Review: Chemical Pathology of Homocysteine. V. Thioretinamide, Thioretinaco, and Cystathionine Synthase Function in Degenerative Diseases

Kilmer S. McCully

Pathology and Laboratory Medicine Service;Veterans Affairs Medical Center;West Roxbury, Massachusetts

Abstract. Hyperhomocysteinemia was first associated with degenerative disease by observation of accelerated arteriosclerosis in children with inherited disorders of cystathionine synthase, methionine synthase, and methylene tetrohydrofolate reductase. The metabolic blockade of sulfate synthesis from homocysteine thiolactone in malignant cells is ascribed to a deficiency of a chemopreventive derivative of homocysteine thiolactone that occurs in normal cells. Its chemical structure was elucidated by the organic synthesis of thioretinamide from retinoic acid and homocysteine thiolactone. Oxidation of the sulfur atom of homocysteine is inhibited in scorbutic guinea pigs, demonstrating ascorbate function in sulfate synthesis from homocysteine. Studies of homocysteine metabolism in protein energy malnutrition led to the conclusion that the biosynthesis of thioretinamide from the retinol of transthyretin is catalyzed by dehydroascorbate and superoxide generated from the heme oxygenase group of cystathionine synthase. Newly synthesized thioretinamide is complexed with cobalamin to form thioretinaco, which is activated by ozone and oxygen to function as the active site of oxidative phosphorylation. In accordance with the trophoblastic theory of cancer, pancreatic enzymes are believed to be oncolytic because they hydrolyze the homocysteinylated proteins, nucleic acids and glycosaminoglycans of malignant tissues. The clonal selection of malignant cells that are deficient in the heme oxygenase function of cystathionine synthase produces cells dependent upon glycolysis for ATP synthesis, since they are deficient in synthesis of thioretinamide, thioretinaco and thioretinaco ozonide. The vulnerable plaque of arteriosclerosis originates from complexes of microbes with homocysteinylated lipoproteins, obstructing *vasa vasorum* narrowed by endothelial dysfunction, causing arterial ischemia, and intimal micro-abscesses. Degenerative diseases may be ameliorated by a proposed therapeutic protocol of thioretinamide with pancreatic enzymes.

Key words: homocysteine, thioretinamide, thioretinaco, cystathionine synthase, ascorbate, nitrilosides, hydrogen sulfide, pancreatic enzymes

Introduction

Since publication of earlier sections of this review [1-4], new discoveries and interpretations concerning the structure and function of cystathionine synthase have increased our understanding of the chemical pathology of homocysteine in such diverse fields as protein energy malnutrition, pancreatic enzyme function in therapy of malignant disease, aerobic glycolysis by malignant cells, the function of ascorbate in oxidation of the sulfur atom of homocysteine, and the metabolic blockade of sulfur oxidation in malignant cells. The purpose of this review is to elucidate the function of the heme oxygenase group of cystathionine synthase in the synthesis of thioretinamide and N-homocysteine thiolactonyl retinamide, and its importance in the formation of thioretinaco, N-homocysteine thiolactonyl retinamido cobalamin, and thioretinaco ozonide in the oxidative metabolism of normal and malignant cells. As explained in earlier sections of this review, the function of thioretinamide, thioretinaco, and thioretinaco ozonide is critical in understanding the metabolic origin of degenerative diseases, including arteriosclerosis, cancer,

Address correspondence to Kilmer S. McCully, M.D.,Veterans Affairs Medical Center, West Roxbury MA 02132 USA; tel 857 203 5990; fax 857 203 5623; e mail kilmer.mccully@va.gov

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dementia, autoimmune diseases, osteoporosis and fracture, venous thrombosis and embolism, accelerated aging, renal failure, metabolic syndrome, protein energy malnutrition, and dietary deficiencies of folate, pyridoxal, and cobalamin. In each of these degenerative diseases and conditions, abnormal homocysteine metabolism has been demonstrated by an increased level of homocysteine bound to plasma proteins by disulfide bonds [5]. This review demonstrates how increased understanding of the function of cystathionine synthase in thioretinamide and thioretinaco synthesis leads to a proposed metabolic program of potential therapeutic benefits in a wide variety of degenerative diseases.

Homocysteine metabolism in arteriosclerosis and cancer

Abnormal homocysteine metabolism was first implicated in the etiology of degenerative diseases by observation of accelerated arteriosclerosis in children with two different inherited enzymatic disorders resulting from the deficiency of cystathionine synthase and methionine synthase [6]. Accelerated arteriosclerosis was subsequently demonstrated in a child with a deficiency of methylene tetrahydrofolate reductase, a third enzymatic disorder of homocysteine metabolism [7]. In all three of these enzymatic disorders, the elevation of blood levels of homocysteine is implicated in the pathogenesis of arteriosclerosis by a direct effect of homocysteine on the metabolic activity of arterial cells and tissues.

