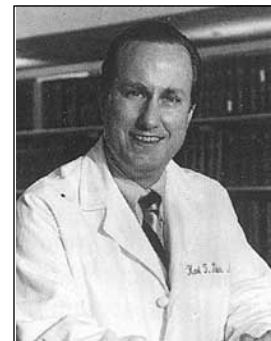


# Aldosteronism in Heart Failure: A Proinflammatory/Fibrogenic Cardiac Phenotype. Search for Biomarkers and Potential Drug Targets

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**Abstract:** Heart failure is a major health problem of epidemic proportions. Irrespective of its etiologic origins, a dysfunction of this normally efficient muscular pump is associated with systemic consequences, a progressive downhill clinical course and poor prognosis. Ventricular dysfunction is ultimately accompanied by neurohormonal system activation that accounts for: the congestive heart failure syndrome; an induction of oxi/nitrosative stress; adverse vascular remodeling; and activation of the immune system that contributes to a wasting syndrome known as cardiac cachexia. Circulating effector hormones of the renin-angiotensin-aldosterone system are an integral feature of this neurohormonal activation; they have systemic consequences. Insights into the pathophysiology of heart failure will identify improved methods of prevention, including biomarkers to aid in its detection and identification of risk, and to the development of specific drug targets. Herein we address one aspect of the neurohormonal profile of heart failure, namely that related to aldosteronism. Our focus is directed at the link between aldosteronism and its adverse influence on coronary vasculature structure, a proinflammatory/fibrogenic cardiac phenotype, which is based on an immunostimulatory state that includes activated peripheral blood mononuclear cells.

## INTRODUCTION

Heart failure is a major health problem of epidemic proportions. Among the elderly, symptomatic heart failure represents the number one cause for hospitalization. Irrespective of its etiologic origins, a dysfunction of this normally efficient muscular pump during either its ejection or filling phases (i.e., systolic and diastolic dysfunction, respectively) is associated with systemic consequences and a progressive downhill clinical course that includes compromised quality of life and poor prognosis. Is this due solely to a failure of the heart? No. Ventricular dysfunction is ultimately accompanied by neurohormonal system activation that accounts for renal and intestinal salt and water retention and the congestive heart failure syndrome that involves lungs, liver and peripheral tissues. Another outcome is a progressive systemic illness that features a wasting of tissues, including bone. Insights into how the pathophysiologic progression of chronic cardiac failure with elevated circulating levels of various neurohormones eventuates in systemic consequences will identify improved methods of prevention, including new biomarkers to aid in its detection and identification of risk, and to newer drug management strategies. This approach would be analogous to the current monitoring of plasma lipid

profile to predict risk of atherosclerosis and which led to the development and introduction into clinical practice of HMG-CoA reductase inhibitors (or statins) that effectively reduce risk through their lipid lowering and nonlipid properties. Herein we address one aspect of the neurohormonal profile of heart failure, namely that related to aldosteronism, defined as chronic elevations in plasma aldosterone (ALDO) inappropriate for dietary Na<sup>+</sup> intake. Our focus will be on the link between aldosteronism and its adverse influence on coronary vasculature structure, a proinflammatory/fibrogenic cardiac phenotype, which is based on an immunostimulatory state that includes activated peripheral blood mononuclear cells (PBMC).

## PATHOPHYSIOLOGY OF HEART FAILURE. BEYOND THE CARDIOCENTRIC PERSPECTIVE

The pathophysiologic scenario that involves systemic organs and tissues originates from a chronic activation of neurohormonal systems of which the renin-angiotensin-aldosterone system (RAAS) and its circulating effector hormones are integral. Aside from their well known influence on vascular tonicity and renal salt and water handling, systemic effects of angiotensin (Ang) II and ALDO include: an induction of oxidative stress, evident within the heart, skeletal muscle, PBMC, and plasma [1-3]; adverse vascular remodeling [4,5]; and activation of the immune system with elevated plasma levels of such proinflammatory cytokines as TNF- that contribute to a

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progressive wasting of bone, fat and lean tissues and which eventuates in a wasting syndrome known as cardiac cachexia [6]. In persons with known ventricular dysfunction, the identification of a) sensitive and specific biomarkers that reliably predict the appearance of this systemic illness, and b) drug targets that would prevent it are therefore of considerable importance.

### **Aldosteronism and Vascular Lesions in Man**

The most common cause of human heart failure is that of ischemic origin, known as an ischemic cardiomyopathy (ICM). Cotran [7] has noted that while an infarct scar is invariably present in ICM, it is a perivascular fibrosis of intramural coronary arteries and microscopic scarring, each appearing at sites remote to the infarct scar, that represents its most characteristic feature. Beltrami *et al.* [8] concluded that it is this accumulation of fibrous tissue remote to the infarct that represents the major component to adverse structural remodeling in the explanted failing human heart with ICM. The cause of such vascular remodeling remote to the infarct raises the specter of a circulating substance(s). Activation of the circulating RAAS is integral to congestive heart failure [4,9-11] and its effector hormones are known to promote such remodeling. To address a role for ALDO in promoting such remodeling Campbell *et al.* [12] examined postmortem tissue obtained from cases of primary aldosteronism and autopsy-proven adrenal adenoma. They found vascular remodeling in the heart and systemic organs. As in ICM, the same perivascular fibrosis involving intramural arteries and arterioles of the heart (and various organs) was found. Experimental animals provide an opportunity to systematically address a role for ALDO and Na<sup>+</sup> in promoting this remodeling.

### **Aldosteronism and Vascular Lesions in Experimental Animals**

In 1946, Hans Selye suggested chronic exposure to environmental stress results in several responses that eventuate in systemic hypertension. He termed it an *adaptation syndrome* [13]. Featured were a structural remodeling of intramural arteries and arterioles of the heart and systemic organs. Because these vascular lesions included inflammatory cells and fibroblasts, he referred to them as “perivascular granulomas.” One form of stress Selye routinely employed to invoke this syndrome was chronic treatment of uninephrectomized rats with a mineralocorticoid (MC), deoxycorticosterone acetate (DOCA), together with dietary NaCl. Multiple observations led him to conclude that it was the Na<sup>+</sup> ion which was essential to “renal and cardiovascular actions of DOCA” and that hypertension was a “secondary” phenomena to these vascular lesions [13-15]. In 1965, Hall and Hall [16] reported a similar, widespread vascular remodeling in uninephrectomized rats treated with ALDO and 1% NaCl. They noted a “polyarteritis” with “infiltrated inflammatory cells” to involve intramural arteries of the heart, pancreas, mesentery, and kidneys, together with nephrosclerosis and glomerular lesions, and found dietary Na<sup>+</sup> as the monovalent cation integral to such iterations in vascular structure. Selye further identified the cardiopro-

TECTIVE role of “antimineralocorticoid-spirolactone” in various stress-related models of cardiac injury [17]. Masson *et al.* [18,19] observed an accelerated vascular remodeling with cardiovascular complications (e.g., hemorrhage), when renin was given in combination with either DOCA or ALDO. Selye concluded that while the properties of MC were prothrombotic, they were normally opposed by the antithrombotic properties of glucocorticoids [13]. From a teleologic perspective, the notion of a reciprocal regulation between these adrenal steroids and inflammation is attractive [20]. In this connection, it further has been suggested that MC have a biologic economy of action in promoting both sodium retention and wound healing after serious tissue injury with hemorrhage [21].

