Ⅰ. Introduction

1. The cardiovascular disease in progress

Cardiovascular disease (CVD) comprises of all diseases and conditions involving the heart and blood vessels, including coronary heart disease (CHD), stroke, peripheral vascular disease, ischemic disease, cerebrovascular disease, and rheumatic heart disease. The main underlying problem in CVD is atherosclerosis, a process that clogs supply blood vessels with deposits containing cholesterol and other substances, often associated with blood clots. A person with atherosclerosis may suffer angina, myocardial infarction (MI) or sudden death in the following situations.

CVD was Taiwan's third leading cause of death in 2003, following malignant tumors and cerebral vascular diseases. According to statistics from Department of Health, Executive Yuan, Taiwan, three of the top ten causes of death are related to vascular disease, including cerebral vascular diseases, heart diseases and hypertensive diseases. Moreover, statistics showed that the cost of hypertension was estimated at NT$ 3.9 million and cardiovascular agents accounted for almost NT$ 2.02 million for this disease in hospitals, clinics and drug stores [International Medications Systems, 1990]. In 1998, medical expense for heart diseases was NT$ 181 thousand for heart disease [Bureau of the National Health Insurance, Taiwan]. Costs for 177,910 inpatients in 2000 had been estimated at NT$ 713 billion, claimed for inpatient Care by Medical Institution and Disease [Department of Health, Executive Yuan, Taiwan]. The economic burden of CVD has a profound impact on health care system worldwide, and this burden continues to grow as the population ages. According to the Centers for Disease Control and Prevention, the cost of CVD in the United States in 1999 was estimated to be $286.5 billion [Collins et al, 2003].

CVD is the main cause of death in the UK (over 240,000 deaths in 2001) [Gibbs et al, 2002; Gibbs et al, 2004]. Half of all deaths from CVD are attributed to CHD, which is the most

common cause of premature death. In the United States, CVD is the number one killer, accounting for 29% of all deaths in 2001. CVD is the leading cause of death among middle age Americans [Ambrose et al, 2004]. Besides, CVD ranks first among all disease categories for women each year. This disease accounted for 44.3% of all female deaths in America in 1996. CVD is also the leading cause of death in New Zealand, accounting for 41% of all deaths in 1999. The estimated expenditure on hospital cardiac services in 1999 was over $150 million. The estimated expenditure on all cardiovascular drugs was over $110 million in 2002. CHD was the major cardiovascular cause of death, accounting for 55% of all such deaths, followed by stroke (24%), heart failure (5%), peripheral vascular disease (4%) and rheumatic fever and rheumatic heart disease (0.5%) [Astin et al, 2004]. Therefore, CVD kills more people than any other disease does. Improving the treatment of patients is the goal for every researcher and scientist.

CVD is due to the following risk factors, including high blood pressure, cigarette smoking, and high blood cholesterol. Overweight and obesity, physical inactivity, and diabetes are additional risk factors for this disease.

2. Treatments of stenosis in CVD

Atherosclerosis is a disease of the arterial intima leading to the formation of atheromatous plaques and occlusion of the lumen, called stenosis. It is most serious when it affects the blood supply to the heart, causing angina or heart attack. There are several medical and surgical procedures, including atherectomy, tissue removing techniques, (laser, rotational atherectomy, directional coronary atherectomy, etc), percutaneous transluminal coronary angioplasty (PTCA), and coronary artery bypass graft (CABG) surgery for the treatment of stenosis

Three commonly used classes of drugs are the nitrates (i.e., isosorbide), β-adrenergic

antagonist (i.e.,propranolol), and calcium-channel blockers (i.e., procardia).

CABG undoubtedly had a major impact on the treatment of obstructive coronary artery disease over the past four decades. CABG surgery is performed to relieve angina in patients whose illness has not responded to medications. CABG is best performed in patients when blockages are located in certain arterial segments, which are not well suited for PTCA. CABG is often also used in patients who have failed to attain long-term success following one or more PTCA procedures. CABG surgery has been shown to improve long-term survival in patients with significant narrowing of the left main coronary artery, and in patients with significant narrowing in multiple arteries, especially in those with decreased heart muscle pump function.

Although many studies have demonstrated the clinical effectiveness and improvement in patients following CABG, there is a limit on the symptomatic improvement following coronary surgery for two principle reasons: (1) progression of native coronary disease and (2) new atherosclerotic disease in bypass conduits. The repeating coronary surgery is also less effective for symptom relief compared to the initial operation because of leading to higher morbidity and mortality [Campeau et al, 1979]. Besides, by-pass surgery is a high-risk procedure and expensive. Cardiologists have preferred less expensive method called PTCA. Advantages and disadvantages between PTCA and CABG are outlined in Table 1.

In 1977 Andreas Gruentzig at the University Hospital of Zurich, Switzerland, inserted a catheter into a patient's coronary artery and inflated a tiny balloon, successfully opening a blockage and restoring blood flow to a human heart. Gruentzig performed the first PTCA in a human [Greuntzig et al, 1979]. The first PTCA procedures were performed in Australia in 1980. The numbers of procedures and units had grown dramatically in Australia, from six units performing 45 procedures in 1981 to 30 units performing 8334 procedures in 1993. To the end of 1993, a total of 39843 PTCA procedures had been carried out in Australia. The

technique of PTCA has evolved rapidly. Some outcomes over five years had been reported by the National Heart, Lung, and Blood Institute's PTCA Registry in the USA, and showed that long-term clinical benefit without increased risk of death or MI can be expected after PTCA [King and Schlumpf, 1993].

PTCA is generally considered when medical treatment has failed to control symptoms. It is most commonly used in single or double vessel diseases. Indications for PTCA have widened. They now include chronic stable angina, unstable angina, patients with stenosed CABG grafts, and patients in whom CABG is inappropriate. PTCA produced better short-term clinical outcomes [Kachel, 1996].

PTCA is proposed as one of major surgical treatments because interventional procedures mechanically disrupt the occlusive thrombus, compress and break the underlying stenosis, and then clear the lumen and rapidly restore blood flow. However, during the procedure the artery may close abruptly, leading to a MI or, in rare cases, death. Abrupt closure during PTCA has been reported in 2 - 10% of patients and this has required emergency CABG backup to be available. In addition, although balloon angioplasty generally has a successful rate, the renarrowing of arterial lumen appears after 5 - 6 months. Prevention of the renarrowing of arterial lumen, called restenosis, after a successful angioplasty remains one of the most challenging issues in the treatment of obstructive coronary diseases.

Table 1. Advantages and disadvantages of surgical treatments：CABG vs. PTCA

CABG	
Advantages	Complete relief from angina in 60 - 90% of patients at 1 year
	A slight decrease in mortality when compared to medical treatment
	Lower revascularization rates after 1 year when compared to PTCA
Disadvantages	Longer hospital stays and convalescence：the mean length of stay post-operatively in uncomplicated cases is 7-10 days
	A slightly higher rate of MI when compared to medical treatment
	Longer hospital recovery after CABG when compared to PTCA
	Some patients not fit to undergo such a major operation
	Higher rate of native coronary artery disease progression following CABG
PTCA	
Advantages	Unnecessary for opening the chest wall so it is useful in those who are poor operative risks
	The short length of stay in hospital
	Carried out as a day case
	People considered not to be fit for a CABG
	No need for prolonged convalescence
Disadvantages	During the procedure the artery may close abruptly, leading to a MI or, in rare cases, death.
	30 - 50% of the renarrowing of arterial lumen[a] after 5 - 6 months

[a], Renarrowing of arterial lumen: restenosis

3. The progression of angioplasty hyperplasia

Angioplasty hyperplasia, well known as restenosis, is due to balloon injury. Restenosis is primarily attributed to migration and proliferation of vascular smooth muscle cells (VSMCs), called neointimal hyperplasia. Neointimal area indicates that the circumference of internal elastic lamina (IEL) subtracts lumen area. Restenosis is the healing response to mechanical injury comprised of four pathologies: elastic recoil, thrombus incorporation, neointimal hyperplasia (i.e., SMC migration and proliferation, extracellular matrix (ECM) deposition), and vessel remodeling.

The main problem of early restenosis (i.e., occurring within less than 1 month) is due to the damage of the arterial wall because balloon dilation alone often leads to immediate recoil. The expanding balloon applies a pressure as high as 20 atmospheres from a few seconds to minutes. That treating arterial narrowing with mechanic endovascular devices is similar to the balloon must cause significant vessel wall damage. After balloon angioplasty, endothelial cells have been denuded.

In response to this wound, inflammatory cells, such as monocytes, macrophages, lymphocytes, etc, accumulate the site of injury. Leukocyte recruitment and infiltration occur rapidly at sites of vessel injury following balloon angioplasty where the lining endothelial cells have been denuded and platelets and fibrin have been deposited. For example, emerging experimental and clinical data indicated that leukocytes might be central to intimal growth after mechanical arterial injury (i.e., PTCA). In animal models of vascular injury, neutrophil and monocyte recruitment precede intimal thickening and inflammatory cell number within the vessel wall is associated with the extent of cellular proliferation and intimal thickening. Infiltration and accumulation of monocyte/macrophages is a dominant pathophysiological response after stent-induced arterial injury, with inflammatory cells comprising up to 60% of neointimal cells in rabbit, porcine, and non-human primate models

and in human autopsy specimens. A sequential adhesion model of leukocyte attachment to and transmigration across surface-adherent platelets has been proposed. For example, the initial tethering and rolling of leukocytes on platelet P-selectin are followed by their firm adhesion and transplatelet migration, processes that are dependent on the leukocyte integrin macrophage-1 (Mac-1) and several platelet receptors, including Glycoprotein Ibα (GP Ibα) and intercellular adhesion molecule-2 (ICAM-2). Activation of platelets causes expression of the Glycoprotein IIb/IIIa (GP IIb/IIIa) receptor. Coagulation pathways result in the formation of fibrinogen, which through the GP IIb/IIIa receptor binds platelet to each other. A thrombus is an aggregate of a network of fibrin, platelets, and blood elements. Thrombosis most often develops on an underlying atherosclerotic plaque when the lesion ruptures. Thrombin formed is a powerful stimulant of platelet activation and stimulus to medial cell proliferation [Waller et al, 1993]. The bridging interaction between platelet GP IIb/IIIa and leukocyte Mac-1 bridged by fibrinogen is also possibly relevant. Therefore, plaques become adherent to the damaged vessel wall within seconds due to the loss of protective endothelium (loss of heparinoids, prostacyclin, endothelium-derived-relaxing-factor (EDRF) and tissue plasminogen activator) and exposure to the platelet of activating subendothelial collagen.