Abnormal homocysteine thiolactone metabolism was demonstrated in cultures of cells from malignant tissues [8]. The results of this study show that cultured malignant cells have a metabolic blockade of the oxidation of the sulfur atom of homocysteine thiolactone to sulfate, leading to accumulation of homocysteine thiolactone within malignant cells. Homocysteine thiolactone reacts with the free amino groups of macromolecules, forming peptide bonds that cause homocysteinylation of the amino groups of proteins, nucleic acids, and glycosaminoglycans. This metabolic blockade within malignant cells is ascribed to a deficiency of a derivative of homocysteine thiolactone that is usually present within normal cells.

The formation of homocysteine thiolactone from methionine in malignant cells is catalyzed by methionyl t-RNA synthase in an error editing reaction [9]. Abnormal homocysteine thiolactone metabolism in malignant cells is hypothesized to result from a deficiency of or a failure to synthesize an N-substituted derivative of homocysteine thiolactone [8]. According to this hypothesis, normal cells contain a chemopreventive derivative that facilitates sulfate formation from homocysteine thiolactone. The concentration of this hypothetical derivative is believed to be diminished during the carcinogenic transformation of normal to malignant cells through the action of carcinogenic chemicals, radiation, microbes, or chronic inflammation. In normal cells, this chemopreventive derivative functions to prevent accumulation of homocysteine thiolactone by catalyzing its conversion to phosphoadenosine phosphosulfate, sulfate esters of glycosaminoglycans, steroids, other compounds, and sulfate ions. Decreased concentration of this chemopreventive derivative in malignant cells leads to the characteristic metabolic abnormalities of malignancy, which are attributable to excessive accumulation of homocysteine thiolactone. According to this concept, the increased growth rate, the aggregation of nucleoproteins, the altered expression of developmentally suppressed genes, the degradation of cellular membranes, and the abnormalities of oxidative metabolism (such as aerobic glycolysis) are attributable to the increased accumulation of homocysteine thiolactone within malignant cells. Treating animals with transplanted malignant neoplasms with homocysteine thiolactone perchlorate causes increased necrosis within malignant neoplasms, presumably due to the increased accumulation of homocysteine thiolactone within malignant tissues [10].

Discovery of thioretinamide, thioretinaco and thioretinaco ozonide

The identity of the N-substituted derivative of homocysteine thiolactone that prevents growth of malignant tumors in animals was elucidated by the organic synthesis of anti-neoplastic compounds

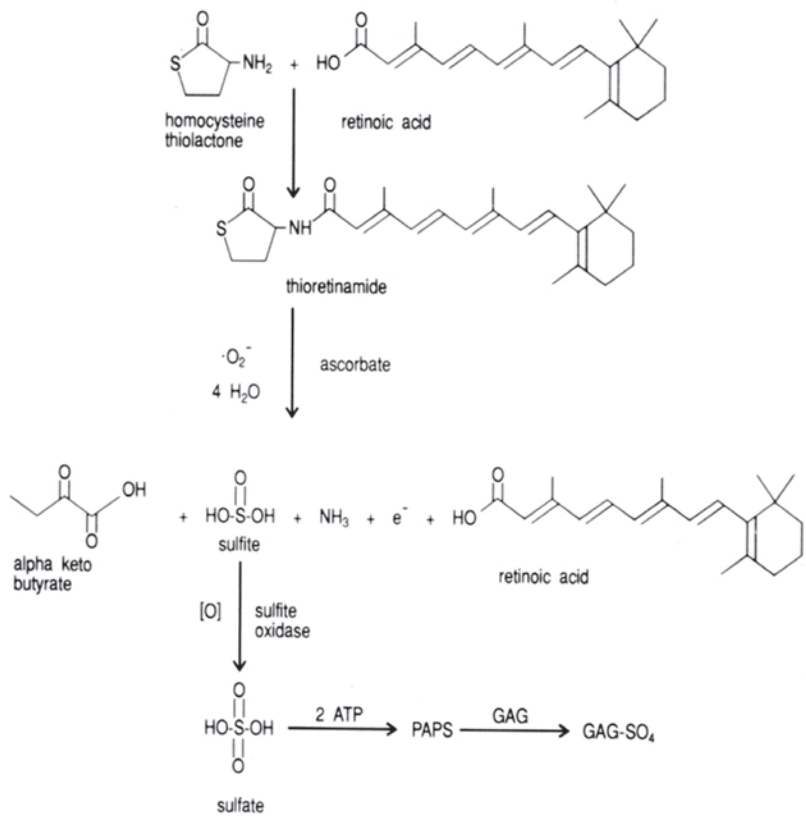

Figure 1. *Hypothetical scheme for synthesis of thioretinamide, oxidation to sulfite and sulfate, and activation of sulfate to phosphoadenosine phosphosulfate, explains sulfation of glycosaminoglycans (GAG) to form sulfate esters [2]. In this scheme superoxide is synthesized from the heme oxygenase group of cystathionine synthase [34], catalyzing retinoic acid synthesis from retinol. Synthesis of thioretinamide from retinoic acid and homocysteine thiolactone is catalyzed by cystathionine synthase and dehydroascorbate.*

