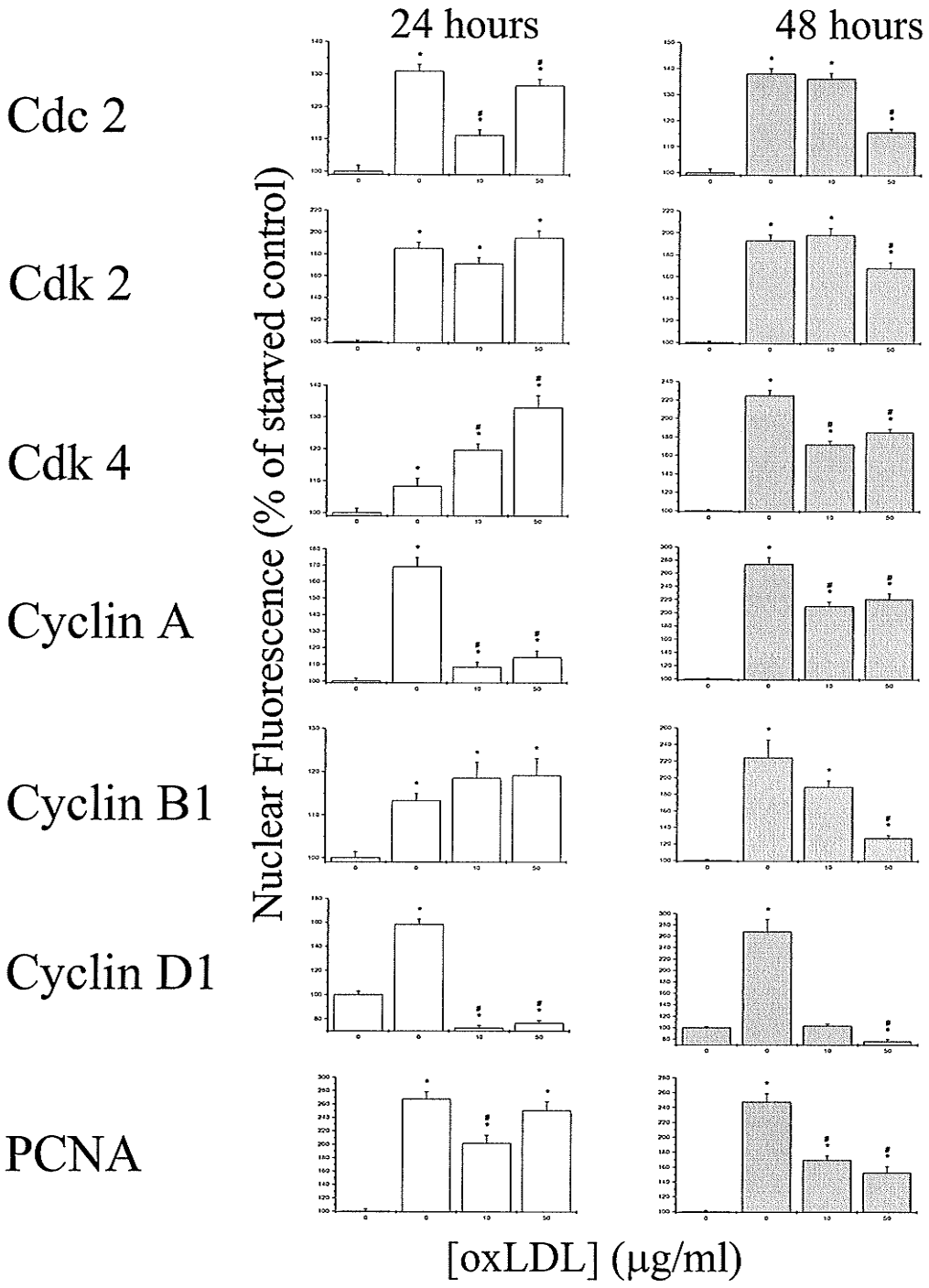
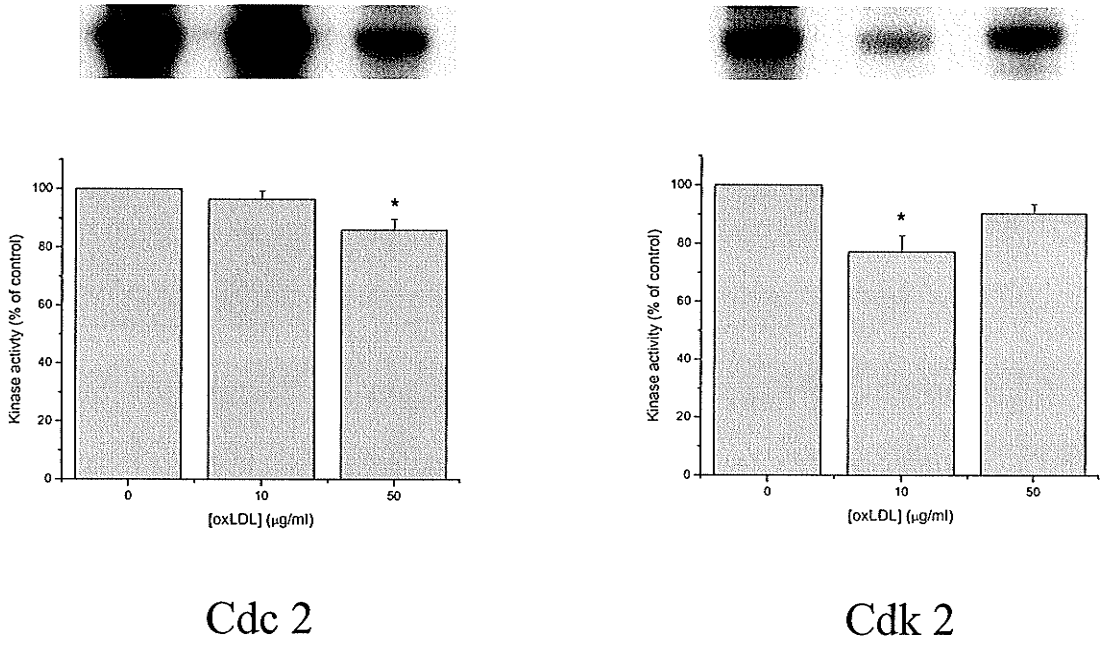


Figure 26. Nuclear fluorescence of Cdc 2, Cdk 2, Cdk 4, Cyclin A, Cyclin B1, Cyclin D1 and PCNA in fibroblasts exposed to serum and 0, 10 or 50 µg/ml oxLDL for 24 or 48 hours, expressed as a percentage of starved control values (first bar on graph). Data represent mean values ± SEM (*$p < 0.05$ vs. starved control, #$p < 0.05$ vs. serum-treated control). All cells were maintained in serum-free media for 6 days preceding treatment. At least 3 independent experiments were performed for each protein.