Except for the stimulation of growth factors from platelets, cell adhesion molecules, adherent thrombin and growth factors from SMCs by themselves all contribute to cellular phenotypic changes observed. The cell cycle changes from G_0 (quiescent phase) to G_1 (proliferate phase). After changing the phenotypes of SMCs, factors such as transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), and the angiotensin receptor on SMCs all contribute to the migration and proliferation in the progression of cell cycle [Grant et al, 1996; O'Brien et al, 1996]. Migration through disrupted IEL into the thin intima is an important prelude to proliferation of SMCs and metalloproteinase, facilitating the

proliferation of SMCs, playing an important role in lesion development [Strauss et al, 1996; Mintz et al, 1997b]. Therefore, endothelial denudation, intimal disruption, and medial layer damage can occur [Carter, 1994; Grant et al, 1996; O'Brien et al, 1996]. In addition, activated platelets release growth factors that alter the medial SMC behavior. Platelet-derived growth factor (PDGF) and TGF-β1 induced proliferation and migration of VSMCs. For example, platelets, activated within the intravascular blood clot, release cytokines such as PDGF. PDGF stimulates the growth of SMCs and myofibroblasts. Besides, TGF-β1 regulates ECM synthesis by SMCs and fibroblasts, increasing the synthesis of fibronectin, thrombospondin, fibrillar collagens, elastin, and proteoglycans. All are present in increased quantities in vessels after injury. Accordingly, substantial evidence implicates TGF-β1 in the pathogenesis of restenosis: (1) increased levels of TGF-β1 occur in injured and restenotic vessels, (2) exposure of arteries to TGF-β1 after injury results in increased neointimal formation, and (3) TGF-β1 induces the phenotypic modulation of adventitial fibroblasts to myofibroblasts, which are responsible for adventitial ECM deposition. Within several weeks to 6 months, the healing site resembles a fibrous plaque, which is the beginning of restenosis.

Inflammatory response can be described as the first step in response to arterial injury. Once in the intima the cells proliferate and form neointima during the first 11 - 14 days following injury. ECM components such as proteoglycans are produced during this phase and increase the bulk of the lesion. The ECM of the restenotic lesion differs from that of atheromatous lesion probably as a consequence of the effects of TGF-β. This is for example the preferential expression of the proteoglycan, biglycan, and collagen types Ⅰ and Ⅲ. By 2 - 3 weeks the proliferation phase is done and retraction of the ECM contracts slightly.

Although the initiation and promotion of the restenotic response is deendothelialization, reendothelialization can result in EDRF-induced SMC inhibition. Neointimal thickening

observed remains essentially unchanged during the subsequent 4 weeks after injury because reendothelialization is not complete. One hypothesis is that intimal surface of a balloon-injured artery could accelerate reendothelialization and reduce neointimal thickening. Evidence showed that the inhibition of these early cellular processes could lead to longer-term inhibition of cellular damage (i.e., formation of reendothelialization).

In addition to neointimal formation, coronary arteries change size, undergoing "remodeling" following coronary angioplasty. Arterial remodeling is measured by the area circumscribed by the external elastic lamina (EEL) or by the perimeter of the EEL. These changes are known as negative remodeling, which is a result of adventitial thickening resulting in vessel shrinkage [Mintz et al, 1997a; Schwartz et al, 1998; Sangiorgi et al, 1999] in Figure 1.

Restenosis reflects a cascade of molecular and cellular events within the vascular wall. Besides, restenosis is the process of growth factor-dependent proliferation of SMCs, migration of those cells from media to the intima, synthesis of ECM, and adventitial scarring. Injury of the blood vessel leads to the release of numerous vasoactive, thrombogenic, and mitogenic factors.

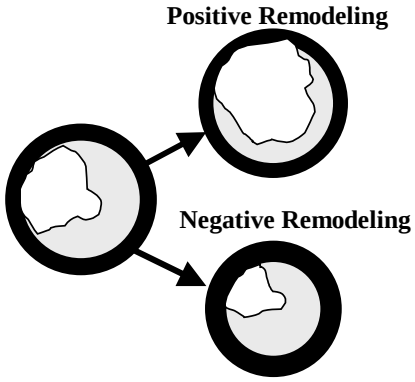

Figure 1. Positive and negative arterial remodeling in response to atherosclerotic plaque formation. This so-called positive remodeling, an increase in lumen diameter, can occur after angioplasty, but negative remodeling can also ensue, contributing to restenosis. [Rajagopal and Rockson, 2003].

4. Treatments of restenosis

4-1. Stenting

The introduction of coronary stents from the mid-1980s was the last major technical development to significantly improve the short- and long-term results of interventional coronary revascularization procedures [Gruntzig et al, 1978; Sigwart et al, 1987]. The clinical series showed that PTCA with stenting was better than PTCA alone [Fischman, et al, 1994]. Among others, the Stent Restenosis Study Investigators compared 520 patients with stable angina and single coronary artery disease: it was shown that stent implantation was better than the standard balloon angioplasty [Serruys et al, 1994].

Compared to CABG surgery, PTCA with the stenting is followed with lower rates of death and MI during long-term follow-up. Thrombotic events usually occur within 2 to 14 days after stenting, often with major clinical complications. Intense anticoagulation and antiplatelet regimen are associated in more hemorrhagic and peripheral vascular complications. Despite lowering the restenosis rate with the implantation of coronary stents

and a decline in the rate of initial complications, restenosis occurs within 6 months after intervention, sometimes necessitating repeating revascularization.

This led to a strategy of coronary stenting in the mid to late 80's that has become a standard treatment modality. A stent is a small, compressible tubular metal lattice that is mounted on a balloon catheter system and then expands at the site of blockage during the angioplasty procedure. There are several factors related to the device's presence in the lumen that reduces rates of restenosis, but do not completely eliminate it. The proliferation of SMCs migrates from the vessel wall to the arterial lumen. This phenomenon can result in a narrowing of the lumen and a 20 - 30% rate of "in-stent restenosis".

Over two million balloon angioplasty and stenting procedures are performed worldwide each year. While these procedures have a high initial rate of success, the chronic problems of restenosis develop in a large percentage of patients.

Stenting has eliminated some of the mechanisms of restenosis such as "elastic recoil", where the walls of the artery tend to spring back immediately after the procedure and "negative remodeling" where the vessel slowly contracts as it heals, producing a smaller lumen. Although stenting prevents negative remodeling, the stent induces greater neointimal formation [Mintz et al, 1997b].

4-2. PTCA with drug-coated stents

Coating stents with pharmaceutical agents deliver a much lower dose of the drug to the target area in which agent achieves in local sites and minimizes the risk of systemic toxicity. The main advantage of the coated stent is its capability to lower the incidence of restenosis from 20% to 5%. The only disadvantage is high cost. Different drugs include anti-inflammation, antiproliferation, antimigration, and reendothelization

Sirolimus elevates levels of p27, a cyclin dependant kinase inhibitor and blocks cell cycle

progression at the G_1 progression at the G_1 - S phase. Besides, it has the ability to inhibit cytokine-mediated and growth-factor-mediated proliferation of lymphocytes and SMCs, thereby acting as a cytostatic agent and reducing neointimal proliferation. The report on 238 patients with angina pectoris (single, primary lesions in native coronary arteries) compared sirolimus-eluting stent with a standard stent follow-up at 6 months. The measured outcome (in-stent late luminal diameter, restenosis, clinical event) were definitely better in the drug-coated group (i.e., with less late luminal loss and with less clinical events [Sousa et al, 2001]. The long-term follow-up of these patients showed the same advantage over uncoated-stents. For example, the effectiveness of drug-coated stents decreases the complex lesions. Other drugs (actinomycin D, estradiol, tacrolimus, dexamethazone, rapamycin) show a potential effect on restenosis and neointimal proliferation in Table 2. Another promising drug is taxol (paclitaxel), regarded as a cytotoxic drug, and its efficacy in reducing neointimal proliferation and restenosis is due to its unique mechanism of action in promoting the assembly of tubulin into extraordinarily stable microtubules, thereby interrupting cellular proliferation, migration and signal transduction [Axel et al, 1997]. It is currently under investigation with two ongoing trials (TAXUS I and TAXUS II). The major problem with drug-coated stents is the price. For example, current cost (Switzerland) of each stent is about $3,500, to be compared with about $900 for normal stent.

4-3. Pharmacological agents

Many agents were tested in different animal models of restenosis for their inhibitory effect on the biological process. Based on such data most of theses agents were then tested in clinical trials. Thus antiplatelet (i.e., aspirin, dipyridamole), antithrombotic (i.e., heparin, hirudin, bivalirudin), anticoagulants (i.e., warfarin), lipid lowering agents, antiproliferative drugs (i.e., trapidil) and angiopeptin (a somatostatin analogue that inhibits IGF-1 and

fibroblast growth factor (FGF), statins, vasodilators and angiotensin converting enzyme (ACE) inhibitors (cilazapril), calcium channel blockers, and β-adrenergic blocking agents have all been tested and have not been shown to beneficially influence restenosis in Table 3 [Schwartz et al, 1990; Hoberg et al, 1991; Serruys et al, 1991; Anonymous 1992; Serruys et al, 1993; Maresta et al, 1994; Emanuelsson et al, 1995; Foley et al, 1996; Karsch et al, 1996]. There were over 30 clinical trials of systemic agents tested in the setting of balloon angioplasty restenosis. In general, the results were disappointing. Besides, there is not a good animal model of atheroma, although many of the changes seen in the animals approximate closely as those seen in human.