containing homocysteine thiolactone. Arachidonoyl homocysteine thiolactone amide and pyridoxal homocysteine thiolactone enamine decrease the growth of transplanted murine mammary adenocarcinoma [11]. N-maleyl homocysteine thiolactone amide, N-maleamide homocysteine thiolactone amide, and rhodium trichloride oxalyl homocysteine thiolactone amide suppress the growth of transplanted neoplasms in animals [12,13]. The encapsulation of N-maleamide homocysteine thiolactone amide within liposomes greatly enhances its anti-neoplastic activity [14]. Structural analysis of these biologically active derivatives of homocysteine thiolactone shows that the hypothetical chemopreventive derivative of homocysteine thiolactone in normal cells is (1) active in a lipid-soluble form, (2) contains a conjugated double bond system with a carbonyl group adjacent to the nitrogen atom of homocysteine thiolactone, and (3) forms a complex with a transition metal atom that enhances anti-neoplastic activity. Homocysteine thiolactone reacts with retinoic acid to form N-homocysteine thiolactonyl retinamide (NHTR), known as thioretinamide, in organic synthesis [15]. Thioretinamide reacts with cobalamin to form N-homocysteine thiolactonyl retinamido cobalamin ((NHTR)2Cbl), known as thioretinaco [16]. Both thioretinamide and thioretinaco have anti-carcinogenic and anti-neoplastic activities in mice treated with a carcinogen and in mice with transplanted neoplasms. The method of synthesis of thioretinamide was significantly improved by use of N-ethyl-N'-(3-dimethyl-aminopropyl) carbodiimide in the reaction mixture [17]. This method replaces the conjugation agent, dicyclohexylcarbodiimide in the reaction mixture of the original method and produces pure thioretinamide in 72% of theoretical yield. This pure thioretinamide and its complex with cobalamin, thioretinaco, have anti-atherogenic activity in rats treated with parenteral homocysteine thiolactone [17].

The anti-carcinogenic, anti-neoplastic, anti-viral, and anti-aging activities of thioretinaco ozonide are enhanced by use of membranergic compositions, specifically the polypeptide cytokines, alpha-interferon, beta-interferon, and gamma-interferon [18]. A therapeutically active composition of thioretinaco ozonide for providing anti-carcinogenic, anti-neoplastic, anti-viral, anti-atherogenic, and anti-aging benefits is formed by thioretinaco ozonide, complexed with adenosine triphosphate and oxygen within an ozone-resistant liposomal carrier [19]. Clinical therapeutic studies with thioretinamide [15], interferons [18], and liposomal delivery systems [19] have not yet proven to be feasible because of potential toxicity of interferons and the complexity of synthesis and liposomal delivery of thioretinaco ozonide in therapy.

Ascorbate, homocysteine thiolactone oxidation, and growth hormone

Studies of homocysteine thiolactone metabolism in the liver of scorbutic guinea pigs that are deprived of dietary ascorbate revealed a failure to oxidize homocysteine thiolactone to homocystine and sulfate, as well as a pathway for synthesis of phosphoadenosine phosphosulfate from the sulfur atom of homocysteine thiolactone [20]. Homocysteic acid, the oxidized sulfonic acid derivative of homocysteine, promotes growth in normal animals and promotes release of insulin-like growth factor, IGF-1, in hypophysectomized animals that are treated with thyroxine [21]. Young animals and hypophysectomized animals convert more homocysteine thiolactone to homocysteic acid and other oxidized homocysteine derivatives than do older or normal animals [22].

Cultured cells that are deficient in cystathionine synthase and unable to convert homocysteine to cystathionine are able to oxidize the sulfur atom of homocysteine thiolactone to sulfate, demonstrating a pathway for sulfate synthesis that is independent of conversion of homocysteine to cystathionine, cysteine and sulfate [23]. As shown in **Figure 1**, the pathway for synthesis of sulfate from homocysteine thiolactone involves synthesis of thioretinamide from homocysteine thiolactone and retinoic acid and subsequent oxidation of thioretinamide to sulfite, alpha-keto-butyrate and retinoic acid by superoxide [2].

In the 1930s ascorbic acid was found to inhibit the growth of cancer in animals, mainly acting to extend their life span [24]. Moreover, the concentration of ascorbic acid in the tissues of animals with cancer is decreased, further suggesting the possibility of a therapeutic action in human patients with cancer. Otto Warburg discovered the role of cytochrome enzymes in cellular respiration [25]. Later he demonstrated that cancer cells and embryonic cells have a characteristic mode of cellular respiration known as aerobic glycolysis in which glucose is converted to lactic acid instead of conversion to carbon dioxide, as seen in normal cells [26]. Ascorbic acid inhibits cellular respiration and glycolysis of cancer cells [27]. Controversial studies