Exposure to 50 μg/ml oxLDL for 48 hours resulted in significant decreases in nuclear levels of Cdc 2, Cdk 2, Cdk 4, Cyclin A, Cyclin D1, Cyclin B1 and PCNA. Therefore, these data demonstrate that exposure of cells to oxLDL in the presence of serum results in decreases in nuclear levels of Cyclin D1, Cdc 2, Cdk 2, Cdk 4, Cyclin A, Cyclin B1 and PCNA. In contrast, nuclear levels of PCNA were increased in fibroblasts and VSMC treated with native LDL and serum, though the increases (up to 6%) were not significant.

4.2.4 Kinase activity following exposure to oxLDL in combination with serum

The cytoplasmic retention of cell cycle proteins suggests that the cyclin/cyclin-dependent kinase complexes are inactive. We directly examined Cdc 2, Cdk 2 and Cdk 4 kinase activity under our experimental conditions. Exposure of cells to 10 μg/ml oxLDL for 24 hours resulted in a 23% decrease in Cdk 2 activity in comparison to control cells (Figure 27), while exposure of cells to 50 μg/ml oxLDL for 24 hours resulted in a 14 % decrease in Cdc 2 activity. Kinase activity of Cdk 4 was unchanged in oxLDL-treated cells as compared to controls.



Cdc 2

Cdk 2

Figure 27. Decreased kinase activity of Cdc 2 and Cdk 2 in serum-treated fibroblasts following exposure to oxLDL. Upper figure: representative autoradiographs showing Cdc 2 activity (left) and Cdk 2 activity (right) with histone H1 as a substrate in whole cell extracts from fibroblasts treated with 0, 10 or 50 µg/ml oxLDL for 24 hours. Lower graphs: densitometric comparisons of Cdc 2 and Cdk 2 activity, expressed as a percentage of control ± SEM (*$p < 0.05$). N=3 for each condition and time point.

4.3 Experiments using atherosclerotic rabbit vessels

Numerous studies have demonstrated that cell cycle proteins are induced in the balloon-injured vessels of animal models of restenosis (3, 42, 168-170). However, the information on cell proliferation in conditions of restenosis is not necessarily transferred to a situation of primary atherosclerosis. To date there have been few comparable studies focused on identifying cell proliferation in conditions of primary atherosclerosis and no studies focused on the response of cell cycle proteins in a model of primary atherosclerosis. Several studies have documented increased thymidine labeling in plaques from atherosclerotic animals (158, 205, 229). This suggested that the plaque region was rich in mitotic activity. More recently, Orekhov and coworkers (187) found a significant increase in PCNA positive cells in lipid rich atherosclerotic plaques and concluded that cell proliferation is stimulated in atherosclerosis. However, Marek et al. (147) and Pickering et al. (193) found little evidence of cell proliferative activity using PCNA as a marker of proliferation in the atheroma. Furthermore, others have advanced the hypothesis that decreased cell death through apoptosis rather than increased cell proliferation may be responsible for the growth of an atherosclerotic plaque (78). A more comprehensive examination of the expression of several different cell cycle proteins would be important supportive evidence that cell proliferation was increased in a primary atherosclerotic plaque. An augmentation in the related cell cycle dependent kinase activity would also be significant supportive evidence of mitotic activity in the plaque.

The purpose of the present study, therefore, was to determine if alterations in the expression of cell cycle proteins and the activity of their related kinases occur in a model of primary atherosclerosis. In addition, it was hypothesized that cell cycle protein

expression may be more accelerated during the early stages of plaque development and then taper off during the later stages of plaque formation in an animal model of atherosclerosis.

4.3.1 Plaque formation in aortas of cholesterol-fed rabbits

A high cholesterol diet was employed for two different durations to induce a moderate and a more severe atherosclerotic plaque formation in the aortae of the rabbit. Atherosclerotic plaque formation was assessed through analysis of digital photos of the aortas (Figure 28). A total of 59 aortas were examined. Animals fed a 0.5% cholesterol diet for 8 or 16 weeks developed significant amounts of plaque in their aortas, compared to animals fed a regular chow diet. Eight weeks of cholesterol feeding resulted in plaque coverage of 36.2% of the aorta, while 16 weeks of feeding resulted in 78.8% plaque coverage (Figure 28). No animals in the control group developed plaque in their aortas.

4.3.2 Cell cycle protein expression in aortas of cholesterol-fed rabbits

To evaluate the status of cell proliferation during the development of the plaque, total protein lysates of aortic tissue from each treatment group were analyzed by Western blotting for the expression of cell cycle proteins. Levels of PCNA were significantly increased in aortas of rabbits fed the cholesterol diet. PCNA expression was increased by 43.9% after 8 weeks versus control, and by 52.0% versus control after 16 weeks of dietary intervention (Figure 29). There was no significant difference between PCNA levels in the 8 week aortas as compared to the 16 week aortas. Similarly, expression of Cdk 4 and Cyclin A were also significantly increased in aortas of cholesterol-fed rabbits.