The model we and others have used to address the effects of ALDO (and Na<sup>+</sup>) on vascular remodeling employs uninephrectomized rats receiving ALDO by an implanted minipump (0.75 µg/h), together with 1% dietary NaCl supplemented with 0.4% KCl to avoid potassium deficiency. This regimen, hereafter referred to as ALDO/salt treatment (ALDOST), raises plasma ALDO levels to those seen in CHF [22]. However, unlike the secondary aldosteronism of human CHF, plasma renin and angiotensin II are rapidly suppressed by ALDOST. This difference notwithstanding, the model offers several advantages. First, it permits an assessment of the consequences of elevated plasma ALDO in the absence of circulating AngII, whose levels are chronically suppressed. Second, the model does not include prior myocardial injury before starting ALDOST. This is contrary to the human scenario of heart failure, but allows us to address the appearance of cardiac pathology secondary to ALDOST and to not have a confounding variable, such as an infarct scar, which we and others have shown to be living tissue that features a dynamic population of myofibroblasts that contributes to an ongoing structural remodeling of myocardium at and remote to the site of previous infarction [23,24]. Third, by avoiding previous cardiomyocyte necrosis with the release and/or exposure of normally sequestered antigenic constituents that can cause a proliferation of antigen-recognizing T cells [25], we can address the interaction of ALDOST with the immune system. Fourth, the model consists of several clinicopathologic stages: during wks 1 and 2, rats are active, eating, drinking and gaining weight (*preclinical* stage); at wk 3, they have become anorectic, lethargic and fail to gain weight (a *clinical* stage); at wk 4, animals remain ill and cardiac lesions now appear (a *pathologic* stage). These lesions include: a proinflammatory vascular phenotype that features monocytes/macrophages and lymphocytes that invade intramural coronary arteries; and phenotypically transformed fibroblasts, termed myofibroblasts, expressing type I and III fibrillar collagens that leads to a perivascular fibrosis [26-30].

### **Vascular Remodeling: Hormonal vs. Hemodynamic Factors**

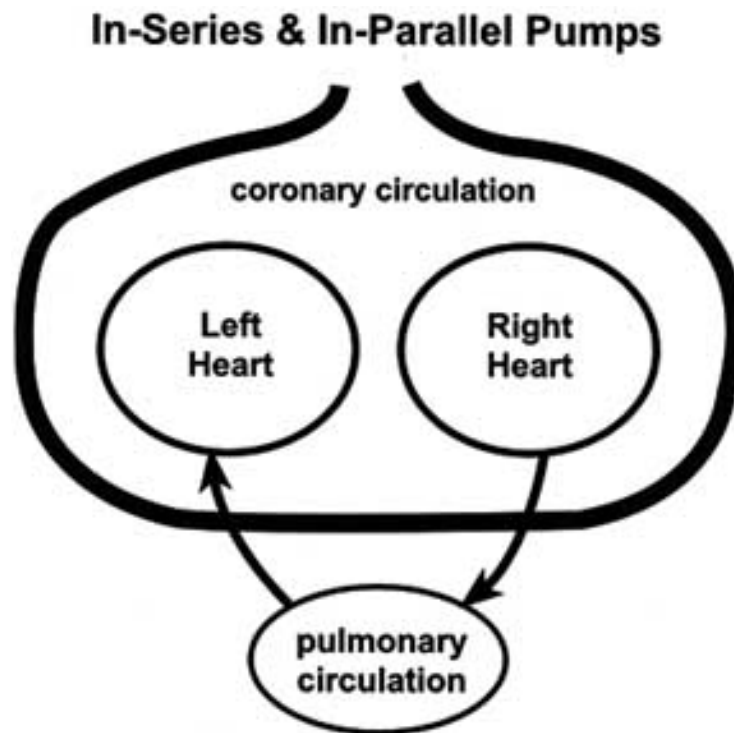
Despite a compelling body of evidence that has accumulated since Selye’s original observations, the importance of ALDOST versus hypertension alone in promoting vascular remodeling continues to be debated. One simple approach that can address this issue is to monitor vascular structure of

the entire heart. The right and left heart, linked by the pulmonary circulation, are arranged *in series* (see Fig. 1). The coronary circulation is common to the right and left heart and establishes their *in-parallel* arrangement. These simple concepts have been used to distinguish the relative importance of circulating hormone vs. hemodynamic factors. ALDO (plus Na<sup>+</sup> loading) reaches all tissues of the right and left heart while hypertension is confined to the left heart sparing the right heart. For those who would suggest elevations in arterial pressure are disseminated throughout the coronary vasculature, it is important to recognize this is true for conduit vessels, but not arterioles, or resistance vessels, involved in vascular remodeling where elevations in intraluminal pressure are dissipated. In this connection, an additional insight relates to examining whether this remodeling affects the *vaso vasorum*, or arterioles, that reside within the adventitia of the aorta and pulmonary artery and which serve to nourish these vessels.

Studies conducted by this and several other laboratories over the past decade examined the structure of right and left atria and ventricles and adventitia of these great vessels to address the role of circulating ALDO vs. hypertension in promoting the adverse vascular remodeling. They have been reviewed in detail elsewhere [31,32]. In brief, findings indicate vascular remodeling involves the normotensive, nonhypertrophied right and left atria and right ventricle, and adventitia of the pulmonary artery as well as the hypertensive, hypertrophied left ventricle and adventitia of the aorta. Lesions are first seen at week 4 of ALDOST. Progressively

more vascular sites are involved with continued treatment and where the initial perivascular fibrous tissue response extends into the contiguous interstitial space [33,34]. Spirolactone (Spiro) co-treatment prevents such remodeling [35-37]. This is true for both nondepressor and depressor doses of Spiro, which respectively do not or do prevent the appearance of hypertension [35]. Finally, the administration of a MC receptor antagonist into the cerebral ventricles prevents the rise in arterial pressure associated with systemic ALDOST, but not coronary vascular remodeling [36]. Hence, it is clear that arterial hypertension and the growth of heart muscle cells, or cardiomyocyte hypertrophy, are not the cause of vascular remodeling. Accordingly, they would not be targets for existing or new drugs.