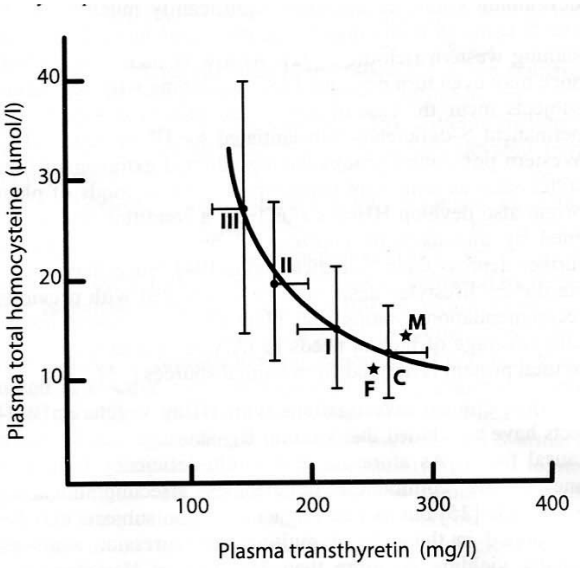

Figure 2. *Correlation between plasma homocysteine and transthyretin concentrations in protein malnutrition. Values are plotted for control subjects (C), both male (M) and female (F), and for subjects with stages I, II and III of protein energy malnutrition, according to WHO criteria. Values are expressed as mean ± standard deviation (horizontal and vertical bars). The figure is reproduced from reference [33] with permission.*

suggested that massive doses of ascorbic acid may cause regression of some advanced human cancers and extend the life span of patients with terminal malignancy, compared with historical controls [28, 29]. Later in the 1980s controlled studies demonstrated that massive doses of ascorbic acid have minor effects on side effects of chemotherapy and radiation and produce a slight increase in survival times of human cancer patients [24].

Biosynthesis of thioretinamide and thioretinaco by cystathionine synthase

Nutritional studies demonstrated that the hyperhomocysteinemia of protein energy malnutrition is associated with reduction in levels of plasma transthyretin, the plasma protein that transports retinol binding protein and thyroxine [30]. The metabolic disorder caused by protein energy malnutrition produces down-regulation of cystathionine synthase activity, leading to hyperhomocysteinemia and decreased synthesis of cystathionine and cysteine from methionine [31]. Transthyretin contains abundant tryptophan, and the plasma level of transthyretin declines in protein energy malnutrition because of dietary deficiency of tryptophan and other essential amino acids, leading to decreased endogenous synthesis of transthyretin.

Plasma transthyretin is a sensitive indicator of protein energy malnutrition [32]. As shown in **Figure 2**, decreased plasma transthyretin in protein energy malnutrition is accompanied by elevation of plasma homocysteine, which is a biomarker for dietary sulfur deficiency [33].

The heme oxygenase function of cystathionine synthase catalyzes the generation of superoxide radical from dioxygen [34]. Retinoic acid enhances the stimulation by thyroid hormone of heme oxygenase activity in the liver of thyroidectomized rats, demonstrating interaction between retinoic acid and the heme group of heme oxygenase [35]. N-(4-hydroxyphenyl)-retinamide, known as fenretinide, induces apoptosis in retinal cells through reactive oxygen species generation and through increased expression of heme oxygenase [36]. Investigation of the properties of fenretinide demonstrates antineoplastic potential, based on animal and clinical studies, because of the ability of this synthetic retinamide to induce apoptosis in malignant cells [37].

Taken together, these observations [30-36] indicate that retinol is delivered to cells by the retinol binding protein component of transthyretin, and the heme oxygenase function of cystathionine synthase is responsible for the oxidation of retinol to retinoic acid by superoxide and the subsequent reaction of retinoic acid with homocysteine thiolactone to produce thioretinamide. This process is catalyzed by binding of dehydroascorbate to the heme group of cystathionine synthase and by the production of superoxide radical from dioxygen. In catalyzing this reaction of retinol with superoxide radical, dehydroascorbate is simultaneously reduced to semidehydroascorbate radical, and thioretinamide is formed by the reaction of homocysteine thiolactone with enzyme-bound retinoic acid. These reactions are illustrated as follows:

(1) retinol + superoxide radical + dehydroascorbate → retinoic acid + semidehydroascorbate radical + water

(2) retinoic acid + homocysteine thiolactone → thioretinamide + water

This formulation of the synthesis of thioretinamide from retinol and homocysteine thiolactone by the heme oxygenase function of cystathionine synthase explains the failure of sulfate synthesis from homocysteine thiolactone in experimental scurvy and the function of dehydroascorbate in sulfate synthesis [20]. Thioretinamide is a precursor of thioretinaco by reaction with cobalamin [16]. Presumably the biosynthesis of thioretinaco is facilitated by interaction of the corrin group of cobalamin with the heme group of cystathionine synthase and subsequent complex formation between newly formed thioretinamide and cobalamin. Thioretinaco ozonide catalyzes the process of oxygen utilization in oxidative phosphorylation by forming a complex with adenosine triphosphate (ATP) [2]. The synthesis of thioretinaco from thioretinamide is facilitated by thyroxine that is transported by plasma transthyretin, explaining how oxidative metabolism is stimulated by thyroxine [35]. Only higher eukaryotes contain cystathionine synthase with a heme functional group, and the cystathionine synthase of prokaryotes, such as yeast and flagellates, contains no heme functional group [38]. Embryonic and malignant cells are deficient in the activity of cystathionine synthase [39]. Hence, this formulation explains why malignant cells are deficient in oxidation of homocysteine thiolactone to sulfate, since they lack the heme oxygenase function of cystathionine synthase.