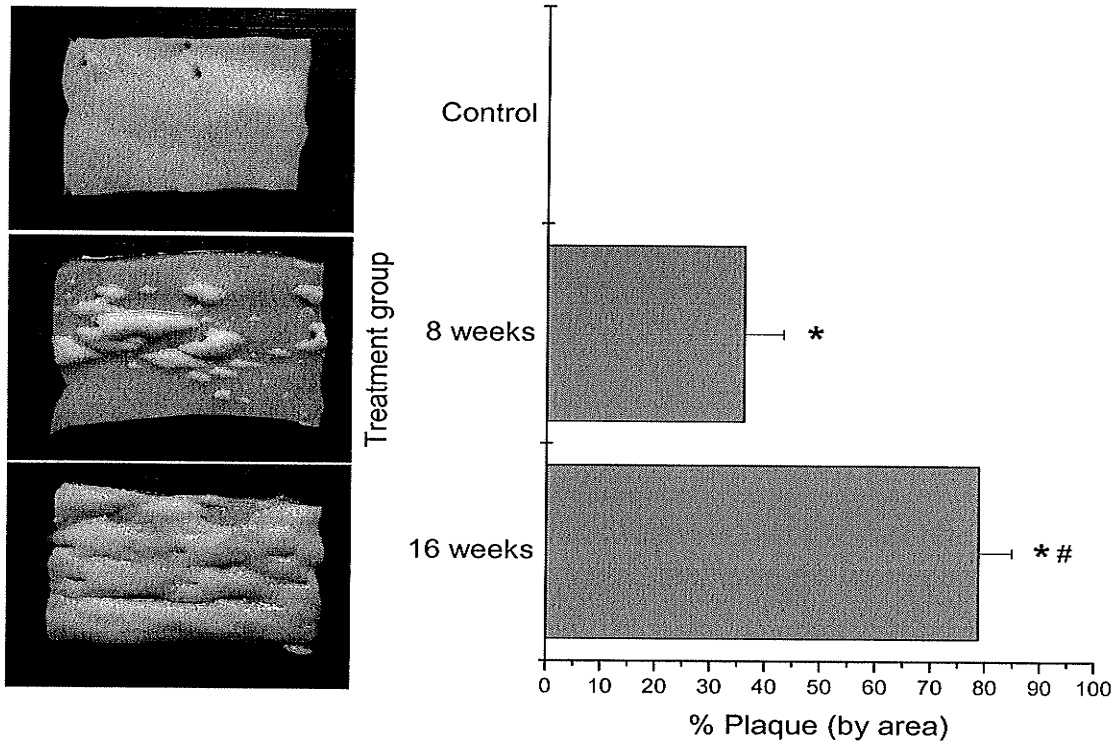


Figure 28. Sections of rabbit aortas showing amount of plaque build-up as a function of weeks of cholesterol feeding. From top to bottom: control, 8 weeks, 16 weeks. Graph shows comparison of the amount of the amount of plaque in the aortas (n= at least 12 aortas per group), expressed as a percentage of the total area, ± SEM, *$p<0.05$ vs. control, #$p<0.05$ vs. 8 weeks.

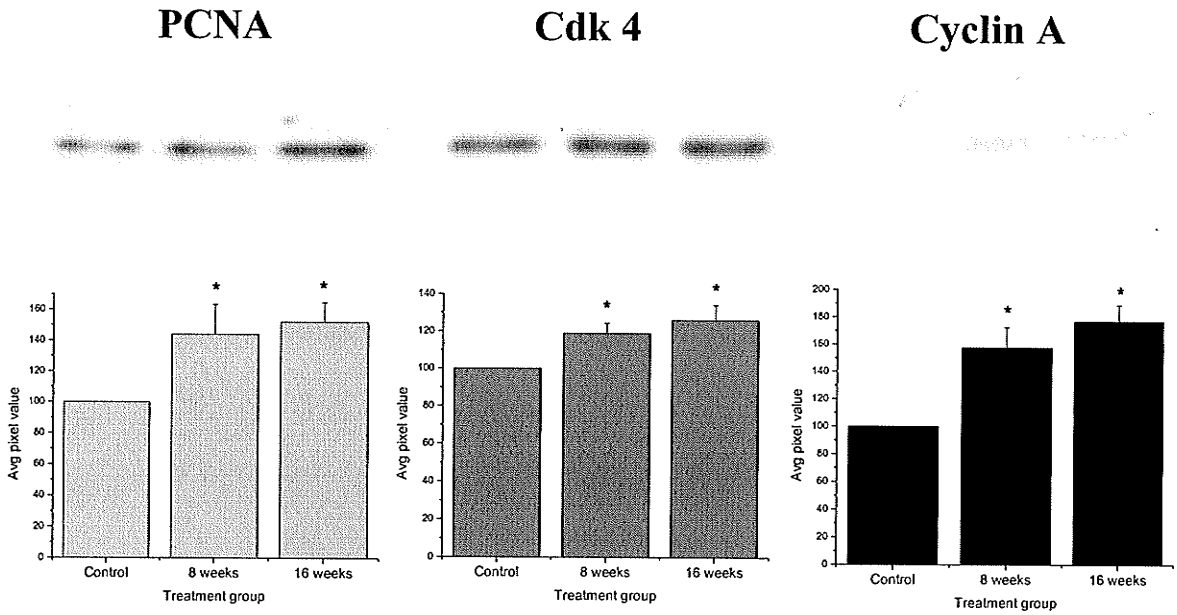


Figure 29. Representative autoradiograms from Western blots showing PCNA, Cdk 4, and Cyclin A expression in aortas from control, 8 week and 16 week cholesterol-fed rabbits. 50 μg of protein was loaded in each well. Densitometric analysis of at least n=6 autoradiograms for each protein. Expression is shown as a percentage of control, ± SEM, *$p<0.05$ vs. control.

Cdk 4 levels were increased by 18.9% in 8 week aortas and 26.0% in 16 week aortas, while Cyclin A expression was increased 57.4% at 8 weeks and 76.7% at 16 weeks (Figure 29). There were no significant differences between expression levels at 8 and 16 weeks for either Cdk 4 or Cyclin A.

The levels of smooth muscle α-actin were also assessed by Western blotting in total protein from the aortic tissue of rabbits from each treatment group to determine its expression relative to that of the cell cycle proteins. Expression of smooth muscle α-actin decreased significantly in both 8 and 16 week aortas (Figure 30). The loss of smooth muscle α-actin may have been a result of accelerated apoptosis in smooth muscle cells in the aortas of cholesterol-fed rabbits. Therefore, PARP (a marker for apoptosis) expression was evaluated in protein from the aortas of animals from each treatment group by Western blot analysis. Although PARP is a marker of early apoptosis, it is reasonable to expect that apoptosis is a continuing process within the tissue, and that an increase in PARP expression would be detectable were apoptosis occurring. No staining for PARP was evident in any of the tissue samples (data not shown). This was not due to a lack of sensitivity in the assay, because a band was present in cultured rabbit vascular smooth muscle cells that were UV-irradiated to induce apoptosis (data not shown).

The amount of plaque differed significantly between 8 and 16 week cholesterol-fed aortas (Figure 28). However, the entire aorta was used for the expression work. Thus, the samples from the animals fed the diet for 8 weeks contained less plaque than the vessels obtained from the animals fed the diet for 16 weeks. The results, therefore, could be viewed as biased and an unfair comparison of expression within the plaque at the two stages of atherosclerosis. It may be a more appropriate comparison to examine expression