Table 2. Summary of current clinical trials involving the use of drug-eluting stents

Drug	Clinical trial	Lesion type	Results	Reference
Sirolimus	FIM[e] (n=45)	De novo	Inhibition of neointimal formation at 24 months	Degertekin et al, 2002
	RAVEL[f] (n=238)	De novo	RS rate at 6 months: 0% (DES) vs. 26.6% (BS[a])	Morice et al, 2002
	SIRIUS[g] (n=1100)	De novo	In-segment RS rate at 8 months: 8.9% (DES[b]) vs. 36.3% (BS)	Moses et al, 2002
Paclitaxel	TAXUS I (n=61)	De novo	RS rate at 6 months: 0% (DES) vs. 10% (BS)	Grube et al, 2003
	TAXUS II (n=536)	De novo	In-segment RS[d] rate at 6 months: 5.5% (DES) vs. 20.1% (BS) in slow-release stent cohort 8.6% (DES) vs. 23.8% (BS) in moderate-release stent cohort	Tanabe et al, 2004
	TAXUS III (n=28)	In-stent restenosis	RS rate at 6 months: 16% (4/25 pts with angiographic FU)	Tanabe et al, 2003
	ASPECT (n=177)	De novo	RS rate at 6 months: 4% (high-dose DES) vs. 27% (BS)	Park et al, 2003
	SCORE	De novo	Trial stopped early due to high rates of MACE[c]	
Dexamethasone	STRIDE	De novo	RS rate of 13.3% at 6 months	Liu et al, 2003
Actinomycin D	ACTION	De novo	Trial halted due to high restenosis rate	

[a] BS, bare stent; [b] DES, drug-eluting stent; [c] MACE, major adverse cardiac event; [d] RS, restenosis; [e] FIM, first-in-man, [f] RAVEL, the randomized study with the sirolimus-eluting bx velocity balloon-expandable stent in the treatment of patients with de novo native coronary artery lesions trial; [g] SIRIUS, multicenter, randomized, double-blind study of the sirolimus-eluting bx velocity balloon-expandable stent [Lim VY and Lim YL, 2003].

Table 3. Pharmacological agents for inhibition of restenosis

Agents	Drugs	Mechanisms
ACE inhibitors	Cilazapril	Lower blood pressure by blocking the production of angiotensin
Anticoagulants agents	Warfarin	Antagonizes vitamin K dependent γ-carboxylation of the procoagulants factor This γ-decarboxylation necessary for normal function of these clotting factors
Antiplatelet agents	Aspirin	Permanent inactivation of a key enzyme in arachidonate metabolism
	Clopidogrel	Structurally related thienpyridine with platelet inhibitory properties Inhibiting ADP-induced platelet aggregation by both agents
Antithrombotic agents	Heparin	Mediated by its interaction with antithrombin III (AT III) inducing a conformation change in ATIII that enhances its antithrombotic activity many folds
Antiproliferative agents	Trapidil	A thromboxane-A2 inhibitor and PDGF receptor antagonist
β-Blockers	Metoprolol	Used as competitive antagonists at the β-adrenergic receptors raise the heart rate.
Calcium channel blockers	Verapamil	Decreasing the heart's pumping strength and being relax blood vessels
Hypolipidemic agents	Statins	Effective for lowering LDL cholesterol levels
Vasodilators	Nitroglycerin	Acting as blood vessel dilators and open vessels by relaxing their muscular walls
Other agents	Angiopeptin	A synthetic cyclic octapeptide analogue of somatostatin, reducing neointimal hyperplasia

4-4. Atherectomy

Some catheter-based devices actually remove the plaque itself, a process known as atherectomy, or ablation. There are four types of atherectomy: laser, rotational, directional, and transluminal extraction.

Laser atherectomy is commonly used to remove enough plaque to allow balloon angioplasty to be performed. In this procedure, a laser attached to the tip of a thin flexible catheter emits short pulses of light that vaporize plaque. Rotational atherectomy typically is used to treat arteries with very long, calcified, or solid blockages. This technique also can be employed to remove plaque that has regrown inside a stent. It uses a high speed-rotating shaver to grind up plaque. Directional atherectomy employs a catheter tipped with a device consisting of a cup-shaped blade and a container. The blade cuts away plaque from the artery and deposits it into the container. When the catheter and device are withdrawn, the plaque is removed from the body. It is no longer commonly used. Transluminal extraction involves a special catheter tipped with a hollow tube and rotating blades. As the blades cut plaque away from the arterial wall, the debris is suctioned out of the body through the tube

4-5. Vascular gene therapy

Despite intensive trials, effective therapy to prevent restenosis had being identified. Engineered inactivation of gene function serves as a gene therapy, such as antisense oligodeoxynucleotides (ODN), ribozymes, and decoys, for treatment of viral infection, cancer and other diseases by aberrant gene expression. Gene function can be inactivated by antisense ODN at the DNA level or by ribozymes at the RNA level. Ribozymes hybridize and cleave target RNAs. Once the target has been cleaved, the ribozyme can dissociate from the cleaved transcript and repeat the process with another RNA molecule. The major advantage of ribozymes is that they can sequence-specifically cleave multiple target mRNA

molecules. Nilsson et al showed that normal, growth-arrested VSMCs did not express PDGF mRNA, whereas cultured VSMCs or VSMCs in atherosclerotic plaques expressed PDGF A-chain homodimer (PDGF-AA) mRNA and secreted PDGF-AA protein, indicating that PDGF-AA contributed to VSMC proliferation in arterial proliferative diseases [Nilsson et al, 1985]. Kotani et al synthesized a chimeric DNA-RNA hammerhead ribozyme that targeted the PDGF AA and found that the cleavage of the rat PDGF-AA mRNA resulted in the reduction of PDGF-AA protein production. A chimeric DNA-RNA hammerhead ribozyme in which ribonucleotides at non-catalytic residues were replaced with deoxyribonucleotides and with phosphorothioate linkages at the 3' terminus for cleavage at the GUC sequence at nucleotide 921 in the loop structure of the rat PDGF-AA mRNA [Kotani et al, 2003].

Nitric oxide (NO) produced by blood platelets and endothelial cells inhibits the aggregation of platelets and the adhesion of platelets and white blood cells to the blood vessels' inner walls, thereby significantly reducing the obstruction of blood vessels that is associated with blood clots. Shears et al proposed that short-term but sustained increased in NO synthesis by an adenoviral vector, AdiNOS, carrying the human NO synthase (NOS) cDNA, achieved with inducible NOS (iNOS) gene transfer at sites of vascular injury. An adenovirus transferred plasmid with the cytomegalovirus (CMV) promoter and an artificial splice sequence was designed to drive the transcription of the iNOS cDNA. iNOS gene transfer leaded to the suppression of neointimal formation rather than elimination of a preestablished neointimal lesion [Shears et al, 1998].

4-6. Brachytherapy

Exposure of cells to ionizing radiation (IR) produces a range of lesions in DNA including base and deoxyribose attack, abasic sites, single-strand breaks (SSBs) and double-strand

breaks (DSBs), plus DNA-DNA and DNA-protein crosslinks [Ward et al, 1990]. IR induces DNA double-stranded breaks that are the most lethal form of DNA damage [Hutchinson et al, 1985].

Each year in the United States nearly 500,000 patients receive radiotherapy to treat cancers [Lichter et al, 1995]. The goals of radiotherapy are either to cure the patient of cancer by killing all of the malignant cells within a tumor or to alleviate symptoms by decreasing local tumor burden. Therefore, brachytherapy have been used in the reduction of restenosis.

Although drug-delivery stents reduce restenosis, various constituents of stents, including the stent backbone, materials used as drug-delivery vehicles, and the physicochemical properties of the pharmacotherapeutic agents have not well established in order to achieve optimization of each of these parameters to provide the greatest efficacy and safety. Besides, the economic effect of the introduction of these stents, which are projected to be 2 to 3 times as expensive as conventional devices.

Over the last several years, intracoronary radiation by percutaneous coronary interventions has been proposed as the most effective treatment of restenosis [Serruys et al, 1994; Casterella and Teirstein, 1999]. The beneficial effect of brachytherapy is to limit the division of SMCs within the artery. Since cells that divide rapidly are sensitive to radiation, brachytherapy has been considered a very promising treatment.

Brachytherapy, meaning “near radiation,” works by inserting radioactive material into the arterial lumen to halt the division of SMCs after angioplasty [Sigwart et al, 2001]. The radiation can be introduced in several different ways including radioisotope-filled balloons, catheter-based systems such as the radioisotope-laced wires, or stents themselves can deliver the radiation.

Most patients with restenosis will be treated with stents, a procedure that combines stenting with the delivery of radiation in one step. The use of radioisotope stents is not as popular

because it is likely related to the occurrence of edge restenosis. Unfortunately, most patients in these studies had de novo lesions. In contrast, catheter-based intravascular brachytherapy (IVBT) can use radiation from either a γ or β source, whereas radioactive stents predominantly use the β radiation.

Clinical trials have demonstrated that IVBT, using either a γ or β source, is effective in reducing restenosis by suppression of neointimal hyperplasia after percutaneous coronary intervention for in-stent restenosis in Table 4 and Table 5 [Leon et al, 2001].

Although brachtherapy is technically simple, it poses several difficulties for widespread applications because of safety for both patient and catheterization lab personnel. Handling radioactive material requires safety precautions, especially for γ radiation. Besides, routine shielding of the cardiac catheterization laboratories is required. Treatment times are 3 - 10 minutes for β and 20 minutes for γ radiation, in addition to time requirement in advance and remove of the catheters [Pavlides et al, 2002].

The β isotopes currently in preclinical or clinical use include Yttrium-90 (^{90}Y), Strontium-90 (^{90}Sr/Y), Phosphorus-32 (^{32}P), Ruthenium-106 (^{106}Ru), Xenon-133 (^{133}Xe), Rhenium-186 (^{186}Re), and Rhenium-188 (^{188}Re). The clinical implication is that β emitters are very effective in smaller vessels (< 4 mm) but may lose their ability to deliver adequate amounts of radiation in larger vessels (≥ 4 mm). β brachytherapy has several advantages; including less shielding requirements, and easier handling of sources have led to more widespread acceptance of β technology [Waksman et al, 2003b].