Retinoids, including all-trans retinoic acid, have been evaluated for prevention of experimental and human carcinogenesis in bronchial and oral tissues [40]. Retinoic acid therapy produces remissions in acute promyelocytic leukemia [41,42]. Synthetic retinoids, such as fenretinide are antiproliferative and induce apoptosis in breast cancer cells [36,43,44]. Fenretinide is bound to retinol binding protein, a component of transthyretin, and is eliminated from plasma by loss of retinol binding protein through glomerular filtration because of decreased affinity of the complex for transthyretin [45,46]. It is likely that thioretinamide can also be bound to retinol binding protein for transport to tissues by plasma transthyretin, but no evidence has been reported to support this suggestion.

The trophoblastic origin of malignant cells

The embryologist John Beard discovered that trophoblastic cells of the embryo, which invade the

uterine endometrium and myometrium during implantation of the fertilized embryo, are related to the asexual cycle of cellular organisms and are converted to placental cytotrophoblastic and syncytiotrophoblastic cells by the action of enzymes produced by the pancreas of the developing fetus [47]. Based on the concept that trophoblastic cells, which are distributed within developing tissues of the fetus, are similar in their cellular behavior to malignant cells, Beard introduced the enzyme treatment of cancer. This treatment consists of injecting enzymes and pro-enzymes extracted from porcine pancreas into patients with various forms of primary or metastatic cancer. The trophoblastic theory of the origin of cancer is based on the assumption that malignant cells arise from adult stem cells that are related to the trophoblastic cells which migrate from the yolk sac of the developing embryo into somatic tissues, as described by Beard.

Human fetal and malignant cells produce small quantities of chorionic gonadotrophin [48]. This hormone is produced in large quantities by the highly malignant tumor of placenta, choriocarcinoma. These observations provide evidence for the trophoblastic origin of malignant cells. Although the origin of adult stem cells in normal human tissues is currently poorly understood, the sensitivity of trophoblastic cells to oncolysis by pancreatic enzymes and pro-enzymes forms the theoretical basis for this therapeutic approach [49,50]. This sensitivity may be related to the accumulation of homocysteinylated enzymes, plasma proteins, and cellular proteins, ribonucleic acid, deoxyribonucleic acid, and glycosaminoglycans by reaction with excess homocysteine thiolactone that accumulates during aging, atherogenesis, carcinogenesis, and autoimmune diseases [2,51]. Pancreatic extracts contain active trypsin, chymotrypsin, hyaluronidase, elastase, amylase, lipase, ribonuclease, and deoxyribonuclease, as well as pro-enzyme precursors of these digestive enzymes. These enzymes are capable of hydrolyzing the homocysteinylated proteins, nucleic acids and glycosaminoglycans that accumulate in malignant tissues and in the tissues of aging persons.

The parenteral enzymatic therapy of cancer failed to realize its potential because of difficulties in preparation of pancreatic enzyme extracts of consistent potency, potential problems with immunological responses to foreign porcine proteins, and disapproval of the use of parenteral preparations of pancreatic enzymes by governmental authorities. Instead the approaches of radical surgery, radiation therapy, and chemotherapy have been the mainstays of cancer therapy for the past century. Recent studies have demonstrated, however, that pancreatic enzymes and pro-enzymes are absorbed through the intestinal mucosa, producing an entero-pancreatic circulation of digestive enzymes and conserving the energy required for their biosynthesis [52,53]. Some success has been claimed recently for enzymatic therapy of experimental and human cancer utilizing oral pancreatic enzyme preparations [49,50].

The enzyme cystathionase (cystathionine γ-lyase) is absent from the liver of the human fetus and premature infants, and the activities of the enzymes, cystathionine synthase and adenosyl methionine synthase are at a level of about 15% to 25% that of adult human liver [54]. This discovery shows that the trans-sulfuration pathway for conversion of homocysteine to cystathionine, cysteine and sulfate is inactive in fetal tissues. Therefore, the pathway for synthesis of sulfate from homocysteine thiolactone, involving synthesis of thioretinamide from retinol and homocysteine thiolactone and subsequent oxidation of thioretinamide to sulfite and sulfate by superoxide, is the source of sulfate groups of glycosaminoglycans utilized in the growth of fetal cells and tissues **(Figure 1)**. The fetal cystathionine synthase assayed in human tissues contains the heme oxygenase function of the enzyme, since sulfate groups of glycosaminoglycans and other molecules are synthesized from the sulfur atom of methionine and homocysteine in embryonic tissues.

Glycolysis, nitrilosides, and hydrogen sulfide in malignant cells

As previously discussed, Warburg discovered in the early 20th century that embryonic tissues and malignant cells preferentially metabolize glucose to lactate as a source of cellular energy in comparison with utilization of oxygen for cellular metabolism [26]. In other studies, Warburg showed that

carcinogenic chemicals decrease normal cellular respiration by irreversible inhibition of oxygenases and by irreversible inhibition of electron transport by cytochrome enzyme systems. These findings are supported by the demonstration of deficient succinic dehydrogenase and cytochrome oxidase activities within malignant tissues [55].