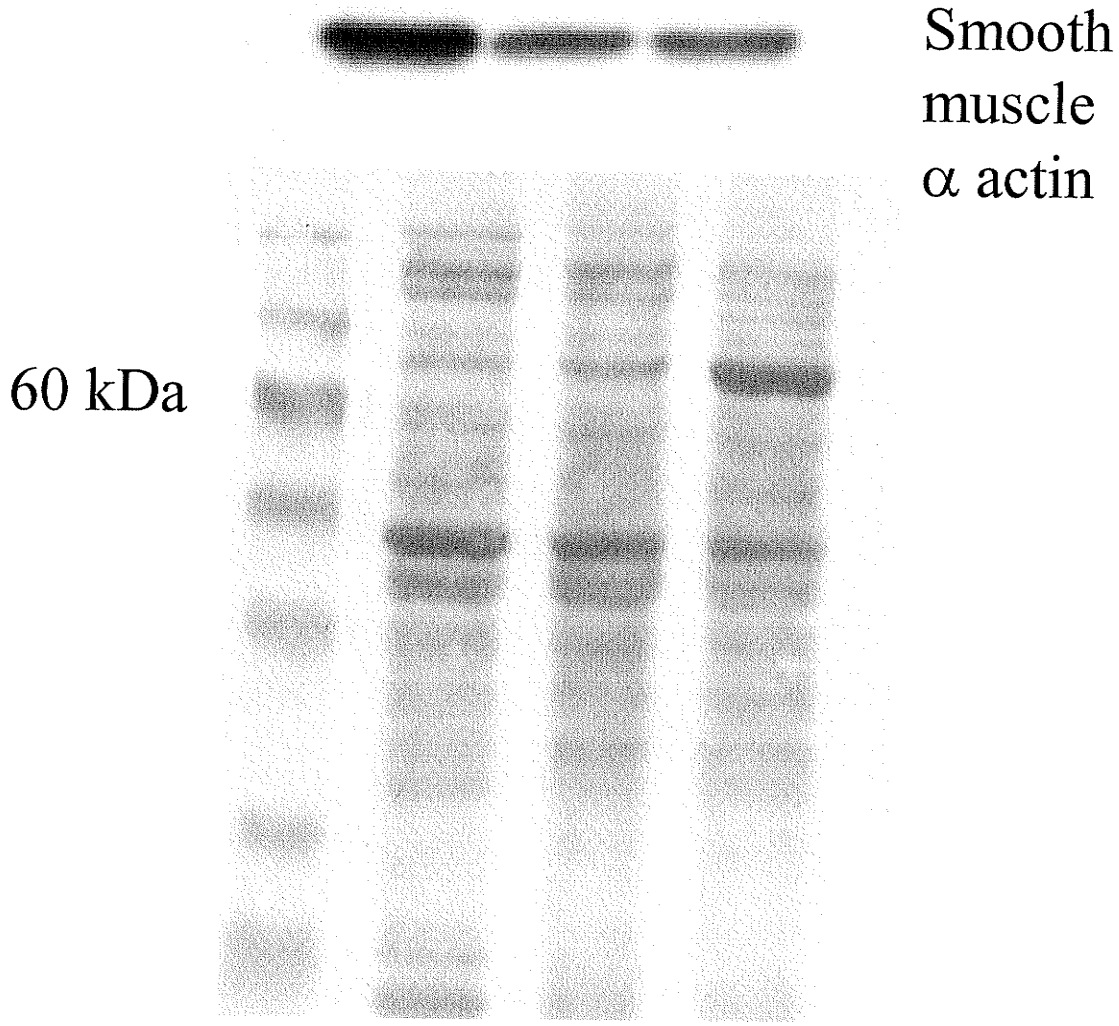


Figure 30. Representative autoradiogram from Western blot showing smooth muscle α-actin expression in aortas from control, 8 week and 16 week cholesterol-fed rabbits. The blot was also stained with Ponceau stain to show that lanes were loaded equally. 50 μg of protein was loaded in each well.

levels of cell cycle proteins specifically from plaque regions in both groups to obtain a better, unbiased analysis of cell proliferation in early and late plaque development, without the confounding influence of the vessel that did not contain any visible plaque. Plaque tissue was carefully dissected away from areas without plaque. This was particularly time-consuming, difficult and generated relatively little tissue in the 8 week aortic samples where approximately 65% of the vessel wall did not contain visible evidence of plaque formation. Because the 16 week samples contained even less tissue without plaque (~20%), we could not complete expression work in non-plaque tissue in this group. The plaque and non-plaque areas of these aortas were homogenized separately and total protein from each was used for Western blots. Expression levels of PCNA, Cdk 4 and Cyclin A were evaluated in these samples. Levels of Cyclin A were increased by 71.5% in the plaque portions of 8 week aortas versus control (Figure 31). As expected, Cyclin A expression was not stimulated as much in the non-plaque regions. However, interestingly, the levels of Cyclin A in non-plaque areas of 8 week aortas were significantly increased (by 36.4%) over controls. Similar qualitative effects were observed for the other cell cycle proteins. For example, in the plaque areas PCNA expression was elevated by 40.7% over controls and by 25.9% in non-plaque areas ($p<0.05$). Levels of Cdk 4 were also significantly increased by 56.5% over control in plaques found within 8 week aortas. Non-plaque areas had Cdk 4 levels 23.1% greater than control ($p<0.05$).

4.3.3 Kinase activity in aortas of cholesterol-fed rabbits

Increased expression of cell cycle proteins would suggest that cell proliferation

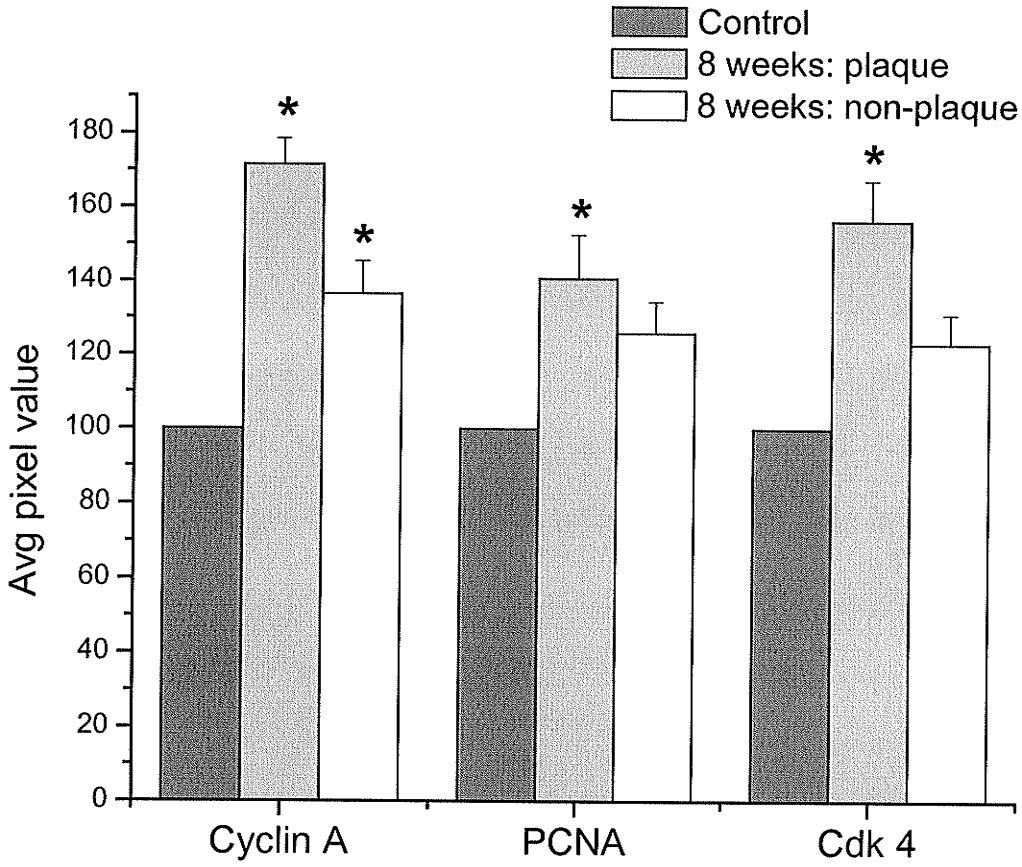


Figure 31. Representative autoradiogram from Western blot showing Cyclin A, PCNA and Cdk 4 expression in rabbit aortas from control, and 8 week plaque and 8 week non-plaque areas from aortas of cholesterol-fed rabbits. 50 μg of protein was loaded in each well. Densitometric analysis of autoradiograms (n=4). Expression is shown as a percentage of control, ± SEM, *$p<0.05$ vs. control.