Long-term follow-up in animals with radioactive stents has been reported. Carter et al described increased neointimal formation and adventitial fibrosis at 6 months using ^{32}P radioactive stents in atherosclerotic porcine coronary arteries [Carter et al, 1999; Taylor et al, 1999]. The delayed neointimal healing at several weeks after radioactive stent implantation in dog coronary arteries were also reported by Taylor et al. However, Farb et al reported a

delayed formation of neointimal proliferation but incomplete vessel wall healing at 1 year after the implantation of ^{32}P radioactive stents in rabbit iliac arteries [Farb et al, 2001]. Compared with radioactive stents, long-term animal data on catheter-based intracoronary radiation are incomplete. Initial studies with ^{192}Ir in balloon-injured pig arteries showed sustained inhibition of neointimal formation at 6 months [Waksman et al, 1995; Wiederman et al, 1995]. Condado et al conducted the first human trial using a ^{192}Ir non-centered wire source in 21 patients (22 lesions) with de novo lesions [Condado et al, 1997]. The prescribed doses were 20 and 25 Gy at a distance of 1.5 mm from the source. The doses were administered immediately post-PTCA. At 3 years follow-up, 19% of treated vessels had developed pseudoaneurysms. Costa et al reported the phenomenon of sudden, late coronary artery had thrombotic events following PTCA and IVBT with β source in 108 patients. 6 of the 91 (6.6%) patients suffered with sudden late thrombotic events at 2 - 15 months after PTCA with and without stenting [Costa et al, 1999]. The authors suggested that the late thrombotic events maybe a result of the treatment of β radiation which lead to impairment of repair system. Recently several studies of intracoronary radiation therapy in patients with in-stent restenosis demonstrated a reduction of binary angiographic restenosis and target lesion revascularization compared with a control group [Waksman et al, 2000]. Angiographic and intravascular ultrasound (IVUS) studies on patients who underwent IVBT showed nearly complete inhibition of the neointima within the stent body but detected stenosis at the edge of the stented segments and the radiation field.

A number of unusual findings have been reported after radiation therapy, including aneurysm formation, and edge effect [Sabate et al, 1999b; Albiero et al, 2000; Sabate et al, 2000]. The edge effect appears and due to the mitotic stimulation of low dose radiation, which occurs at the edges of the radiating segment of the catheter. Besides, data on β-radiation therapy of the nonstented lesion has shown that there was significant positive

remodeling (an increase of EEM area) [Sabate et al, 1999b]. In addition, the delayed healing, and the increased thrombogenicity were recognized [Vodovotz et al, 1999; Waksman et al, 1999]. The mechanism of thrombosis is not entirely clear in humans. In experimental studies, it was found that delayed endothelial regeneration leaving uncovered the thrombogenic stent surface were most likely responsible for thrombosis. Therefore, understanding of stent endothelialization after brachytherapy not only inhibits the growth of SMCs but the growth and regeneration of the cells of all layers of the arterial wall. Therefore, remodeling pattern or change of EEM area after radiation therapy might determine the occurrence of such kinds of clinical events.

It is obvious that brachytherapy is one of the proven therapy for restenosis today, but without the capability to eliminate this problem entirely.

Table 4. Clinical trials of intracoronary radiation for in-stent restenosis.

Trial	Dosage	Target lesions	Results	References
γ-Radiation (^{192}Ir)				
WRIST[a]	15 Gy at 2.0 mm from the radiation source	Restenosis, lesion length < 47 mm	Death, MI, or target lesion revascularization at 6 months	Waksman et al, 2000
Long WRIST	14–15 Gy at 2.0 mm from source	Native or vein graft, 3.0 – 5.0 mm reference, < 47 mm length		Waksman et al, 2003a
Gamma-One	8 to 30 Gy	In-stent restenosis, lesion length < 45 mm	Death, MI, or target lesion revascularization at 9 months	Leon et al, 2001
β-Radiation				
4R	15 Gy of ^{188}Re-MAG3 at 1 mm into the vessel wall.	Six-month binary angiographic restenosis rate was 10.4%. Two potential limitations of this technology include reduced dosing at the balloon margins (edge effect) and the risks of balloon rupture with radiation spill.	10.4% restenosis rate at 6 months	Park et al, 2001
INHIBIT[b]	20 Gy of ^{32}P at 1.0 mm in the vessel wall	The binary restenosis by 67% in stented segment and by 50% in analysis segments. There were no differences in the edge effect rates between the treated and the control groups.at 9 months	Completed. Demonstrated reduction of 50% in restenosis (analysis segment) and 55% in MACE.	Waksman et al, 2003c

[a] WRIST, Washington radiation for in-stent restenosis trial; [b] INHIBIT, intimal hyperplasia inhibition with β in-stent trial.

Table 5. Clinical trials of intracoronary radiation with β emitters for de novo lesions

Trial	Dosage	Target lesions	Results	References
β-Radiation				
BETA-Cath	14 or 18 Gy of ^{90}Sr/Y at 2.0 mm from the center	240-day target vessel failure rate was 14.2% with 31% restenosis rate.	A single lesion and single vessel intervention with a de novo or restenotic lesion >50% in vessels between 2.7 and 4.0 mm reference vessel diameter	Williams et al, 2002
Dose-Finding Study Group	9 (n = 45), 12 (n = 45), 15 (n = 46), or 18 (n = 45) Gy of ^{90}Y at a tissue depth of 1.0 mm	Abrupt thrombosis or late occlusion of the target vessel in 4 out of 120 (3.3%) patients who were treated with only balloon angioplasty and in 7 of the 49 (14.3%) patients who received new stents	Significant dose-dependent benefit was evident at 6 months follow-up.	Verin et al, 2001
ECRIS	22.5 Gy of ^{188}Re at 0.5 mm	After 6 months follow-up, late loss was significantly lower in the irradiated group compared to the control group.	TVR was 6.3% and late loss was significantly lower at 6 months follow-up.	Hoher et al, 2003
SVG BRITE	20 Gy of ^{32}P at 1.0 mm from balloon surface	De novo lesions were encouraging with only 8% 12-month TVR (target vessel revascularization) rate and 13% angiographic restenosis rate from the entire analysis segment (allowing for edge effects) after β brachytherapy of de novo SVG lesions.	8% TVR and 13% restenosis rates at 1 year	Stankovic et al, 2003

5. Molecular character and function

5-1. Structural components, synthesis and degradation in the family of NF-κB

NF-κB was originally identified as a transcription factor that binds to the κB site in the intronic enhancer of the immunoglobulin κ light-chain gene in B-lymphocytes before 15 years [Sen and Baltimore, 1986]. It was later found in all mammalian cells as a heterodimer whose subunits, p50 and p65, belong to the multigene Rel family [Baeuerle et al, 1994; Siebenlist et al, 1994]. In mature B-lymphocytes and some monocytes and macrophages, NF-κB is constitutively active in non-hematopoietic cells, whereas in most other cells, NF-κB is inactive [Thompson et al, 1995]. However, SMCs differ from endothelial cells in exhibiting basal constitutive NF-κB activity [Lawrence et al, 1994; Peng et al, 1995].

NF-κB is a family of proteins, conserved from Drosophila to humans [Hoffmann et al, 1999]. NF-κB is composed of homo and heterodimers of five members of the Rel family including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (Rel) in Figure 2 Hetero and homodimerization of NF-κB proteins exhibit differential binding specificities including p50/RelA, p50/c-Rel, p52/c-Rel, p65/c-Rel, RelA/RelA, p50/p50, p52/p52, RelB/p50 and RelB/p52. All the Rel proteins contain a conserved N-terminal region, called the Rel homology domain (RHD). The N-terminal part of the RHD contains the DNA-binding domain, whereas the dimerization domain is located in the C-terminal region of the RHD. Close to the C-terminal end of the RHD exists the nuclear localization signal (NLS), which is essential for the transport of active NF-κB complexes into the nucleus.

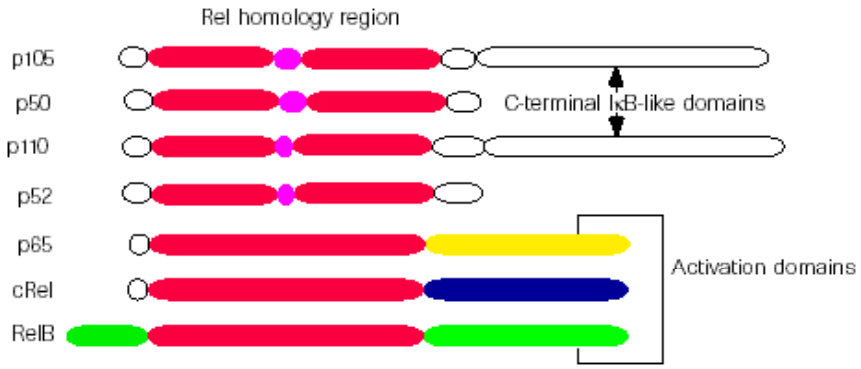

Figure 2. Sequence motifs in the Rel/NF-κB family of proteins. p50 and p52 are proteolytic products of p105 and p110, respectively, in which their inhibitors (IκB-like domains) are removed. Regions of high homology exist among the family members (RHD). p105 (p50) and p110 (p52) also contain 32- and 18- amino-acid inserts, respectively, in the RHR (ovals in the middle of RHM). Non-homologous transactivation domains exist in p65, c-Rel, and RelB **[**Huxford et al, 1998**]**.