It was the observation that a small hemodynamically insignificant dose of Spiro had cardioprotective effects that served as a scientific underpinning to a controlled, international trial (RALES) conducted in 19 countries on 5 continents in over 1660 patients with advanced heart failure. A small, oral dose of Spiro (25 mg) daily, in combination with an ACE inhibitor and loop diuretic, reduced risk by 30% for such adverse events as all-cause mortality, cardiac mortality and hospitalization due to symptomatic heart failure [38]. In a substudy to the RALES trial, elevated levels of serologic markers of type I and III collagen synthesis, presumptive biomarkers of ongoing vascular fibrosis, were reduced in the Spiro-treated group. This response accounted for the observed survival benefit [39]. In another substudy [10], the beneficial neurohormonal profile of Spiro in these



**Fig. (1).** The right and left heart are muscular pumps arranged in-series by their connection to the pulmonary circulation and in-parallel by their common coronary circulation.

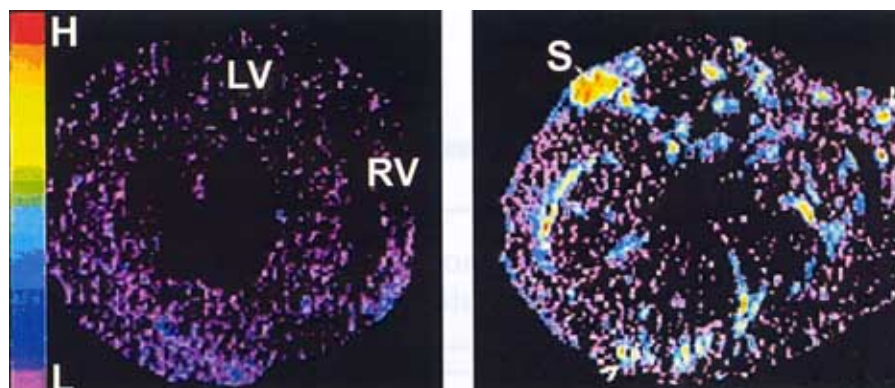
patients was shown to include: a reduction in plasma natriuretic peptides; and an elevation in circulating AngII and ALDO. Given that patients randomized to ACE inhibitor, diuretic and Spiro improved so dramatically while circulating levels of AngII were elevated and not negated by co-treatment with an AT<sub>1</sub> receptor antagonist, suggests ALDO may be a primary component of the adverse neurohormonal cascade seen with chronic cardiac failure.

### Vascular Remodeling: Inflammatory Cells & Oxidative Stress

The lesions of the intramural coronary vasculature include inflammatory cells that invade the perivascular space [26, 27]. Nicoletti and Michel [40] postulated that inflammatory mechanisms triggered within the vascular wall were responsible for the homing of these cells to this segment of the vasculature and their migration through it. They proposed a paradigm that included the respective expression of adhesion molecules, chemoattractant cytokines and proinflammatory cytokines and demonstrated the presence of T helper and T cytotoxic lymphocytes and macrophages within the perivascular space co-localized with fibroblast-like cells expressing type I collagen. Sun *et al.* [34,41] had previously observed an upregulation in the binding density of ACE and bradykinin receptors at these vascular sites in response to ALDOST. This raised the prospect that once present, the *de novo* generation of stimulators (e.g., AngII and endothelin-1), together with ACE-mediated degradation of inhibitors (e.g., bradykinin and AcSDKP), would serve to promote an activation of monocytes/macrophages and growth and differentiation of fibroblasts to myofibroblasts [5]. But what had induced the proinflammatory vascular phenotype? Was it the vascular wall, as Nicoletti and Michel suggested? Was it immune cells that targeted the coronary vasculature? Or were both responses operative?

Nuclear transcription factor- $\kappa$ B (NF  $\kappa$ B) is integral to inflammation. It regulates the expression of a cascade of mediators that include adhesion molecules, chemokines and proinflammatory cytokines. NF  $\kappa$ B is redox-sensitive and normally held in check by inhibitory kinases. It is activated by oxidative stress, which in the case of immune cells and

vascular tissue is determined by the activity of NADPH oxidase found in leukocytes, adventitial fibroblasts and endothelial cells [42,43]. To test the hypothesis that the vascular remodeling seen with ALDOST is related to the induction of oxi/nitrosative stress and activation of NF  $\kappa$ B, Sun *et al.* [44] used immunohistochemistry to detect the activation and to localize: gp91<sup>phox</sup>, a NADPH subunit specific to leukocytes; 3-nitrotyrosine, a stable tyrosine residue of short-lived peroxynitrite formation that is generated by the interaction of superoxide with nitric oxide; and NF  $\kappa$ B. *In situ* hybridization was used to address and localize the mRNA expression of intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)- $\alpha$ . At wk 3 of treatment, there was neither evidence of inflammatory cell invasion at coronary vascular sites nor oxidative stress. At wk 4, however, this was the case and remained so at wk 5 of continued ALDOST indicating the persistent induction of oxi/nitrosative stress that occurred within inflammatory cells that invaded the perivascular space and in endothelial cells of the involved coronary vasculature. These events occurred in both the right and left ventricles. Coincident in time and space was the activation of the RelA subunit of NF  $\kappa$ B and the increased expression of ICAM-1 (see Fig. 2), MCP-1 and TNF- $\alpha$ . VCAM-1 expression, however, was not upregulated. Rocha *et al.* [30] recently reported that another MC receptor antagonist, eplerenone, was able to attenuate the upregulated expression of MCP-1 and osteopontin, a chemoattractant cytokine, and to reduce the vascular inflammatory response that appeared in the left ventricle at wk 4 of ALDOST; the right heart was not examined. The selective upregulation of ICAM-1 within the affected intramural coronary circulation may offer an opportunity for tissue-specific drug delivery (*vide infra*). Cell proliferation, as detected by BrdU labeling, an indicator of the S phase of the cell cycle, was likewise evident at wks 4 and 5 of ALDOST [44]. This cascade of proinflammatory events, the appearance of invading inflammatory cells (i.e., ED<sub>1</sub>-positive macrophages and lymphocytes) and replication of myofibroblasts responsible for the fibrogenic phenotype, expressed as a perivascular fibrosis, were each prevented by co-treatment with Spiro or antioxidant (either pyrrolidine dithiocarbamate or N-acetylcysteine) [44].