has been induced. However, in order to confirm this, the activity of cyclin dependent kinases was examined. Activities of Cdk 4, Cdk 2 and Cdc 2 were assessed in protein extracts from aortas of control and cholesterol-fed rabbits (Figure 32). Cdk 4 activity was significantly increased in both 8 and 16 week aortas relative to controls (26.8% and 24.4% above control, respectively). Activity of Cdk 2 was elevated by 40.2% in 8 week aortas, and 16.4% in 16 week aortas. Finally, Cdc 2 activity increased by 51.7% at 8 weeks and 58.7% at 16 weeks of cholesterol feeding, as compared to aortas from control animals. Levels of activity did not differ significantly between 8 and 16 weeks in cholesterol fed aortas for any of the cyclin dependent kinases studied.

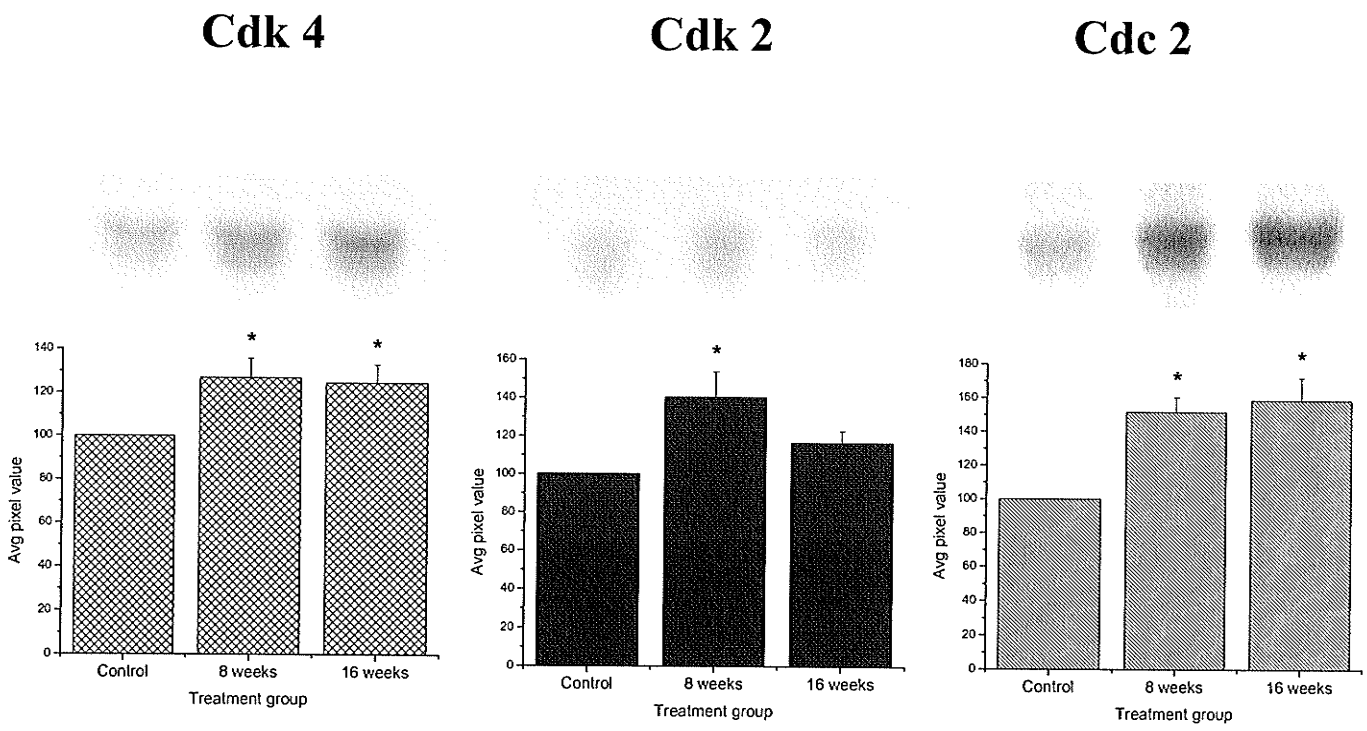


Figure 32. Representative autoradiograms from kinase assays showing Cdk 4, Cdk 2, and Cdc 2 activity in rabbit aortas from control, 8 week and 16 week cholesterol-fed. Densitometric analysis of at least n=4 autoradiograms for each kinase. Activity is shown as a percentage of control, ± SEM, *$p<0.05$ vs. control.

CHAPTER 5: DISCUSSION

5.1 Oxidized low density lipoprotein stimulates cell proliferation through a general induction of cell cycle proteins

OxLDL induced a significant increase in the total number of cells in culture, in the absence of any other cytokines or growth factors. Therefore, this study identifies oxLDL as a compound capable of inducing proliferation in the absence of any other mitogenic factors. The mitogenic action of oxLDL was similar to bFGF but did not maintain as large or as sustained a proliferative effect as bFGF. This mitogenic effect was specific to oxidized LDL (native LDL did not have the same magnitude of effect) and showed time and dose dependency. The effect was not dependent upon cell type, as both fibroblasts and VSMC responded in a similar manner. We may safely conclude that oxLDL acts as an independent mitogen, as shown by others previously (36, 149). Although this finding is not physiologically significant, it does identify oxLDL's mitogenic potential.