Taken together these early observations [26,47,55] can be interpreted as examples of the clonal selection of malignant cells from trophoblastic stem cells that are deficient in the heme oxidase activity of cystathionine synthase. The resulting failure of oxidation of retinol to retinoic acid and failure of reaction of retinoic acid with homocysteine thiolactone to produce thioretinamide by malignant cell clones lacking the heme oxygenase activity of cystathionine synthase will lead to deficient formation of thioretinaco and the failure of oxidative phosphorylation catalyzed by thioretinaco ozonide [2]. The failure of oxidative phosphorylation by these malignant cell clones will lead to an embryonic form of metabolism in malignant tissues in which ATP synthesis is dependent upon the production of lactate from glucose, known as aerobic glycolysis.

Nitrilosides are substances containing nitrile groups produced by plants [56]. Nitrilosides are widely distributed in grasses, roots, legumes, fruits and berries. An important plant nitriloside is amygdalin (mandelonitrile β-diglucoside), found in apricot and fruit kernels. The nitrilosides dhurrin (hydroxymandelonitrile β-glucoside), lotaustralin (methylethyl-ketone-cyanohydrin β-glucoside), and linamarin (acetone-cyanohydrin β-glucoside) are cyanogens found in tropical plants such as cassava roots [56].

Both normal and malignant cells contain glucosidase, the enzyme that metabolizes amygdalin and other nitrilosides to cyanide [56]. Normal cells contain rhodanese, a sulfotransferase enzyme that catalyzes thiocyanate synthesis from cyanide and thiosulfate. Thiocyanate is relatively non-toxic, causing inhibition of thyroid function only with prolonged dietary or therapeutic exposure. Malignant cells contain insufficient rodanese to catalyze thiocyanate synthesis from cyanide and thiosulfate, theoretically leading to a toxic accumulation of cyanide within these cells [56]. Cyanide from consumption of inadequately processed cassava is implicated in konzo, a toxic upper motor neuron myelopathy associated with protein energy malnutrition [57]. The conversion of cyanide to thiocyanate is inhibited in those with a dietary deficiency of sulfur amino acids, and cyanide is instead converted to cyanate, a known neurotoxin, as shown in experimental studies [58].

The prevention and control of growth of malignant cells and tissues by dietary nitrilosides are attributable to the accumulation of cyanide within malignant cells because of a deficiency of rhodanese [56]. The reaction of cyanide with the cobalt atom of thioretinaco inactivates thioretinaco ozonide, thereby preventing oxidative phosphorylation [4]. The growth of malignant cells in animals is inhibited by hydrogen cyanide [56], presumably because of decreased concentrations of mitochondrial thioretinaco ozonide [2]. Thus this system of chemical surveillance against the growth of trophoblastic malignant cell clones is promoted by dietary or supplemental consumption of amygdalin and other plant nitrilosides as a source of cyanide.

Hydrogen sulfide is generated from homocysteine by cystathionine synthase and from cystathionine by cystathionase, and low levels of hydrogen sulfide decrease oxidative stress and ameliorate pathological conditions such as ischemia-reperfusion injury, hypertension, and renal failure [59]. Hydrogen sulfide is a key gasotransmitter in sensing oxygen availability in tissues [60]. The reducing properties of hydrogen sulfide are responsible for scavenging the reactive oxygen species production induced by increased blood levels of homocysteine, inhibiting myocardial injury [61]. Increased production of hydrogen sulfide from homocysteine, metabolized from homocysteinylated proteins, nucleic acids, and glycosaminoglycans of apoptotic cells by pancreatic enzymes, will presumably promote catabolism of homocysteine and conversion of the sulfur atom of homocysteine to thiocyanate by the reaction of thiosulfate with the cyanide generated from dietary nitrilosides. Cyanide is converted to thiocyanate by reacting with thiosulfate, catalyzed by rhodanese; thiosulfate is produced by oxidation of hydrogen sulfide, catalyzed by sulfide oxidase [62].

Homocysteine, thioretinamide, and thioretinaco in degenerative diseases

Since the discovery of the atherogenic properties of homocysteine in 1969 [6], an elevated level of homocysteine has been demonstrated in the plasma of persons with a wide variety of chronic degenerative diseases [5]. A partial list of these conditions includes arteriosclerosis, stroke, acute coronary syndrome, cancer, osteoporosis and fracture, dementia and other neurodegenerative diseases, autoimmune diseases such as lupus erythematosus, ulcerative colitis, thyroiditis, rheumatoid arthritis and pernicious anemia, venous thrombosis and pulmonary embolism, retinal vein thrombosis, hypothyroidism, accelerated aging, renal failure and uremia, diabetes mellitus, metabolic syndrome, macular degeneration, psoriasis, organ transplantation with therapeutic immune suppression, protein energy malnutrition, familial or spontaneous amyloidosis, dietary vitamin deficiencies of folate, pyridoxal, and cobalamin, complications of pregnancy such as pre-ecclampsia and placenta previa, and congenital birth defects, including neural tube defects, cleft palate, and congenital heart disease.