The NF-κB family can be divided into two groups, based on differences in their structures, functions, and modes of synthesis [Baeuerle et al, 1994; Siebenlist et al, 1994]. Members of one group (p105, p100, and Drosophila Relish) have long C-terminal domains that contain multiple copies of ankyrin repeats, which is found in inhibitory factor kappa B (IκB) family members, including IκBα, IκBβ, IκBγ, Bcl3, and Drosophila cactus in Figure 3. NF-κB1 and NF-κB2, having a molecular weight of 105 and 100 kDa respectively, are initially considered as large precursors of p50 and p52 of NF-κB complexes in Figure 4 [Lin et al, 1996; Lin et al, 1998; Orian et al, 1999; Karin et al, 2000; Lin et al, 2000; Lee et al, 2001]. In the N-terminal region of NF-κB1, there is a RHD composed of ~300 amino acids that are responsible for DNA binding, dimerization with other Rel family members, and interaction with IκB proteins. A glycine-rich region (GRR) is directly adjacent to the RHD, and a string

of 33 to 35 amino acids (Fig. 4). The C-terminal region of NF-κB1 contains multiple copies of the so-called ankyrin repeats. The C-terminal portion of p105 is identical to IκBγ, which is a product of an alternatively spliced RNA found in certain mouse pre-B cells. These precursors do not bind to DNA but can interact with other Rel-related proteins and function in an IκB-like fashion [Rice et al, 1992; Mercurio et al, 1993]. Proteolytic degradation of the p105 and p100 precursors leads to the production of mature DNA-binding subunits and the release of active Rel-related protein dimers, containing p50 and p52, respectively [Fan et al, 1991; Mercurio et al, 1992]. The rate of processing of both precursors appears to be regulated and can be increased in response of extracellular stimuli [Mercurio et al, 1993].

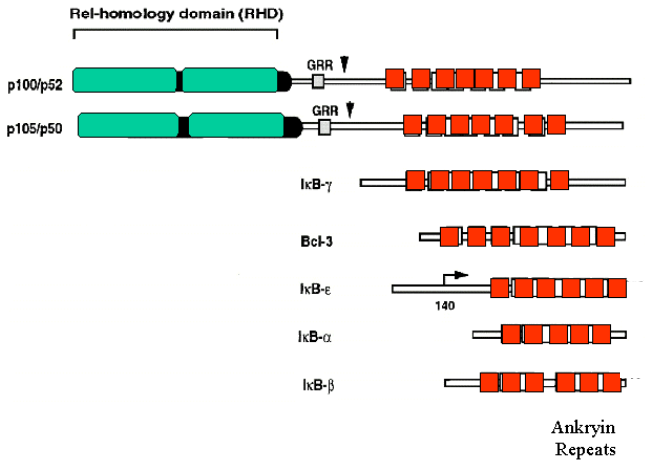

Figure 3. The family of NF-κB and IκB, evolutionarily conserved mediator in the immune response. All of the IκB inhibitors have five-seven ankyrin (30aa) repeats domains [Ghosh et al, 1998]

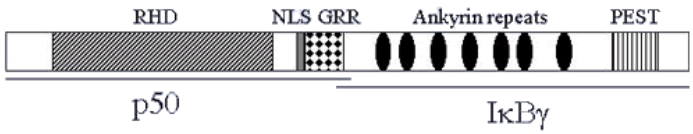

Figure 4. Overview of human NF-κB1 (p105). Known functional domains are indicated [NCBI]

Members of this group do not function as transcription activators, except when they form dimers with members of the second group, which include p65 (RelA), Rel (c-Rel), RelB, and the Drosophila Rel proteins Dorsal and Dif [Baeuerle et al, 1996]. These proteins are not synthesized as precursors, and in addition to the N-terminal of RHD, they possess one or more C-terminal transcriptional activation domains. NF-κB was the original name for the p50-p65 heterodimer. In unstimulated cells, NF-κB-family proteins exist as heterodimers or homodimers that are located in the cytoplasm by their association with a member of the IκB family of inhibitory proteins [Baeuerle et al, 1996]. From biochemical studies and direct structural determinations, it is clear that IκB makes multiple contacts with NF-κB. These interactions mask the NLS of NF-κB and interfere with sequences important for DNA binding [Ghosh et al, 1995; Cramer et al, 1997].

5-2. The biological function of NF-κB

A large variety of bacteria and viruses can lead to the activation of NF-κB which in turn controls the expression of many inflammatory cytokines, chemokines, immune receptors, and cell surface adhesion molecules in Figure 5 [Pahl, 1999; Li and Stark, 2002]. Therefore, NF-κB historically has been considered as a central mediator of the innate immune response. However, recent studies have shown that NF-κB may function more generally as a central regulator of stress response in different stressful conditions, including physical stress, oxidative stress, and exposure to certain chemicals, leading to the activation of NF-κB [Pahl, 1999].

NF-κB can be activated by exposure of cells to lipopolysaccharides (LPSs), mitogens, growth factors, hormones, inflammatory cytokines, such as tumor necrosis factor (TNF-α) or Interleukin-1 (IL-1), lymphokines, oxidant-free radicals, viral infection or expression of certain viral or bacterial gene products, ultraviolet (UV) irradiation, B or T cell activation, and

other physiological and non physiological stimuli. The most potent NF-κB activators are the proinflammatory cytokines IL-1 and TNF-α, which cause rapid phosphorylation of κBs at two sites within their N-terminal regulatory domain. TNF binds to its receptor and recruits a protein called TNF-associated receptor death domain (TRADD). TRADD binds to the TNF-receptor-associated factor 2 (TRAF2) that recruits NF-κB-inducible kinase (NIK), which phosphorylates IKK.

5-3. The mechanisms of NF-κB activation

5-3-1. The role of IκB kinases

Phosphorylation of IκB by the IKK (IκB kinase Complex) leads to translocation of NF-κB. The IKK is composed of three subunits: two of them, IKKα (IKK1) and IKKβ (IKK2), are kinases, while the third, IKKγ (NEMO), has no catalytic activity but plays a critical regulatory role. IKKα is the predominant IKK.

5-3-2. The NF-κB signaling pathway

More than 150 extracellular signals can lead to activation through the dissociation of NF-κB from the IκB proteins. These signal transduction pathways lead to the activation of the IKK, which in turn phosphorylates two specific serine residuals on IκB proteins (S32 and S36) [Ghosh et al, 1998]. Phospho-IκB is then recognized by the β-transducin repeat-containing protein (β-TrCP)-containing skp1-cullin 1-f-box protein complex (SCF) ubiquitin ligase complex ($SCF^{\beta\text{-}TrCP}$), leading to its ubiquitination and degradation by the proteasome. The destruction of IκB unmasks the NLS of NF-κB, leading to its nuclear translocation and binding to the promoters of target genes [Read et al, 2000]. Recently, more and more evidence suggests that the phosphorylation and degradation of IκB and the

consequent liberation of NF-κB are not sufficient to activate NF-κB-dependent transcription. A second level of regulation of NF-κB activity relies on phosphorylation of members of the second group of NF-κB proteins (p65/RelA, RelB, and c-Rel) which results in the activation of transcriptional activity of NF-κB [Zhong et al, 1997; Wang et al, 1998; Zhong et al, 1998; Sizemore et al, 1999; Madrid et al, 2000; Wang et al, 2000; Madrid et al, 2001; Silverman et al, 2001].

5-3-3. The regulation of NF-κB transcriptional activity

In addition to the signal-induced liberation of NF-κB from IκB and the consequent nuclear localization of NF-κB, the transcriptional activity of NF-κB is also regulated in response to stimulation. The NF-κB p65 protein has a site for phosphorylation by protein kinase A (PKA) on serine 276, and phosphorylation of this residue is required for efficient binding to the transcriptional activator protein cAMP response element binding (CREB)-binding protein (CBP). The catalytic subunit of PKA was shown to be bound to inactive NF-κB complexes which leave from IκB and, to phosphorylate p65, resulting in a conformational change of p65 and consequent interaction with CBP [Zhong et al, 1997]. Moreover, TNF-α treatment of cells results in phosphorylation of Ser 529 in the transactivation domain of p65, resulting in activation of its transcriptional activity. Recently, casein kinase II (CKII) was implicated in the TNF-α-dependent phosphorylation of Ser 529 [Wang et al, 1998; Wang et al, 2000]. IκBα protects p65 from phosphorylation by constitutively active CKII, but signal-dependent degradation of IκB exposes the p65 phosphorylation site to CKII activity. Thus, once released from IκB, at least two kinases, PKA and CKII, phosphorylate p65 at different serine residues, to increase its transcriptional activity.

On the other hand, phospatidylinositol-3-kinase (PI3K) and Akt have been shown to be

required for IL-1- and TNF-α-induced NF-κB activity. IL-1 and TNF-α induce the activation of PI3K and Akt, which lead to the phosphorylation and activation of p65. Akt has also been implicated in ras-induced NF-κB activation, through the activation of IKKβ and phosphorylation of p65 at Ser 529 and 536 [Madrid et al, 2000; Madrid et al, 2001]. Recently, IL-1-stimulated PI3K/Akt activation has also been implicated in the phosphorylation of the p50 NF-κB subunit, which increases the DNA-binding capacity of the NF-κB complex [Koul et al, 2001].

Radiation induces the activation of NF-κB through IKK-dependent pathway. After γ rays results in degradation of IκB and nuclear entry of NF-κB. The radiation depends on the phosphorylation of IκB at Ser 32 and 36, the IKK phosphoacceptor sites of IκBα. Therefore, radiation leads to IKK activation [Prasad et al, 1994; Li and Karin, 1998]. Stress-induced NF-κB signaling pathway is shown in Figure 6.