The present investigation has also identified several components of the cellular signaling pathway associated with the proliferative effects of oxLDL. We have identified both cell surface and intracellular sites of action. The scavenger receptor blocker polyinosinic acid prevented oxLDL-induced increases in cell number. This suggests that oxLDL induces its proliferative action through an interaction with the scavenger receptor. If so, one would suspect that the receptor stimulation would lead to activation of an intracellular signaling pathway. Signaling pathways previously linked to the activation of the scavenger receptor include the PI 3-kinase pathway (142) and the MAP kinase

pathway (49). Our data would suggest that the PI 3-kinase pathway appears to be involved in the proliferative effects of oxLDL. This is consistent with results reported previously (149). The PLC pathway and the intracellular signaling molecule IP_3 also appear to be involved. The association of oxLDL, proliferation and PLC has not been identified previously. However, lysophosphatidylcholine (LPC) (a component with oxLDL) has been identified as an activator of PLC (15). The observation that PD98059, a selective MEK1/2 inhibitor, was less effective in blocking proliferation in response to higher concentrations of oxLDL is somewhat surprising, given that numerous studies have shown activation of the MAPK pathway following exposure to oxLDL (38, 55, 115, 123). However, it is possible that the inhibition by PD98059 of the MAPK pathway is incomplete and the activation by higher concentrations of oxLDL simply overwhelms the inhibitory effect. Furthermore, activation of MEK1/2 does not necessarily imply its involvement in growth (244). Similarly, the inability of PD98059 to block oxLDL's mitogenic effect does not rule out the participation of other members of the MAPK family (115).

The most important and surprising observation in the present study is that oxLDL induced the simultaneous induction of both cell cycle activators and suppressors. In a state where cell proliferation is stimulated, one would have expected an increased expression of proteins responsible for the activation of the cell cycle and/or and inhibition of cell cycle inhibitory proteins. This is the case in other conditions of rapid cell proliferation like cancer or in development. Malignant cell growth is typically characterized by high levels of one or more cell cycle inducers, and low levels (or a complete absence) of functional cell cycle inhibitors (220). However, this seemingly

contradictory situation has previously been observed in other disease states, such as liver regeneration (5). It has been hypothesized that, by activating both inducers and inhibitors simultaneously, the cell effectively regulates its own growth. More recently, p53 and $p21^{cip1}$ (both of which would be expected to be downregulated under growth conditions) were observed to be upregulated in quiescent VSMC stimulated with serum (161). P53 was proposed to be acting as a "growthostat" in these cells, upregulating $p21^{cip1}$ so as to prevent inappropriate proliferation (161). Induction of $p21^{cip1}$ serves to regulate the rate of progression through G1, whereas $p27^{kip1}$ modulates Cdk 2 activity before and after S phase (5). The cell cycle will proceed forward (presumably due to an imbalance of inducers over inhibitors), but high levels of inhibitors ensure that it may be shut down rapidly in response to changes in the cellular environment. This cooperation between cell cycle regulators is proposed to lead to a precisely controlled type of growth (5). This observation of a controlled proliferative response due to a generalized induction of all cell cycle proteins is consistent with the slower, non-malignant cell growth typical of an atherosclerotic or restenotic plaque. The time dependency that we observed is consistent with this observation and further demonstrates that the expression of activators and inhibitors of the cell cycle are not exactly "simultaneous". The induction of a cell cycle activator like Cyclin D1 occurred faster and to a greater degree than the induction of an inhibitory protein like $p21^{cip1}$. One may conclude, therefore, that the $p21^{cip1}$ expression represents an adaptive response that may regulate the initial proliferative effects.

An increase in the expression of cell cycle proteins does not necessarily mean that functional changes exist. Translocation of cell cycle proteins into the nucleus is thought to activate cyclin/cyclin-dependent kinase complexes (83). Movement of cell cycle

proteins into the nucleus would, therefore, represent strong indirect evidence in support of an activation of the cell cycle. In the present study, the increases in the total levels of these proteins, as determined by Western blot analysis, were generally accompanied by increases in the levels of cell cycle proteins in the nucleus. Increases in the total cellular levels of these proteins, as determined by Western blot analysis, were generally accompanied by increases in the levels of cell cycle proteins in the nucleus. One surprising observation obtained in the present study concerns the simultaneous induction by oxLDL of both cell cycle inducers and suppressors. Nuclear localization of the cell cycle inducers Cdc 2, Cdk 2, Cdk 4, Cyclin A, Cyclin B1, Cyclin D1, Cyclin E and PCNA were all significantly increased with respect to controls following oxLDL treatment. Direct analysis of Cdk 4 activity confirmed the hypothesis that the kinase complexes were not only importing into the nucleus but that they were active and associated with the proliferative event. Consistent with the expression data, the cell cycle inhibitors $p21^{cip1}$, $p27^{kip1}$, p53 and Rb were all found in greater concentrations in the nucleus of the cell. These data are consistent with the hypothesis that oxLDL is inducing a proliferative event by increasing the expression and nuclear translocation of both inhibitors and activators of the cell cycle.

The mechanism responsible for the movement of cell cycle proteins into the nucleus of the cell by oxLDL is unclear from the results of the present study. Several possibilities exist based upon previously published reports. It may occur through a PLC or PI 3-kinase pathway as indicated above. However, no study has yet examined the potential for these pathways to regulate nuclear protein import. Alternatively, two other studies have demonstrated that nuclear protein import is sensitive to oxidative reactions

and LPC (52, 234). LPC is a major by-product of the oxidation of LDL and has proliferative action (33). It is possible that its entry into the cell may have altered nuclear translocation of the cell cycle proteins. This awaits further experimentation.

In summary, oxLDL was capable of inducing proliferation in fibroblasts and smooth muscle cells in the absence of other added mitogens. We may conclude that oxLDL is an independent mitogenic factor. Under some conditions, oxLDL can be cytotoxic. OxLDL was not cytotoxic under any of the conditions used in the present study. The mitogenic effect of oxLDL occurred through an interaction of the oxLDL with scavenger receptors on the cell surface and an augmentation of intracellular signaling through the PI 3-kinase and PLC pathways. The stimulation was accompanied by a significant increase in the total cellular expression of cell cycle proteins, as well as a re-distribution of the cell cycle proteins into the nucleus of the cell. Our results provide the first demonstration that a known atherogenic lipoprotein, oxLDL, can induce changes in cell cycle protein distribution and expression characteristic of a controlled, adaptive response to a chronic pathological condition. These effects may play an important role during the early proliferative phases of atherosclerotic and restenotic vascular disease.

5.2 Oxidized low density lipoprotein retards the growth of proliferating cells by inhibiting translocation of cell cycle proteins

The purpose of the present study was to determine the mitogenic potential of oxLDL when the cells were under the simultaneous mitogenic influence of other growth factors and cytokines in serum. That oxLDL reduced, rather than amplified, the proliferative response of cells to serum was unexpected (11, 22, 39, 273). However, the observation that oxLDL functions to inhibit cell proliferation is not entirely without precedent. OxLDL has been shown by Henry's laboratory to inhibit cell proliferation by altering the expression of mitogens (40, 41). Similarly, prostaglandin E2 has been previously described by Yau et al. as a compound that stimulates quiescent cells and inhibits growing ones (267). In the present study, three lines of evidence support our observation of an inhibitory effect of oxLDL on cell proliferation. First, oxLDL reduced the total number of serum-treated fibroblasts entering the cell cycle. Second, oxLDL reduced the total number of cells completing the cell cycle in serum-treated cultures. Third, oxLDL caused a decrease in nuclear levels of cell cycle proteins in serum-treated cells. These effects were not specific to cell type: both fibroblasts and VSMC exhibited similar responses.