The etiology of many of these degenerative diseases and conditions is incompletely understood. However, susceptibility to many of these chronic degenerative diseases is correlated with the aging process. The importance of deficiencies of thioretinaco ozonide in cells of aging tissues is related to the accumulation of homocysteine thiolactone and to the homocysteinylation of macromolecules [2,3]. Regardless of etiology, however, elevated plasma homocysteine levels and the homocysteinylation of macromolecules in chronic degenerative diseases are susceptible to therapeutic intervention by the preservation of cellular oxidative metabolism through increased production of thioretinaco ozonide and by enhanced catabolism of homocysteine produced by enzymatic degradation of homocysteinylated macromolecules. Moreover, preservation of cellular thioretinaco ozonide by membranergic proteins and by the liposomal complex of ATP and oxygen with thioretinaco may prolong survival and counteract the aging process [18,19].

Infections, homocysteine, ozone and the vulnerable plaque

Increasing evidence supports the role of infectious organisms in the pathogenesis of arteriosclerotic plaques [63]. Remnants of infectious micro-organisms, such as Staphylococcus, Streptococcus, Salmonella, Herpes simplex, Escherichia coli, Chlamydia pneumoniae, Mycoplasma pneumonia, Porphyromonas, other dental organisms, Helicobacter pylori, and Archeae, are detected within plaques by immunohistochemistry, by electron microscopy, or by hybridization with DNA oligonucleotides directed against microbial nucleic acids. In the case of Chlamydia pneumoniae, live organisms have been cultured from plaques. The lipoproteins of the plasma constitute an innate immune system capable of inactivating a wide variety of infectious organisms and their toxins by the aggregation of microbes with lipoproteins [63].

Homocysteine thiolactone reacts with the free amino groups of the apoB protein of low-density lipoproteins to form aggregates that undergo spontaneous precipitation in vitro [64]. Vulnerable plaques of arteries in atherosclerosis are hypothesized to originate from the obstruction of *vasa vasorum* of arterial walls by aggregates formed from lipoproteins complexed with microbial remnants, homocysteinylated and oxidized lipoproteins, and lipoprotein autoantibodies in areas of high tissue pressure, causing ischemia, degeneration of arterial wall cells and rupture into arterial intima to form a micro - abscess [63]. The obstruction of *vasa vasorum* by lipoprotein aggregates may be exacerbated by swelling and hyperplasia of endothelial cells, as well as by fibrin deposition in the walls of arterioles [6]. These changes in endothelial cell structure and function are manifestations of the endothelial dysfunction caused by hyperhomocysteinemia [4]. Increasing evidence also implicates the presence of microbial remnants within the extracellular amyloid plaques and neurofibrillary tangles within neurons as a factor in the pathogenesis of dementia and neurodegenerative diseases [65].

The evidence that human arteriosclerotic plaques contain ozone [66] supports the theoretical role of

ozone in the activation of thioretinaco to form thioretinaco ozonide as the active site for oxidative phosphorylation [2]. The demonstration of ozone and ozonolysis products from cholesterol oxidation in plaques supports the function of ozone in atherogenesis [66]. Cholesterol oxides are atherogenic in animals and accumulate during human atherogenesis [67]. However, the significance of the oxidation of cholesterol and lipoproteins in atherogenesis is less clear [63]. The plasma concentration of oxidized lipoproteins or cholesterol oxides predicts coronary artery disease in healthy men [68] and in patients with acute coronary syndrome [69]. Anti-inflammatory drugs exacerbate cardiovascular disease, showing that suppression of inflammation by these drugs does not inhibit atherogenesis. If oxidized lipoproteins or cholesterol oxides were responsible for inciting the inflammation leading to atherogenesis, these drugs should suppress rather than exacerbate this process. The presence of ozone in plaques, however, may be a response to the presence of microbes, remnants of which have been demonstrated in vulnerable plaques [63].

Insulin resistance, obesity, fatty liver disease, and carcinogenesis

Autopsy studies show that children with homocystinuria frequently develop fatty liver, regardless of which enzyme deficiency is the cause of hyperhomocysteinemia [6,7,70]. Although hyperinsulinemia and insulin resistance occur in some individuals with homocystinuria [71], diabetes is not a usual complication of the natural history of homocystinuria [72]. However, hyperhomocysteinemia is a characteristic finding in diabetic subjects with microalbuminuria and renal failure, and hyperhomocysteinemia is associated with insulin resistance, obesity and hypertension, as shown by the Framingham Offspring Study [73]. An experimental study demonstrated hyperhomocysteinemia, hyperinsulinemia, and obesity in rats fed a high fat-sucrose diet, associated with decreased cystathionine synthase activity and increased methylenetetrahydrofolate reductase activity [74].

Increasing evidence implicates fructose consumption from sucrose and high fructose corn syrup in the etiology of metabolic syndrome and non-alcoholic fatty liver disease [75,76]. Both dietary fructose and ethanol generate reactive oxygen radicals in the form of superoxide. Moreover, fructose combines with free amino groups of protein, resulting in fructosylation of macromolecules in a manner similar to the glucosylation of the free amino groups of hemoglobin to produce hemoglobin A1C. Thus dietary fructose increases oxidative stress and causes fructosylation of macromolecules in a process analogous to the oxidative stress and homocysteinylation of free amino groups of macromolecules caused by hyperhomocysteinemia in aging and degenerative diseases [3,51]. Deficiency of thioretinaco ozonide produced by aging, atherogenesis, or carcinogenesis theoretically reduces the anti-oxidant capacity of mitochondria and leads to the oxidation reactions that are characteristic of diseases of aging [3]. Moreover, the decreased capacity of oxidative phosphorylation associated with a deficiency of thioretinaco ozonide function presumably leads to the increased biosynthesis of fatty acids and cholesterol from the resulting excess acetyl-coenzyme A, which is characteristic of fatty liver and the metabolic syndrome [3,75,76].