**Fig. (2).** *In situ* hybridization of mRNA expression for intercellular adhesion molecule (ICAM)-1 in control rat right and left ventricles (left panel) and at 4 wks ALDOST (right panel). Low-density expression is present in control heart and markedly increased in response to ALDOST. Reproduced with permission from Sun, *Am J Pathol* '02.

Thus, evidence to date would suggest the coronary vascular remodeling associated with ALDOST is mediated by both an activation of inflammatory cells and endothelial cells that appears in response to the induction of oxidative stress within these cells of both normotensive and hypertensive ventricles. ALDO receptor antagonists and several antioxidants proved cardioprotective. Factors responsible for the induction of this oxi/nitrosative stress within each cell population remain to be elucidated and would appear to be additional drug targets.

### IDENTIFYING BIOMARKERS OF HEART FAILURE

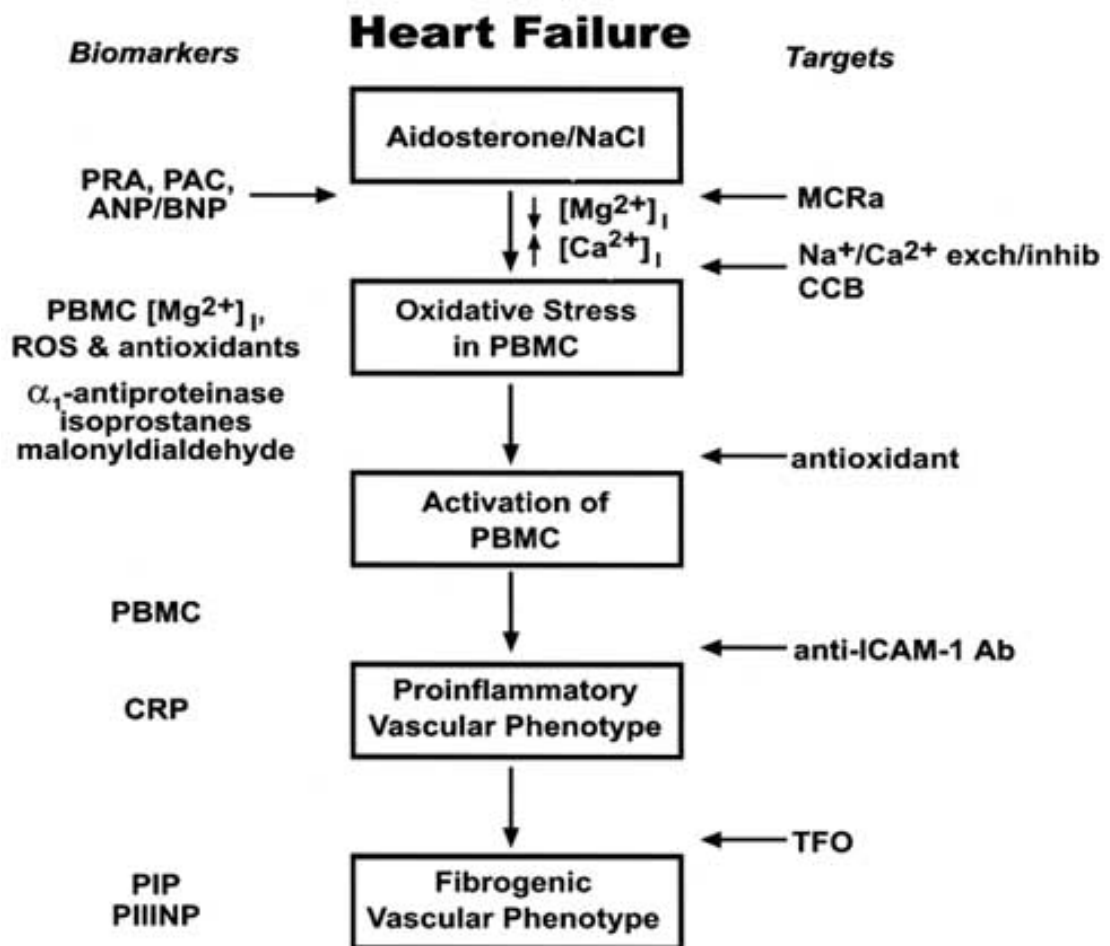
Many gaps in our knowledge remain, including factors responsible for the induction of oxi/nitrosative stress within PBMC and endothelial cells in response to the aldosteronism that accompanies chronic cardiac failure. Nonetheless, the framework upon which to undertake research pertinent to the identification of biomarkers and potential drug targets would appear to be at hand as the biomedical community turns

more and more to the prevention of the heart failure epidemic. In this connection, we are of the opinion that it is time to "think outside the box" and explore new venues while mindful of prior knowledge.

### Potential Biomarkers

A number of biomarkers of heart failure are currently under evaluation. They include: brain natriuretic peptide released by atria and ventricles of the right and left heart in response to chamber distention [45]; and such markers of inflammation as C-reactive protein, an acute phase reactant, and ICAM-1 [46].

Fig. (3) depicts a theoretical paradigm linking aldosteronism with the associated proinflammatory/fibrogenic cardiac phenotype. ALDO is involved in mono- and divalent cation exchange. ALDO-related vascular remodeling is both  $\text{Na}^+$ - and time-dependent. One might therefore suppose  $\text{Na}^+$ ,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  and their exchange mechanisms are somehow



**Fig. (3).** A paradigm of the proinflammatory/fibrogenic cardiac phenotype that accompanies aldosteronism in heart failure. Potential biomarkers are shown on the left and drug targets on the right. PRA, plasma renin activity; PAC, plasma aldosterone concentration; PBMC, peripheral blood mononuclear cells;  $[\text{Mg}^{2+}]_i$ , cytosolic free  $\text{Mg}^{2+}$  concentration; ROS & antioxi, reactive oxygen species and antioxidant reserves; plasma CRP, C-reactive protein; plasma PIP and PIIINP, serologic markers of type I and III collagen synthesis; MCRa, mineralocorticoid receptor antagonist;  $\text{Na}^+/\text{Ca}^{2+}$  exch/inhib,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger inhibitor; CCB,  $\text{Ca}^{2+}$  channel blocker; anti-ICAM-1 Ab, intercellular adhesion molecule-1 antibody; TFO, triplex forming oligonucleotide.