This inhibition of proliferation was not due to cell death. While oxLDL has been shown to induce apoptosis (22), no apoptotic cells were observed under the present experimental conditions, nor did LDH levels increase after any experimental intervention (data not shown). This would argue strongly against cell death by necrosis. In addition, although oxLDL inhibited the proliferative effects of serum, cell numbers continued to increase, although not nearly as fast as in the absence of oxLDL. The most likely

conclusion, therefore, is that oxLDL slowed the proliferative response through an effect on the cell cycle, not through an induction of cell death.

We focused our research, therefore, upon the cell cycle to understand the mechanism responsible for the observed inhibition of proliferation by oxLDL. The expression of cell cycle proteins in cells treated with oxLDL and serum was inconsistent with the observed decreased entry into the cell cycle and reduction in cell number. For example, expression of Cyclin D1 (required for movement out of G0/G1 into the cell cycle) was unchanged in cells treated with all [oxLDL] and at all time points despite a time and dose-dependent inhibition of release from growth arrest and a significant reduction in cell numbers. In addition, although 10 μg/ml oxLDL reduced cell numbers at 24 and 48 hours by 16% and 20% respectively, the expression of only one cell cycle protein was altered. This does not argue in favor of a strong association between cell cycle protein expression and growth under our conditions.

Alternatively, the functional ability of the cell cycle proteins depends upon their translocation to the nucleus. A dramatic reduction in the nuclear levels of all cell cycle proteins was observed in cells treated with oxLDL plus serum as compared to serum alone. All cell cycle proteins were affected, although the effects were dependent upon the concentration of and the duration of exposure to oxLDL. Significantly, the nuclear translocation of Cyclin D1 was consistently inhibited by 10 μg/ml oxLDL. The nuclear import of other proteins critical for progression through the cell cycle (Cdc 2, Cdk 4, Cyclin A, and PCNA) was also inhibited by 10 μg/ml oxLDL in a manner consistent with growth arrest. The failure of the cell cycle proteins to enter the nucleus would necessarily result in the formation of fewer active cyclin/cyclin-dependent kinase complexes. Cdc 2

and Cdk 2 kinase activities were significantly reduced in cells treated with oxLDL in combination with serum, as compared to cells treated with serum alone. Together these data point to an inhibition in the nuclear translocation of cell cycle proteins as a key mechanism for the attenuated proliferative effects of oxLDL.

The surprising observation that oxLDL in combination with serum results in a diminished, rather than enhanced, proliferative response would seem to conflict with published observations by Auge and colleagues (11). In their experiments, the combination of oxLDL and serum produced an enhanced proliferative response in cultured bovine aortic smooth muscle cells. In their experiments, the combination of oxLDL and serum produced an enhanced proliferative response in cultured bovine aortic smooth muscle cells. However, the growth promoting effects of serum are known to vary considerably as a function of its composition. This can vary depending upon the batch obtained and the company that produces it. Auge and co-workers have used a lipoprotein-depleted serum (11). Thus, their serum may be different than ours not only in its lipid composition but also in many other undefined constituents (i.e. aFGF, etc.) In our investigations into the specific components of serum that may be interacting with oxLDL, we found that aFGF (but not bFGF or TGF-β1) inhibited cell growth when given in combination with oxLDL. Interestingly, the recent findings of Ananyeva et al. (6) suggest that oxLDL complexes with aFGF and inhibits its growth-promoting function *in vitro*. Thus aFGF may be responsible, at least in part, for the diminished growth seen in cells treated with serum plus oxLDL.

The mechanism whereby the oxLDL-mediated inhibition of nuclear translocation occurs is unclear. However, it is possible that the MAP kinase pathway is involved.

OxLDL can stimulate MAP kinase activity (52, 115, 123). Whereas the MAP kinase pathway is commonly associated with cell growth (52, 115, 123), it is also clear that chronic activation (hours) of the MAP kinase cascade results in an inhibition of DNA synthesis, cell cycle progression and Cdk 2 activity (244). Chronic activation of MAP kinase would be expected under our experimental conditions. MAP kinase activation can inhibit or stimulate nuclear protein import (52, 234). This may occur through an alteration in Ran cycling that would be expected to alter the import of any protein into the nucleus (52). Therefore, oxLDL-induced activation of MAP kinase would inhibit the nuclear import of cell cycle proteins. We tested this possibility directly with the use of drugs that inhibit MAP kinase activity. Unfortunately, both of the commonly used blockers of the MAP kinase pathway that we employed (PD98059 and SB203580) were cytotoxic under our extended experimental conditions (data not shown). However, U73122 is an effective blocker of PLC, which is known to be upstream of the MAP kinase pathway (115). U73122 restored cell proliferation to control levels and negated the inhibitory effects of oxLDL (Figure 22), while completely knocking out ERK1/ERK2 activity (Figure 23). Therefore, it is reasonable to argue that the oxLDL-induced stimulation of the MAP kinase pathway (which has already been shown to inhibit DNA synthesis (244), reduce Cdk 2 activity (244), and depress the import of marker proteins into the nucleus (52)) was inhibited by U73122 and this resulted in a restoration of the cellular proliferative response. We cannot discount the possibility that U73122 is inhibiting other pathways downstream of PLC. We may conclude that the depressed cell cycle protein translocation induced by oxLDL occurs at least in part via activation of the MAP kinase pathway. At present it is unknown whether the effects of aFGF on oxLDL

are mediated through the MAP kinase signaling pathway. Further investigation into this possibility may be the subject of a future study.

The findings of this paper challenge prevailing notions about the role of oxLDL in atherogenesis. OxLDL has been suggested to participate in the development of atherosclerosis partly by promoting the growth of vascular cells (39, 121, 207). However, most studies investigating proliferative activity in human atherectomy tissue have found little evidence for active cell replication (typically <1%) (82, 183) despite the presence of oxLDL in the vascular environment (125, 268). Clinically significant stenoses take several decades to develop. In the complex environment in which these plaques are formed, factors that negatively modulate the proliferative response of vascular cells (or at least slow its progression) must come into play to explain the relatively slow cell growth in atherosclerosis. Apoptosis may be one factor (78), but the present experiments demonstrate that oxLDL itself can negatively modulate the response of cells under some conditions. Its action *in vivo* may be far more complex than originally anticipated and may alter dramatically dependent upon the proliferative state of the vasculature and the mitogenic environment.