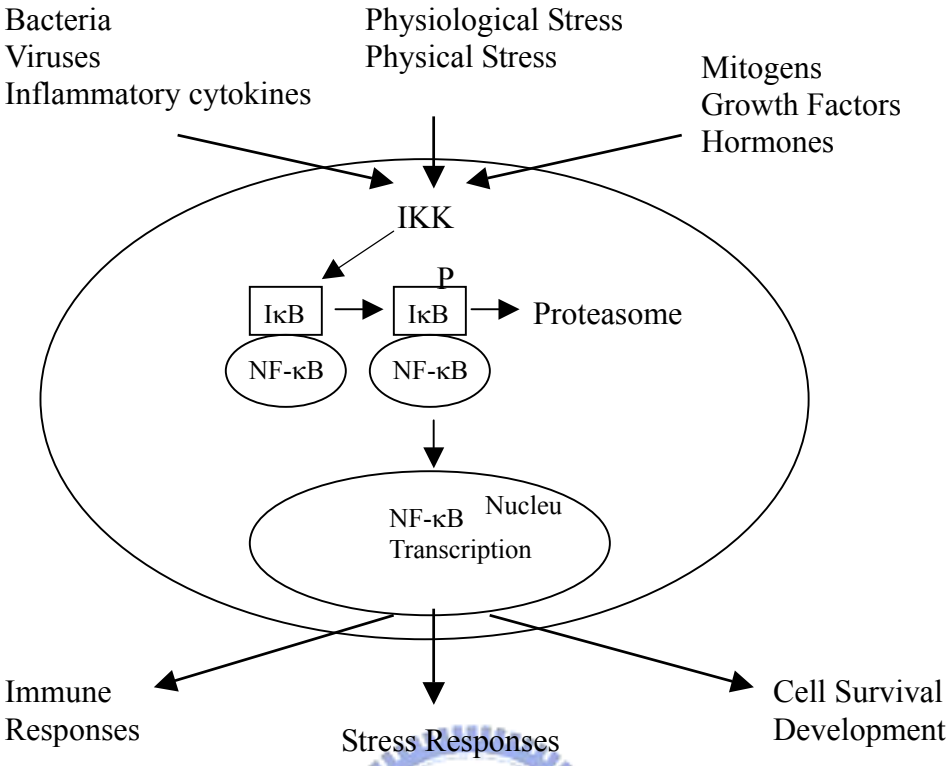

Figure 5. Some of the inflammatory stimuli activate NF-κB, and NF-κB itself induces various kinds of responses [Li and Stark, 2002]**.**

It is established by the interactions of endothelial cells and leukocytes via cell adhesion molecules play an important role for leukocyte recruitment in atherogenesis. Besides selectins, the adhesion molecules of the immunoglobulin family ICAM-1 and VCAM-1 are involved in the inflammatory responses.

5-4. Adhesion molecules

5-4-1. The biological function of ICAM-1

ICAMs bind to lymphocyte function-associated antigen-1 (LFA-1) [Staunton et al, 1990] or Mac-1 [Smith et al, 1989] and mediate the adhesion of monocytes, lymphocytes, and

neutrophils to endothelial cells. Five ICAM molecules have been identified. These Ig-like domains are expressed on the extracellular portion of the protein. In general, ICAMs span the cell membrane and contain only a short cytoplasmic tail [Springer et al, 1990].

Sequence analysis of the ICAM-1 from -227 to -136 of the promoter revealed potential binding sites for four transcription factors, such as Sp1 at the region from -206 to -201 of ICAM-1 [Dynan et al, 1983], CAAT/enhancer-binding protein (C/EBP) from -198 to -195 [Graves et al, 1986], ets from -153 to -150 [Macleod et al, 1992].

5-4-2. The biological function of VCAM-1

The binding of VCAM-1 to very late antigen-4 (VLA4) ($\alpha_4\beta_1$) has been implicated in numerous physiologic and pathophysiologic processes involving cell-cell adhesion [Springer et al, 1990]. On activated endothelium, VCAM-1 supports the adhesion of lymphocytes [Carlos et al, 1990; Elices et al, 1990], monocytes, eosinophils, basophils, natural killer cells, and certain tumor cells, particularly malignant melanoma and osteosarcoma [Allavena et al, 1991; Bochner et al, 1991; Lauri et al, 1991]. Adhesion of VLA4-expressing cells to endothelium has been implicated in the pathogenesis of many diseases and disease processes, including rheumatoid arthritis, osteoarthritis, allogeneic graft rejection, graft-versus-host disease, encephalomyelitis, delayed-type hypersensitivity reactions, tumor metastasis, and atherogenesis [Cybulsky et al, 1991; Morales-Ducret et al, 1992; Norton et al, 1992; Pelletier et al, 1992; Yednock et al, 1992]. VCAM-1 has also been identified on a variety of nonvascular cell types, including follicular dendritic cells, bone marrow stromal cells, secondary myoblasts, some macrophages, certain renal epithelial cells, fibroblast-like synoviocytes, reactive mesothelial cells, stimulated astrocytes, and stimulated cortical neurons [Rice et al, 1991; Rosen et al, 1992; Simmons et al, 1992]. Roles for VCAM-1 in germinal center development, lymphopoiesis, and myogenesis have been suggested [Freedman et al,

1990].

The VCAM-1 promoter contains two tandem κB sites located in close promixity to an IFN-stimulated response element (ISRE) site [Iademarco et al, 1992; Neish et al, 1992]. Recent studies have shown that interferon regulatory factor 1 (IRF-1) synergizes with NF-κB in transactivating the VCAM-1 gene [Neish et al, 1995].

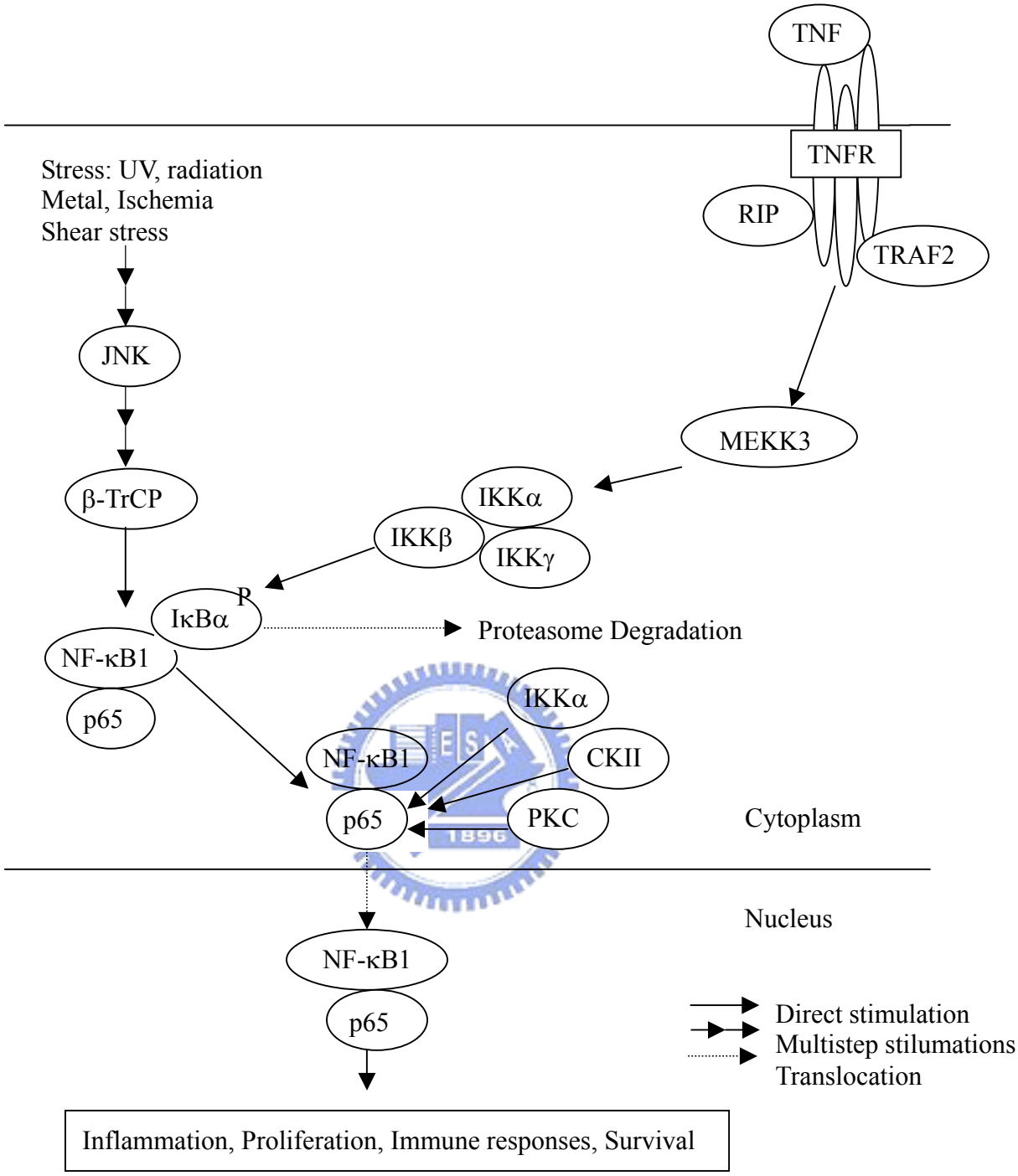

Figure 6. Stress-induced NF-κB signaling pathway. Proinflammatory cytokines, LPS, growth factors and antigen receptors activate IKK complexes, which phosphorylate IκB proteins. Phosphorylation of IκB leads to its ubiquitination and proteasomal degradation and translocation of NF-κB/Rel complexes, either alone or in combination with other transcription factor families. [http://www.cellsignal.com/]

5-5. Characteristics of AP-1 regulation

5-5-1. Components of AP-1 activation

The proto-oncogenes c-Fos and c-Jun encode nuclear transcription factors involved in the transmission of inter and intracellular information through multiple signal transduction pathways [Karin and Smeal, 1992; Kerppola and Curran, 1995]. c-Fos and c-Jun are members of a multigene family implicated in a number of signal transduction cascades associated with growth, differentiation, neuronal excitation, and cellular stress. AP-1 is a sequence-specific transcription factor composed of either homo or heterodimers between members within the c-Jun (c-Jun, c-JunB, and c-JunD) and c-Fos (c-Fos, FosB, Fral, and Fra2) families [Kerppola and Curran, 1995]. Gene products from the Fos and Jun families are known as leucine zipper proteins, as they dimerize via a cluster of basic leucine residues [Turner et al, 1989]. Various AP-1 proteins have different transcriptional properties because its specific activation and repression domains of the AP-1 complex may contribute to its regulatory function. In addition, binding by c-Jun homodimers or c-Fos-c-Jun heterodimers produces distinct DNA bending resulting in specific protein-protein interactions among the AP-1 complex and other promoter-bound transcription complexes [Abate et al, 1991]. Furthermore, the c-Jun and c-Fos proteins contain several transcriptionally active regions, including several autonomous transactivation domains, a C-terminal transrepression domain, and a region that interacts with the TATA box binding protein [McBride and Nemer, 1998]. The exact mechanisms that regulate assembly, targeting, and functional specificity of the different AP-1 complexes remain unclear, although differential gene expression, post translational modification, conformational alterations, and altered DNA-binding specifities of heterodimers may all be involved [Xanthoudakis and Curran, 1996].