involved in the appearance of this phenotype. Delva *et al.* [47] have identified a  $\text{Na}^+$ -dependent, ALDO-mediated efflux of cytosolic free  $[\text{Mg}^{2+}]_i$  from cultured human lymphocytes that is blocked by ALDO/receptor antagonist (canrenoic acid) and by inhibitors of transcription and protein synthesis. A  $\text{Na}^+/\text{Mg}^{2+}$  exchange site has therefore been implicated. Such a reduction in  $[\text{Mg}^{2+}]_i$  would attenuate the activity of  $\text{Mg}^{2+}$ -dependent Na, K ATPase and lead to an accompanying rise in intracellular  $\text{Na}^+$  and to intracellular  $\text{Ca}^{2+}$  loading via  $\text{Na}^+/\text{Ca}^{2+}$  exchanger.  $\text{Ca}^{2+}$  overload, in turn, would account for an induction of oxidative stress and it is these activated cells that invade the intramural coronary circulation. In preliminary studies using the rodent ALDOST model, we observed a reduction in  $[\text{Mg}^{2+}]_i$  and elevation in  $[\text{Ca}^{2+}]_i$  prior to the appearance of the proinflammatory cardiac phenotype [48]. If this scenario proves the case then noninvasive harvesting of blood and examination of PBMC divalent cation composition, specifically  $[\text{Mg}^{2+}]_i$  and  $[\text{Ca}^{2+}]_i$ , would provide biomarkers of early triggering mechanisms involved in the appearance of oxidative stress and activation of PBMC with impending cardiac pathology. The molecular phenotype of these cells, i.e., their transcriptome and proteome, may provide additional insights into exact molecular pathways and potential new drug targets (*vide infra*).

### PBMC Transcriptome

Newer technological advances applied to genetics will enhance our understanding of the genes underlying susceptibility to disease. Most common diseases, however, have a substantial environmental component and therefore other approaches will be needed. The environment influences gene expression and modifies gene products in ways that initiate, accelerate, or retard progression of pathogenic processes. This does not change the genome, but inevitably changes the complement of transcribed genes (the transcriptome) and the composition of proteins translated from them (the proteome) in affected tissues. Just as the term *genome* covers all genes, the terms *transcriptome* and *proteome* respectively cover all transcribed mRNA and all proteins present in a given individual, tissue, or cell. The tools are now in hand with which to comprehensively interrogate and unravel molecular events.

Gene array technology is used to make expression arrays that simultaneously evaluate the relative levels of large numbers of different mRNA gene transcripts [49]. In a typical comparative expression array analysis experiment, mRNA is isolated and converted to the complementary DNA (cDNA) sequence. Each sample is labeled with different fluorescent dyes, e.g., Cy3 and Cy5. The labeled cDNA from the 2 samples is mixed and incubated on a "gene chip," then washed to remove nonbinding cDNA. The gene chip has been arrayed with a large number of small DNA spots, each containing the DNA sequence of a different gene. Each labeled cDNA (derived from each expressed mRNA in the original samples) will bind to the spot containing the corresponding gene (DNA). Levels of each of the 2 dyes, on each gene spot on the gene chip, are determined in a fluorescent reader. For each gene spot, the relative intensity of the 2 colors indicates if the expression level of that gene is increased or decreased in one sample relative to the other.

Several variations on this principle and protocol are being used for determination of gene expression levels. By comparing transcriptomes from diseased and non-diseased tissue, it will be possible to produce a comprehensive "definition" of the specific disease state at the mRNA level.

Using gene array technology and PBMC obtained from man or rats during preclinical, clinical and pathologic phases of aldosteronism, such as those seen in heart failure, new biomarkers of risk may be identified and prevention strategies implemented. In preliminary studies conducted on the PBMC transcriptome, obtained from rats receiving ALDOST for 1, 2, 3 or 4 wks, we observed a progressive rise in gene expression: 205, 292, 313 and 431 genes/-expressed sequence tags (EST), respectively. A total of 822 genes/ESTs were differentially expressed at one or more times. Whereas only 20% (42/205) of genes differentially expressed at wk 1 were unique to this time point, 68% (291/431) of differentially expressed genes at wk 4 were specific here suggesting new pathologic processes had been initiated. Clusters of PBMC genes affected by ALDOST included those affected by or dependent on mono- and divalent cations, genes associated with the induction of or counteracting oxi/nitrosative stress, inflammatory response activation and specific immune responses.

### PBMC Proteome

Proteomics enables the accurate, rapid, comprehensive, large-scale, and systematic analysis of proteins that participate in biologic events. For heart failure, proteomics provides a powerful tool to identify complex molecular pathways, protein modifications and interactions, and the time-course of protein expression that underlie its progressive and systemic nature. Such information could lead to the development of biomarkers that would prove useful in clinical detection of risk and progression and which would aid in the development of management strategies. One could approach the proteomics of heart failure through the use of myocardial tissue to address molecular mechanisms of ventricular dysfunction. Jungblut [50] and Dunn [51] have chosen this approach to address how proteins contribute to altered cellular functions that result in ventricular dysfunction in cardiomyopathies of idiopathic origins. This approach assumes the heart is homogenous tissue. It is not. Cardiac myocytes are large; they occupy 75% of the heart's tissue space. They, however, represent but one-third of all cells that comprise the myocardium [52]. Other, more plentiful constitutive cells (e.g., endothelial and vascular smooth muscle cells, fibroblasts) could obfuscate meaningful analysis. Moreover, endomyocardial biopsy provides only limited amounts of tissue. Finally, explanted failing human hearts offer larger amounts of tissue, but are in limited supply. Therefore, these investigators have established an international registry to obtain the large number of human tissue samples needed with standardization of sample preparation.