5.3 Cell cycle protein expression and kinase activity is augmented in atherosclerotic rabbit vessels

Proliferation of cells within the vascular wall has long been assumed to be an important component of the atherosclerotic process (94, 207). Cell proliferation would necessarily involve an upregulation of the components of the cell cycle machinery. The present investigation demonstrates for the first time that both the expression of cell cycle proteins (Figure 29) and the activity of their related kinases (Figure 32) were elevated in the aortas of cholesterol-fed rabbits. This would strongly support the hypothesis that cell proliferation was stimulated in the vessel wall during atherosclerosis. This conclusion would support the work of Orekhov and co-workers (187) but is in opposition to the work of Marek and colleagues (147) and Pickering et al. (193). Previous studies have used PCNA as the sole marker of cell proliferation. Using PCNA as the only marker of cell proliferation has limitations that may lead to false positive values (147). We used several different cell cycle proteins that are induced at different points within the cell cycle as markers of cell cycle progression. This avoids the concern that false positive results may be obtained from cells entering and exiting the cell cycle at different times. In addition, the measurement of kinase activity avoids any fixation or antibody artifacts associated with PCNA (147) that may have produced inaccurate results. The activities of all of these cell cycle kinases were elevated within the atherosclerotic vessels. This lends further strong support to the contention that cell proliferation is stimulated within the atherosclerotic vessel wall. The decrease in smooth muscle α-actin in both the 8 and 16 week cholesterol-fed animals (Figure 30) is also consistent with a change in the phenotype of the vascular smooth muscle cells from a contractile state to a proliferative

one (189, 242). Furthermore, the decreased expression of α-actin in the same tissue used to detect increases in cell cycle protein expression demonstrates the specificity of the changes in protein expression in the developing plaque.

In the present study, we employed the cholesterol-fed New Zealand white rabbit as a model for primary atherosclerosis. When maintained on a 0.5% cholesterol diet, these rabbits consistently develop plaques in their aortas and the duration of the dietary intervention can be used to manipulate the severity of the atheroma. Eight weeks on the 0.5% cholesterol-supplemented diet resulted in moderate plaque development, and a 16 week dietary intervention induced more severe plaque formation. Our initial results using the entire vessel to obtain cell extracts demonstrated that the expression of cell cycle proteins was similar during the early and late stages of atherogenesis. This was surprising. We had expected to detect augmented cell proliferation during the early stages of atherosclerosis. However, it was possible that the results in the 8 week samples were biased by a greater "contamination" of the vessels with tissue that did not contain any plaque. If expression was normal in the area of the vessel that was not infiltrated by the plaque, this would effectively dilute the values and artificially produce lower expression values. Therefore, areas of visible plaque in 8 week aortas were separated from non-plaque areas and analyzed independently. Expression of the cell cycle proteins was consistently greater in the plaque than it was in the neighboring sections of the vessel that did not contain a plaque. It was, however, similar in the plaque-containing samples from the 8 week vessels to that found in the later stage atherosclerotic plaques in aortae from rabbits that underwent the 16 week dietary intervention. This would suggest that cell cycle protein expression remains relatively constant from moderate to more severe stages

of atherosclerosis. Interestingly, non-plaque areas of early stage atherosclerotic aortas also expressed higher levels of cell cycle protein than control samples. It would appear that these "pre-atherosclerotic" areas may be initiating cell proliferation as a first step in the generation of a plaque.

The mechanism responsible for the augmentation of cell proliferation during atherosclerosis is likely complex and multi-factorial. Vascular cell proliferation has been shown to be induced by bFGF (12), aFGF (6), insulin-like growth factor (179), vascular endothelial growth factor (199), native LDL (273), oxidized LDL (33, 273), lysophosphatidylcholine (33), adrenomedullin (113), ATP and ADP (253), via phospholipase D (177), MAP kinase activation (122, 160), and phosphatidylinositol 3-kinase activation (62) to identify just a few. Therefore, the mechanisms responsible for inducing cell cycle protein expression and the related kinase activity in the present study may be any one or more of these factors. For example, oxLDL can increase the expression and activation of cell cycle proteins in vascular cells (273). The status of the cell within the cell cycle may also influence the response of cell cycle proteins to mitogens like oxidized LDL (271). The precise mechanism that induces cell cycle expression *in vivo* in atherosclerosis may prove to be exceedingly difficult to separate out and clearly identify. However, this should not detract from its significance. Strategies that target specific cell cycle proteins have proven valuable in restenosis (47) and may be worthy of study in primary atherosclerosis now that we are certain that they are altered in the atherosclerotic lesion.

The present investigation provides the first evidence that several cell cycle proteins have an elevated expression and activation in atherosclerotic lesions of

cholesterol-fed rabbits. These data strengthen the contention that cell proliferation is augmented during plaque initiation and development. This investigation also demonstrates that the augmentation of cell proliferation is independent of the stage of the atherosclerotic plaque. Interestingly, even the area of the atherosclerotic vessel that does not contain visible plaque formation appears to be in the process of initiating cell proliferation. Our results provide important new insights into the involvement of cell cycle proteins and cell proliferation in the process of primary atherogenesis.

CHAPTER 6: CONCLUSIONS

1. OxLDL is an independent mitogen for quiescent vascular cells, and exerts its proliferative effect through a pathway involving the scavenger receptor, PI 3-kinase and PLC.
2. OxLDL-stimulated proliferation in quiescent vascular cells is accompanied by increases in the expression and nuclear translocation of cell cycle proteins, as well as the activity of their related kinases.
3. The simultaneous upregulation of both cell cycle inducers and inhibitors in oxLDL-treated quiescent vascular cells suggests a controlled, adaptive type of growth consistent with the development of a primary atherosclerotic plaque.
4. OxLDL inhibited the proliferative response of vascular cells to serum, through a mechanism involving the PLC pathway.
5. The reduced vascular cell growth in cells treated with a combination of oxLDL and serum was associated with a reduction in nuclear translocation of cell cycle proteins.
6. OxLDL had differential effects on vascular cell growth in the presence of other growth factors, suggesting that oxLDL may positively or negatively modulate proliferation depending on the local cellular environment.
7. Cell cycle protein expression and activation were elevated in aortae from cholesterol-fed rabbits compared with controls.

8. The increased cell cycle protein expression and activation in atherosclerotic rabbit aortae was associated with a decrease in SM α-actin, consistent with the change from a contractile to synthetic VSMC phenotype during proliferation.
9. The expression and activity of cell cycle proteins did not differ between whole aortae from animals with early and late stage atherosclerotic disease, but was more concentrated in early stage plaques.
10. Tissue with no visible plaque in early stage atherosclerotic vessels expressed increased levels of cell cycle proteins compared with controls, suggesting that this tissue was "pre-atherosclerotic" and that the initial stages of proliferation had already begun.

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APPENDIX

Published manuscripts from this Thesis:

1. **Zettler ME and Pierce GN.** Cell cycle proteins and atherosclerosis. *Herz* 25: 100-107, 2000.
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