An interesting observation in Figure 7 is that c-Fos is a downstream gene for the signal transducer and activator transcription (STAT), CREB, ternary complex factor (TCF) and

AP-1 transcription factors. Unlike NF-κB, STAT, CREB and TCF transcription factors which are constitutively present within cells, activation of the AP-1 family of transcription factors is regulated at the transcriptional level. NF-κB lies in the cytoplasm but is passed from the cytoplasm to the nucleus after stimulation. In contrast, the c-Fos-c-Jun family proteins are restricted to the nucleus. The activation of AP-1 involves in mitogen activated protein kinase (MAPK) pathways through messages between these subcellular compartments [Karin and Smeal, 1992]. In addition to phosphorylation, this may be achieved via a series of redox sensitive factors. Thus, it appears that cytoplasmic kinase and protein phosphorylation are involved in the activation of AP-1 [Xanthoudakis et al, 1994].

The Fos and Jun components of AP-1 transcription factors are known as early response genes because they are rapidly, but transiently transcribed in response to extracellular stimuli such as growth factors and cell stress. As shown in Figure 7, there are four regulatory elements in the c-Fos promoter, the cis-inducible element (SIE), CRE, serum response element (SRE) which binds to TCF transcription factors, and the 12-O-tetradecanoyl-phorbol-13-acetate response element (TRE) to which AP-1 itself binds [Wang and Howells, 1994]. For the c-Jun gene, which represents the first identified member of the Jun gene family, there are two TREs in the promoter that preferentially bind a heterodimeric protein consisting of activating transcription factor (ATF) and c-Jun.

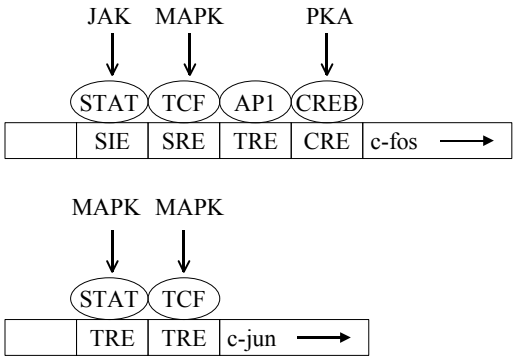

Figure 7. Promoter regions of c-Fos and c-Jun genes showing response elements and the protein kinase/transcription factors that activate each element [Yates and Rayner, 2002**].**

5-5-2. The regulation of AP-1 in response to extracellular stimulus

5-5-2-1 The role of AP-1 in wound healing

AP-1 is used as a wound-induced transcription factor [Okada et al, 1996]. Many downstream genes containing the AP-1-binding TREs in their promoters produce key proteins involved in wound healing, and more specifically reepithelialization. These include TGF-β1, matrix metalloproteinases (MMPs) such as collagenase, stromelysin and gelatinase B, and the adhesion molecules α_2 integrin, α_6 integrin, β_4 integrin and laminin α_3A [Gum et al, 1997; Lewis et al, 1999]. In previous study rapid activation of the AP-1 transcription factor occurs following injury, potentially via Ca^{2+} and the MAPKs, in keratinocytes.

5-5-2-2. The redox regulation of NF-κB and AP-1

Similar to NF-κB, it appears that chemicals or agents that alter the cellular oxidation/reduction status also affect the activity of the AP-1 transcriptional complex [Mohan and Meltz, 1994; Sen and Packer, 1996]. While initial work suggested that oxidants induce AP-1 and NF-κB, AP-1 is also strongly induced by some antioxidants [Dalton et al, 1996]. For example, pretreatment of cells with the antioxidants N-acetyl-L-cysteine or pyrrolidine dithiocarbamate (PDTC) prevents cytokine-induced NF-κB activation.

The same transcription factors are also found to be regulated by oxidative stress, although the evidence has shown both positive and negative regulation of NF-κB. Besides, transcription factor NF-κB is regulated through interaction with inhibitory proteins, the IκBs. Many diverse stimuli including TNF-α, IL-1, LPS and IR activate NF-κB through induction of IκB phosphorylation, they all operate through a common protein (IKK). Stress-induced activation of these early response genes appears on changes in intracellular oxidation/reduction (redox).

5-5-2-3. The role of AP-1 in other stimuli

Changes in the expression of the c-Jun gene have been observed in a number of experimental systems. For example, it is elevated in cells following serum withdrawal and in cells irradiated with UV or IR [Gillardon et al, 1994; Hallahan et al, 1995]. In addition, a number of chemotherapeutic drugs, as well as TNF-α, all of which trigger apoptosis, also induce an early increase in c-Jun [Singh et al, 1995]. Furthermore, c-Jun levels are elevated in irradiated neurons in the developing brain and in neurons treated with amyloid peptide [Anderson et al, 1995].

5-5-3. Signaling pathways that regulate AP-1 activity

AP-1 activity is induced by growth factors, cytokines, neurotransmitters, polypeptide hormones, cell-matrix interactions, bacterial and viral infections, and a variety of physical and chemical stresses. These stimuli activate MAPK cascades that enhance AP-1 activity through the phosphorylation of distinct substrates. That serum and growth factors potently induce AP-1 by activating the extracellular-signal-regulated kinase (ERK) subgroup of MAPKs leading to the transcriptional activity of TCFs and binding to Fos promoters [Hill et al, 1994]. Furthermore, the ERKs directly phosphorylate Fra1 and 2 in response to serum stimulation, possibly enhancing their DNA binding in conjunction with c-Jun [Gruda et al, 1994]. An additional contribution to AP-1 induction by serum growth factors comes from ERK5-induced phosphorylation of the transcription factor monocyte specific enhancer binding factor 2c (MEF2C), whose activation increases c-Jun expression [Kato et al, 1997].

The induction of AP-1 by proinflammatory cytokines and genotoxic stress is mostly mediated by the c-Jun amino terminal kinase (JNK) and p38 MAPK cascades. Once activated, the JNKs translocate to the nucleus, where they phosphorylate c-Jun and thereby enhance its transcriptional activity [Karin, 1995]. The JNKs also phosphorylate and

potentiate the activity of ATF2**,** which heterodimerizes with c-Jun to bind divergent AP-1 sites in the c-Jun promoter. Importantly, the induction of c-Jun expression by certain genotoxic stresses, such as short-wavelength UV radiation, is more persistent than the induction seen after mitogenic stimulation [Shaulian et al, 2000].

Therefore, AP-1 activation has an important role in the control of cell proliferation and differentiation. However, activation of NF-κB can lead to gene expression involved in inflammatory responses.

6. Expression of molecules on SMCs in human diseases

Increased levels of ICAM-1 and VCAM-1 are a promising feature of the formation of the atherosclerotic plaque. A functional NF-κB binding site has been shown to be necessary for optimal expression of the adhesion molecules [Collins, 1993]. An activated form of NF-κB has been identified in SMCs within atherosclerotic lesions [Brand et al, 1996]. Inhibition of NF-κB activation can alleviate expression of VCAM-1 in endothelial cells after the treatment of antioxidant [Marui et al, 1993]. One working hypothesis is that activation of NF-κB by oxidative mechanisms and inflammatory cytokines may serve to initiate and perpetuate atherosclerotic lesions.

ICAM-1 expression could not be detected on SMCs in the normal adult aorta, whereas expression of this adhesion molecule was observed on SMCs in the intima of atherosclerotic lesions [Printseva et al, 1992; Davies et al, 1993]. In addition, ICAM-1 expression was occasionally seen in some medial SMCs adjacent to atherosclerotic plaques. Similarly VCAM-1 expression was detected on intimal SMCs in atherosclerotic coronary arteries as well as in aorta and carotid arteries [O'Brien et al, 1993; Bobryshev et al, 1996]. Several investigations demonstrated a correlation between ICAM-1 and VCAM-1 expression on SMCs and mononuclear cell infiltration suggesting that mediators derived from these

mononuclear cells may contribute to the induction of adhesion molecules in SMC in vivo [O'Brien et al, 1993; Bobryshev et al, 1996; O'Brien et al, 1996]. ICAM-1 and VCAM-1 expression on SMCs in the intima and media were most prominent in fibrous plaques and advanced atherosclerotic lesions.

In the rabbit aorta, induction of ICAM-1 expression on endothelial cells was observed 2 days after balloon injury. In neointimal SMCs, however, a marked expression of ICAM-1 was detected after 5 to 10 days and remained constant for at least 30 days after the injury [Tanaka et al, 1993]. In contrast to the strong endothelial expression of VCAM-1 in this model, neointimal SMCs demonstrated only a weak immunostaining.

Balloon injury of rat carotid arteries resulted in a strong expression of ICAM-1 on medial SMCs after 1 and 2 days [Yasukawa et al, 1997]. The expression of ICAM-1 on neoinitimal SMCs and regenerating endothelial cells was observed later, 5 to 7 days after the injury. At this time-point, medial ICAM-1 expression was decreased [Yasukawa et al, 1997]. Therefore, data on the expression of adhesion molecules on SMCs in restenosis came exclusively from animal studies.

7. NF-κB, ICAM-1 and VCAM-1 in response to radiation

Cellular responses to IR include activation of signal transduction cascades that may originate at the plasma membrane, cytoplasm, or nucleus. The cell's success in dealing with radiation "stress" determines its survival or death. Activation of transcription factor NF-κB, an immediate early response after exposure to IR, functions to protect cells from apoptosis (programmed cell death).