An alternate approach, independent of myocardial tissue and the diverse etiologic origins of heart failure, would therefore have advantages. Irrespective of causality, genetic predisposition and environmental factors, the common ground to the progressive, systemic nature of heart failure

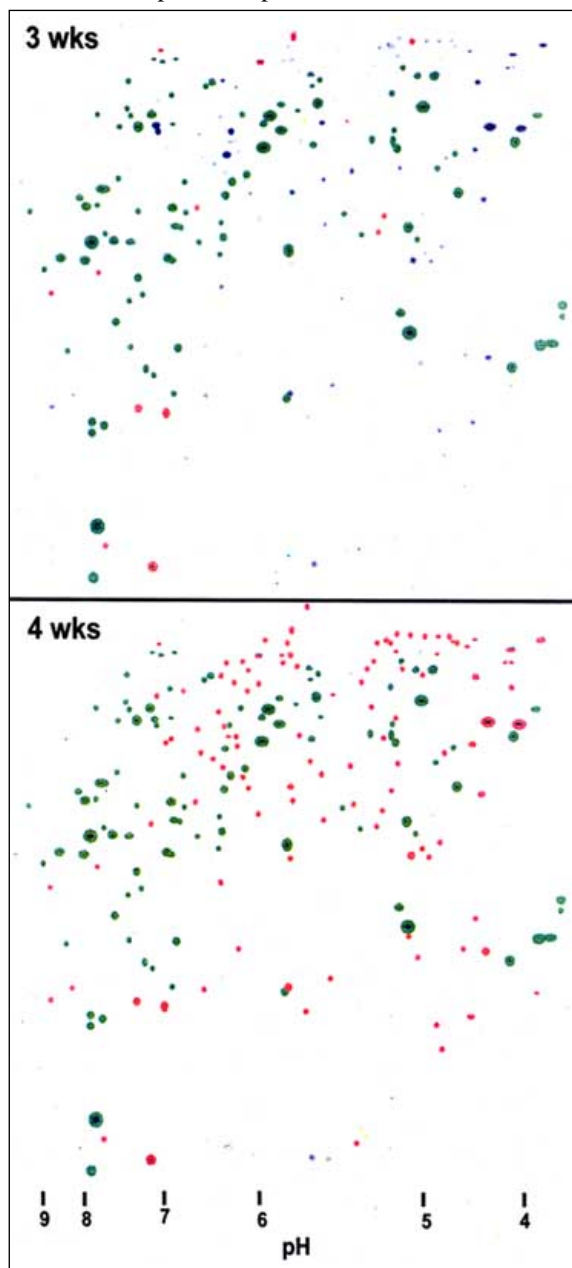
resides in neurohormonal activation with more recently appreciated effects that include a state of oxidative stress and subsequent proinflammatory/fibrogenic cardiac phenotype. Circulating cells of the mononuclear-phagocyte system invoke this phenotype. The proinflammatory heart failure paradigm is akin to that proposed for atherosclerosis, where chronic inflammation is now recognized as a hallmark of the atherosclerotic plaque. Monocytes/macrophages of atheromatous lesions and autocrine/paracrine properties of AngII generated by these cells are inextricably linked to the pathogenesis of atherosclerosis and to the pathophysiology of its clinical presentation [53]. In particular, pro-oxidative and proinflammatory properties of AngII trigger oxidative stress, including oxidative-induced iterations of DNA, lipids and proteins, via activation of NAD(P)H oxidase in monocytes/macrophages and endothelial cells. These are pressor-independent actions of AngII. In heart failure, the influence of the RAAS is systemic based on endocrine properties of its circulating hormones, which act as proinflammatory signals on mononuclear cells. We, therefore, hypothesize that what begins with cardiac injury (of diverse causality) and ventricular dysfunction becomes a progressive, systemic illness involving the heart and systemic organs and whose origins are rooted in an immunostimulatory state created by oxidative stress in immune cells and transduced by effector hormones of the RAAS. Circulating mononuclear cells serve as “signatures” of its proinflammatory phenotype.

Proteomics is often used to search for novel protein targets—in a mode of discovery—without any concern for preconceived hypothesis. However, the information developed from traditional, reductionist experiments can be useful and guide the direction of proteomic studies. The simplistic paradigm of inflammation shown in Fig. (3) and identified by linear-based protein studies involves several “proteins” (e.g., NADPH oxidase and NF B) that serve as a unifying signaling system for oxidative stress and inflammatory stimuli. This “backbone” paradigm identifies candidate proteins of interest to the heart failure phenotype and key signaling elements that will provide a rational basis to interrogate more complex protein reactions and interactions.

By using circulating PBMC as “signatures” of this phenotype, we gain the significant advantage of ready access to large “tissue” samples with an important ability to noninvasively obtain serial samples for a given individual and, very significantly, to interrogate the proteome in culture. Moreover, by using PBMC, together with cell-enrichment technologies (e.g., flow cytometry and antibody-based magnetic bead separation), we can focus on specific cell populations. After their selection (e.g., by negative phenotype), these cells can be studied in culture or *ex vivo* under controlled experimental conditions. Subcellular compartments, including cell membranes, can be examined for the distribution and location of multiple signaling molecules and coordinated action of these elements at a specific intracellular site. These latter studies could prove useful in the development of pharmacologic interventions, including ALDO receptor antagonists and antioxidants.

In preliminary studies, we have examined the proteome of PBMC obtained from rodents with ALDOST for 3 and 4

wks. Untreated age-/gender-matched rats served as controls. We selected these time points because rodents become lethargic and anorectic (reduced food and water intake) at 3wks treatment before the proinflammatory phenotype appears in the heart at 4wks treatment. We found 13 proteins (see upper panel, Fig. 4) that were differentially expressed by PBMC at 3wks ALDOST, and which were associated with the clinical appearance of illness, but prior to NF B activation and the appearance of inflammatory cells in the heart. Differential protein expression rose to an additional 92



**Fig. (4).** Differential expression of proteins by mononuclear cells (red) obtained from rodents treated with ALDO for 3 and 4 wks (upper and lower panels, respectively). A combination of mass spectrometry (peptide mass fingerprinting) and database searching (SWISS-PROT) was used to identify several of the differentially expressed proteins (red) and those common to both treated and untreated controls (green).

proteins at 4wks ALDOST (see lower panel, Fig. 4) when the proinflammatory phenotype was found in the heart and rodents remained ill. These early findings are consonant with proof-of-concept with regard to PBMC activation by ALDO and  $\text{Na}^+$ . Moreover, they underscore the potential value of the circulating mononuclear cell proteome, a noninvasive strategy, in accurately predicting the initial clinical appearance of the systemic illness and subsequently the proinflammatory cardiac tissue phenotype.

The ultimate goal of proteome analysis is to obtain information about the identity, expression level, subcellular location, posttranslational modifications, and binding partners of each protein expressed in a biological sample of interest. Whereas current technology can easily obtain such information for individual proteins expressed at high levels, the challenge for the field of proteomics is to obtain information for *all* proteins expressed at any level. The central problem for this challenge is detection sensitivity. One mole contains  $6 \times 10^{23}$  molecules. To measure a protein expressed at 10 copy/cell (cpc), in a sample of 600 cells (i.e., 6000 molecules), we need a method with a detection sensitivity at the  $10^{-20}$  mole level (Table 1). Currently, the detection sensitivity of protein stains and routine mass spectrometry instruments used for 2D gel-based proteomics studies is at the  $10^{-14}$  mole level (10fmol). At this level of sensitivity, one would need a sample size of 600 million cells to detect proteins expressed at 10cpc. Providing for sample loss during preparation, at least twice that number of cells would be needed. For most tissues, this sample size is prohibitively large and, therefore, we need to improve detection sensitivity by orders of magnitude.