NF-κB is induced after exposure to IR as well as UV radiation. For example, previous studies had shown that almost all cells studied in vitro response to radiation increased the expression of NF-κB. However, in vivo studies have been undertaken concerning

long-lasting effects of radiation on the transcription of NF-κB. Besides, in vivo exposure of mice to various doses of whole body radiation does not cause a global activation of NF-κB [Zhou et al, 1999]. Recently an in vitro study showed different dose and time dependent expression patterns of various adhesion molecules on endothelial cells during IR.

8. Gene expression by microarray

It is technically impossible to verify all the expression data by other means such as northern blotting, RNase protection assay or PCR. Microarray is suitable for studying the expression patterns of large numbers of genes; however, it is a costly technique. Except for high throughput technology, competitive hybridization of dual labeled microarray samples (Cy5/Cy3) is a predominant method employed in gene expression analysis. This technique eliminates variables associated with multiple microarrays, hybridizations, and washings associated with single channel expression array techniques. In addition, the different intensity of dual-color samples is examined by the log ratio in intra-array and inter-array. These comparisons display the relative merits of the two different labeling systems with regard to data reproducibility and confidence.

Every cell on organism contains a full set of chromosomes and identical genes with a few exceptions. Only a subset of these genes is active at a given time. These gene expressions define the unique properties of a cell type. Gene expression describes the transcription of information and translation of process in which messenger RNA (mRNA) molecules that are then translated into proteins, performing most of the critical functions of cells. Biologists study various kinds and amounts of mRNA produced by a cell in order to learn which genes are expressed. Gene expression is a complex regulated web that allows a cell to respond dynamically under different environmental stress conditions. Therefore, it is necessary to control the level of expression of particular genes in specific cells. The study of gene

expression helps to understand fundamental aspects of growth and development and underlying genetic causes of many human diseases [http://www.microarrays.org]. Microarrays help to monitor the expression of many genes in parallel [Schena et al, 1995].

8-1. The introduction of microarray

A microarray employs the ability of a given mRNA molecule to bind specifically to, or hybridize to, the DNA template from which it is originated. It contains many DNA sequences, and the expression levels of thousands of genes can be determined in a single experiment by measuring the amount of mRNA bound to each site on the array [http://www.tigr.org]. Microarrays are small glass slides or nylon membranes onto which the gene sequences are printed, spotted or synthesized. The sequences can be DNA, cDNA or oligonucleotides.

The relative abundance of each in two RNA samples (test and reference) may be estimated by fluorescently labeling the samples. After the step of labeling, samples are mixed, and the mixture hybrid to the sequences on the glass slides. The two samples of mRNA from cells (target) are reverse transcribed into cDNA, and labelled using two different dyes (red fluorescent dye, Cy5, and green fluorescent dye, Cy3, in general). Usually the reference sample is labeled with Cy3 and the test sample with Cy5. The mixture reacts with the spotted cDNA sequences (probes). This, called competitive hybridization, results in probes which are base-pairing with targets. After this hybridization step is complete, the microarray is placed in a scanner, consisting of lasers with different wavelengths, a microscope and a camera. Laser light excites the fluorescent dyes (Cy3 is excited by green laser light at 532nm and Cy5 is excited by red laser light at 635nm). Usually, the raw wavelength images are collected at 16-bit resolution, giving fluorescence intensity measurements for each sample for each spot. The channel intensities for any spot should be proportional to the amount of

mRNA from the corresponding gene in the respective sample. Because the absolute channel intensities are usually less reproducible, the intensity ratios of Cy5/Cy3 generated from hybridization are indicative of gene expression level and are used for further analysis.

Microarray experiments are large-scale experiments and costly. Careful design is important if the results should be maximally informative, given the effort and the resources. Which issues need to be addressed, and which features have an impact on the resulting measurements are important.

A survey of the programming tools and an overall view of visualization, normalization, analysis and organization of microarray data are as follows.

Since microarray experiments include multiple slides, the data should be treated with experimental design. The user should be able to organize the uploaded slides into experiment classes (i.e., biological conditions and different time-points) and to provide all the information needed for normalization. Before starting all steps of normalization, files are prepared from image analysis software, containing all essential data. Files will contain information and will report in the format prescribed by the GAL. Result of files from different software vendors (e.g. GenePix, Agilent) should be supported.

The goal of image analysis is to provide foreground and background intensity values for the red and green channels for each spot on a microarray [http://www.axon.com]. Besides, quality measures of each spot are collected for marking weak or unreliable spots. Some issues of image acquisition highly affect further analytic steps like normalization [Smyth et al, 2003].

An optical scanner scans the array, recording the fluorescence emissions at each point on the slide. One scan for each channel (Cy5 and Cy3) is performed and the data is stored into two 16-bit TIFF (Tagged Image File Format) images. To avoid saturated pixels, emitting more photons than the photomultiplier tube (PMT), the PMT-voltage can be adjusted so that

the brightest pixels are below the scanner saturation. However, PMT settings are sometimes a compromise between avoiding saturated pixels and getting weak sensitivity for less intense pixels.

Certain spots should be ignored during later analysis because of defects on the slide, saturation, small signal intensities etc. Most microarray acquisition programs have the ability to flag such spots. For example, GenePix Pro lets the user define special flagging criteria which can be saved to be applied to every microarray. Spots not suitable to the conditions would be marked as bad and therefore excluded for later steps [Axon Instruments].

In order to accurately and precisely measure gene expression, it is important to consider control experimental and systematic variations that can occur in every microarray experiment. For example, a source of systematic variations can be biases associated with different fluorescent dyes.

The purpose of the normalization step is to identify and remove any systematic bias in the measured fluorescence intensities, arising from variation in the microarray process rather than from biological differences between the RNA samples. From the sources of systematic variation the most important bias is associated with the different fluorescent dyes. Dye biases can stem from a variety of factors, including physical properties (heat, light sensitivity, half life), efficiency of dye incorporation, or scanner settings. Other artifacts result from the robotic printing process, hybridization including contamination in background and any other spatial effects that are introduced during the production and use of the microarrays. All these factors make distinctive effects expression of genes. Therefore, biological differences can be easily distinguished, as well as to allow the comparison of expression levels across slides. A wide choice of common normalization methods should be offered to the user to remove the systematic errors within and between arrays (e.g. Lowess fit, using control spots, scaling and averaging within and between slides).

Because visualization of microarray data is the best help for choosing the way to normalize experiments, common used plots (e.g. scatterplot, histogram, MA plot, boxplot) should be implemented. Besides, one aim of microarray technology is to search new target genes.

8-2. The application of DNA microarray in CVDs

Inflammation is an inherent response of the organism that permits its survival despite constant environmental challenges. The process normally leads to recovery from injury to healing. The role of inflammatory cytokines or growth factors with inflammatory reactivity has gained increasing attention in atherosclerosis and restenosis [Ross, 1999; Libby et al, 2002].

There are several examples, which show various kinds of genes involved in vascular injury by microarray and transitional methods in previous studies. Table 6 gives the summary of applications of expression profiling to CVDs analyzed by cDNA microarray. The main contributors to the risk for atherosclerosis include lipoprotein, homocysteine, hypertension, diabetes, and oxidant stress [Libby et al, 2002]. In the present study, IL-1β contributed to VSMC proliferation and lesion progression in atherosclerosis [Nathe et al, 2002]. Allograft inflammatory factor-1 (AIF-1) was associated with VSMC activation and vascular injury [Chen et al, 2004]. Besides, apolipoprotein J (ApoJ) was expressed during tissue injury and remodeling and has been implicated in VSMC differentiation [Sivamurthy et al, 2001]. Early growth response factor 1 (Egr-1) is a key transcriptional factor to mediate gene expression after vascular injury in endothelial cells. In addition, functionally related genes for tristetraproline (TTP), selectin E, MMP 9, and TNF-α were highly upregulated at the late phase of myocardial ischemic preconditioning by microarray [Zubakov et al, 2003].

The mechanism of restenosis still remains unclear for several decades. In this study, we had taken advantage of large-scale gene expression arrays to detect gene expression and

identify upregulatedly- and downregulatedly-candidate genes potentially responsive to angioplasty, in the exposure of brachytherapy, angioplasty prior to brachytherapy, and brachytherapy after angioplasty. According to my data, the technology of microarray will reveal novel genes related to surgical treatments, including PTCA and/or barchytherapy. We plan to understand the mechanisms of restenosis in details detected by cDNA microarray and select the interest genes for further analysis. After the double check of RT-PCR or Northern blot, we may understand restenosis in the field of CVDs and will have more applications to improve CVDs in the future.

Table 6. Summary of applications of expression profiling to CVDs by cDNA microarray

Diseases	Examples of genes identified by expression profiling	Tissue culture	Reference
Atherosclerosis	↑ Expression of IL-8,MCP-1,VCAM-1, PAI-1, VE-cadherin and Gro-α in activated compared to quiescent ECs	SAGE analysis of human ECs activated with conditioned monocyte medium	De Waard et al, 1999
	↑ Expression following incubation with oxidized LDL: adipophilin, heparin-binding epidermal growth factor like growth actor, thrombomodulin, CD73 and nuclear receptors LXRα,RXRα, and PPARγ	The human THP-1 macrophage cell line response to oxidized LDL was characterized using a DNA array.	Shiffman et al, 2000
Restenosis	↑ Expression: manganese superoxide dismutase and cyclooxygenase-2 in response to laminar shear stress	Differential display analysis of ECs exposed to laminar shear stress.	Topper et al, 1996
	↑ Expression with strain: cyclooxygenase-1, tenascin-C and plasminogen activator. ↓ Expression of MMP-1 and thrombomodulin	DNA array analysis of human VSMC responses to biaxial cyclic strain	Feng et al, 1999
	Identification and cloning of gene: 2A3-2, homologous to human translational elongation factor in highly proliferating VSMCs	Subtractive hybridization analysis was employed to compare a highly proliferating rat VSMC line with primary VSMC in culture.	Zibara et al, 1999