## POTENTIAL DRUG TARGETS

### Drug Delivery and Vascular Remodeling

The activation of PBMC and endothelial cells of the intramural coronary circulation during aldosteronism raises the prospect for tissue-specific, targeted drug delivery using nanosphere technology and adhesion molecule-based drug delivery. Movement of leukocytes from within the vasculature to the extravascular space is mediated, in part, by

adhesive bonds which form between glycoproteins (ligands) present on the leukocytes and cognate glycoproteins (receptors) present on the endothelium [54-57]. A key paradigm in this adhesion cascade is that certain endothelial cell adhesion molecules are inducible. That is, they are expressed at a low level, if at all, on endothelium within normal tissue (see left panel, Fig. 2), but dramatically upregulated in response to ALDOST (see right panel, Fig. 2). Leukocytes attach to the endothelium, for the most part via selectins (e.g., E-selectin, CD62E and P-selectin, CD62P) and begin to translate along the vessel wall (roll) at a velocity which is significantly lower than leukocytes in the free stream [57]. As leukocytes roll they become activated in response to chemokines and firmly adhere to the endothelium *via* integrins and proceed to migrate between adjacent endothelial cells into the extravascular space [57,58].

As noted above, a key component of leukocyte emigration is endothelial cell activation wherein the adhesion molecule profile on the luminal surface of the endothelium is altered. Recognition of these drastically different endothelial surfaces has led to the concept of *endothelial cell adhesion molecule-mediated drug delivery* [59-64]. However, in many cases this targeting scheme is not successful because the differential in molecule expression between normal and targeted tissues is not large enough [65]. Aldosterone-induced upregulation of ICAM-1 by endothelial cells of intramural coronary arteries provides a potential avenue for targeting drugs to the affected vasculature similar to that used to target drugs and/or other molecules to irradiated tissue [44,66]. In this manner, the upregulation of an endothelial cell adhesion molecule(s) within the diseased heart is used as a target to deliver therapeutic agents (drugs, genes, etc.) selectively to that tissue. In this model the drug carrier would contain a therapeutic agent (e.g., see targets in Fig. 3) and, on its outer surface, a recognition molecule (ligand) for a cognate molecule (receptor) that is expressed selectively (e.g., ICAM-1) on the luminal surface of the endothelium within the coronary circulation.

Particle targeting via upregulated adhesion molecules can also be used to quantify upregulation of adhesion molecules in various tissue *in vivo* and *in vitro* [66-68]. In addition to being easy and reproducible, this technique can be used to

**Table 1. Minimum Sample Size, Corresponding to Different Method Sensitivities and Expression Levels**

Sensitivity	1000 Copies/Cell	100 Copies/Cell	10 Copies/Cell
10 Femtomole ( $10 \times 10^{-15}$ ) (Protein Stains) (Current MS)	<b><math>6 \times 10^6</math> Cells</b> Platelets: 20 $\mu$ L of blood Mononuclear Cells: 1mL	<b><math>60 \times 10^6</math> Cells</b> Platelets: 200 $\mu$ L of blood Mononuclear Cells: 10mL	<b><math>600 \times 10^6</math> Cells</b> Platelets: 2mL of blood Mononuclear Cells: 100mL
10 Attomole ( $10 \times 10^{-18}$ ) (Radio-labeling) (Cutting Edge MS)	<b><math>6 \times 10^3</math> Cells</b> Platelets: <1 $\mu$ L of blood Mononuclear Cells: 1 $\mu$ L	<b><math>60 \times 10^3</math> Cells</b> Platelets: <1 $\mu$ L of blood Mononuclear Cells: 10 $\mu$ L	<b><math>600 \times 10^3</math> Cells</b> Platelets: 2 $\mu$ L of blood Mononuclear Cells: 100 $\mu$ L
10 Zeptomole ( $10 \times 10^{-21}$ ) (Future MS)	<b>6 Cells</b> Platelets: <1 $\mu$ L of blood Mononuclear Cells: <1 $\mu$ L	<b>60 Cells</b> Platelets: <1 $\mu$ L of blood Mononuclear Cells: <1 $\mu$ L	<b>600 Cells</b> Platelets: <1 $\mu$ L of blood Mononuclear Cells: <1 $\mu$ L

determine which adhesion molecules are upregulated to a functional level, i.e., the level of protein upregulation on the luminal surface of endothelial cells is high enough to support significant leukocyte attachment. Application of this technique usually involves injection of 1–2  $\mu\text{m}$  particles (micron-sized particles are generally used because they are easier to visualize under a microscope) which bear ligands to adhesion molecule(s) of interest on their surfaces. The number of particles adhering to the tissue of interest is then proportional to the level of upregulation of adhesion molecule(s) of interest. In this approach the dynamic interaction of these particles with the vessel wall (i.e., rolling, adhesion) can be directly observed in real time via intravital microscopy and can be recorded on videotape for off-line analysis [66-68].

Drug carriers for targeted drug delivery can be generated using several different types of material. Well-made and well-characterized small unilamellar liposomal formulations of smaller liposomes (<200nm) are non-toxic, non-hemolytic, non-immunogenic, even upon repeated injections, are biocompatible/biodegradable, and can be designed to avoid clearance mechanisms (reticulo-endothelial system, RES, renal clearance, chemical or enzymatic inactivation) [65,69]. Depending on the formulation design, liposomes can effectively control the pharmacokinetics and biodistribution of the drug, and specific tissues can be avoided or targeted, and thus therapeutic index enhancement can be achieved in principle both via toxicity reduction and efficacy enhancement. Drug carriers can also be generated using biodegradable nanospheres (PLA-PEG) [63,70,71] or poly(amidoamine) (PAMAM) dendrimers [72], microbubbles [73], polymersomes [74], or synthetic secretory granules [75]. Erythrocytes (red blood cells) coated with antibodies (immunoerythrocytes) have also been used for drug targeting [76,77]. These immunoerythrocytes offer the intriguing possibility of using native particles for delivering drugs to tissue, thereby ensuring biocompatibility, and this approach may be extended for targeting other particles to tissue, e.g. targeting “immuno-stem cells” to damaged myocardium. However, at the current time it is not clear how the properties of erythrocytes, or other cells, may be altered by antibody coating.

### Triplex-Forming Oligonucleotides

In the last 5 years there has been an exponential growth in the application of antigene strategies, especially triplex DNA, to regulate expression of various genes. The ability to design a specific triplex-forming oligonucleotide (TFO) that recognizes a unique site within megabase segments of genomic DNA, offers a novel approach to down-regulate the expression of the target gene. For instance, in an unabated wound healing response, relentless fibrosis in organs such as liver, kidney, heart and lung, results in the loss of structure and function. Fibrosis is largely due to abnormal accumulation of type I collagen in the organ's extracellular space. If one could specifically repress transcription of either *I* or *2* genes of type I collagen, it might be possible to halt the progressive deposition of collagen and restore the function of damaged organs, such as occurs with aldosteronism in heart failure. Intensive efforts are underway to develop novel triplex

strategies that target specific genes known to cause or contribute to the initiation or progression of different diseases. Several recent developments on the use of TFOs include: a) site-directed mutagens; b) agents that cleave target DNA sequences; and c) antigene therapeutic molecules that specifically and selectively down-regulate target genes.

Normally DNA exists in a duplex form. However, under certain circumstances, DNA can assume triple-helical (triplex) structures, which are either intra- or intermolecular. Intermolecular triplexes, formed by the addition of a sequence-specific third strand to the major groove of the duplex DNA, are shown to have enormous potential as site-directed mutagens, repressors of transcription, and inhibitors of replication. TFOs specifically recognize duplex homopoly-purine:homopolypyrimidine sequences present in a number of eukaryotic genes.

Ever since the discovery of triplex-forming sequences, a major effort has gone into using them as specific reagents to deliver DNA damaging chemicals that will induce site-specific damage in a target sequence in the genome [78,79]. For example, introduction of interstrand crosslinks into specific regions of the chromosomes can interfere with vital processes of DNA replication and transcription. Psoralen is one such damaging molecule, which is relatively non-toxic to cells. However, when it is activated by long-wave UV light (UVA), it can form adducts and interstrand crosslinks [80]. Several groups used psoralen-linked TFOs to stimulate homologous recombination in yeast and in mammalian targets and to increase mutation frequencies up to 2500-fold compared to the control non-triplex-forming oligonucleotides [81-88]. It should be pointed out that TFOs, themselves, can induce mutation frequency, as demonstrated in mice by Vasquez *et al.* [89]. By using a TFO designed to form a triplex with the polypurine site in the *supFG1* gene, they showed induction of mutations only in the *supFG1* gene but not in other genes. This study not only showed the general uptake of the TFO by most of the tissues following intraperitoneal administration but also demonstrated formation of triplexes with the target sequence. Similar studies using a psoralen-conjugated TFO designed to bind to a transcription factor in the upstream sequence of human gamma-globin gene, mutation frequencies as high as 20% were reported when a psoralen-conjugated TFO was used [90]. This type of strategy can be used not only to mutate a gene, but also to correct a gene. A short DNA sequence of about 40–44 nucleotides is tethered to a TFO, which forms a triplex with the sequence in the targeted gene. Upon transfection of this tethered TFO, gene-specific correction was observed [91]. TFOs, specifically developed to form triplexes with the adenosine deaminase gene and *p53* genes, corrected mutations in ADA-deficient human lymphocytes and *p53* mutant human glioblastoma cells with a frequency of 1–2% [92]. In most of these studies, longer TFOs (20–30 nucleotides) were used. Recently, it has been shown that shorter (10 bp) target sites in a gene can be achieved by using cationic phosphoramidate linkages. For example *N,N*-diethylethylenediamine and *N,N*-dimethylaminopropylamine phosphodiester TFOs increase the efficiency of targeted mutagenesis by 6–10-fold [93]. Double-strand breaks in the target gene could be induced by TFOs containing radioactive isotopes [94,95]. TFOs containing non-radioactive chemicals

have been successfully used to cleave target DNA at specific sites [96-100]. TFOs, in conjunction with laser-induced two-photon excitation, have been used to damage DNA at specific sequences [101]. Camptothecin, an antitumor drug, acts by interfering with DNA topoisomerase I. Camptothecin-conjugated TFOs, upon binding to the duplex target DNA, selectively positions the drug at the triplex site where it stimulates topoisomerase I-mediated DNA cleavage at this site [102]. This approach not only offers a novel route of targeting oncogenes, but also provides a powerful tool to study the interactions between topoisomerase I and DNA.

TFO-mediated down-regulation of a variety of genes is now widely appreciated. [90,96,103-122]. It has been shown that the human *c-src* promoter contains sites for the ubiquitous transcription factors Sp1 and a novel pyrimidine-binding factor SPy. Interestingly, the SPy-binding site appears to assume a non-B DNA (H) structure and deletions in both Sp1 and SPy motifs drastically reduce transcription [103]. Of greater significance is the demonstration that TFOs designed to target both Sp1 and SPy-binding sites down-regulate *c-src* expression. Similar studies indicate that the expression of a chemokine, MCP-1, which is expressed in a variety of tissues in response to inflammatory stimuli such as ALDOST could be blocked by a TFO specific for the Sp1 site in the human MCP-1 gene promoter [96]. Inhibition of expression of a gene can be achieved by using TFOs specific for regions other than the promoter or the enhancer of the gene. For example, a TFO targeted to the 3'-untranslated region (3'-UTR) of the *bcl-2* proto-oncogene effectively inhibited synthesis of *bcl-2* protein as demonstrated by Western blotting [110]. Sequence-specific targeting by TFOs coupled with induction of highly localized damage of the target site by <sup>125</sup>I was also shown to be an effective way of destroying gene transcription [114].

One novel approach worthy of mention is the generation of TFOs inside a cell to specifically suppress a gene's function. Specific uptake or introduction of TFOs into cells and tissues is a formidable problem for rational application of the TFO strategy for antigene activity. *De novo* generation of such TFOs within the target cell is one logical approach to overcome these problems. Cogoi *et al.* [107] constructed three different plasmid vectors carrying a 76 bp triplex-forming sequence specific for the c-Ki-*ras* promoter, driven to expression by a human small nuclear *U6* gene promoter. Upon transfection into human 293 cells, these vectors could endogenously generate three different TFOs of 76 nucleotides, each one of which could form a triplex with the target sequence. They found significant inhibition of transcription of the *ras* gene in these cells [98]. Similar approaches using ribozyme hammerheads can be employed to generate these TFO sequences within the target cells.

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