Levels of serum retinol binding protein (RBP4) are increased in mice with insulin resistance caused by deletion of the adipocyte GLUT4 glucose transporter genes [77]. Serum RBP4 levels are increased in human subjects with metabolic syndrome, including obesity, insulin resistance, elevated serum triglyceride levels, elevated systolic blood pressure, and decreased HDL levels [77,78]. The synthetic retinoid fenretinide lowers serum levels of RBP4 by increasing urinary excretion of RBP4 and decreases insulin resistance both in experimental animals [77] and in human obese premenopausal women with early breast cancer [79]. RBP4 is a limiting factor for plasma transport of retinol because of its binding to transthyretin [80], and retinol is a precursor of thioretinamide, as previously discussed. Thioretinamide has a preventive effect on arteriosclerotic plaques produced by parenteral homocysteine thiolactone in experimental animals [17], but there are no published reports concerning the possible effect of thioretinamide on insulin resistance either in experimental animals or human subjects. The elevation of RBP4 levels in metabolic syndrome and hyperhomocysteinemia may reflect

decreased endogenous synthesis of thioretinamide from retinol bound to RBP4.

Non-alcoholic fatty liver disease (NAFLD) is increasing in incidence in many populations, and this condition progresses to non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma in a significant number of individuals [81]. In addition to obesity and the consumption of fructose and fats, NAFLD may be related to exposure to industrial toxins, drugs like amiodarone or tamoxifen, total parenteral nutrition, and jejuno-ileal bypass surgery [82].

Oxidative stress is well documented in NASH, as demonstrated by increased immunohistochemical staining for 3-nitrotyrosine, increased levels of thioredoxin, mitochondrial dysfunction, and lipid peroxidation [82]. Adenosyl methionine is deficient in many types of liver disease, and decreased synthesis of adenosyl methionine, cysteine, and glutathione is demonstrated in alcoholic liver disease (ALD) [83]. Null mice for methionine adenosyl transferase (MAT1) are predisposed to steatohepatitis and the increased expression of cellular proliferation genes [84].

Insulin resistance and metabolic syndrome are associated with NAFLD and NASH [82,85]. NAFLD is associated with endothelial dysfunction in newly diagnosed subjects with type II diabetes [86], and intimal medial thickness is positively correlated with RPB4 and negatively correlated with retinol in subjects with metabolic syndrome [87]. In addition, low plasma retinol predicts coronary events in healthy men [88].

A role for retinol, retinoic acid, and thioretinamide in insulin resistance is suggested by the observation of weight loss, increased insulin sensitivity, decreased fasting plasma glucose, decreased plasma retinol and RBP4 by the administration of retinoic acid in obese mice [89,90]. Retinoic acid activates the nuclear receptor peroxisome proliferation-activated receptor β/δ (PPARβ/δ) and retinoic acid receptors (RARs) and restores these suppressed receptors in obese mice [89]. These results suggest that retinoic acid may be useful in the treatment of metabolic syndrome and type II diabetes. The decreased retinol observed in these experiments is compatible with the function of retinol as a precursor of thioretinamide, as previously discussed. In addition, thioretinamide may prove to be useful in increasing insulin sensitivity in metabolic syndrome.

The demonstration of retinaldehyde in the adipose tissue of obese mice and the repression of adipogenesis and diet-induced obesity by retinaldehyde in mice [91] are compatible with a role for thioretinamide in the regulation of insulin sensitivity and in the stimulation of oxidative metabolism through conversion to thioretinaco [3]. The elevation of RBP4 in obese children and adolescents with impaired glucose tolerance suggests that decreased synthesis of thioretinamide from retinol may contribute to insulin resistance [92].

Hepatocellular carcinoma incidence increased significantly in the USA during the past two decades, and diabetes increases the risk of this malignancy [93]. Many cases of hepatocellular carcinoma that are unassociated with known risk factors for this disease, including hepatitis B, hepatitis C, and alcoholism, are attributed to nonalcoholic fatty liver disease (NAFLD), which is the hepatic manifestation of the metabolic syndrome [94]. Moreover, many cases of hepatocellular carcinoma develop in NAFLD without evidence of the development of cirrhosis [95-97]. A recent report demonstrated increased RBP4 expression in cultured cell lines and increased RBP4 levels in the serum from patients with hepatocellular carcinoma [98]. Serum RBP4 was identified as an independent factor for overall survival and disease-free survival in patients with hepatocellular carcinoma. These findings are compatible with the interpretation of the decreased synthesis of thioretinamide from retinol and the decreased conversion of thioretinamide to thioretinaco as critical factors in metabolic syndrome, NAFLD, and hepatic carcinogenesis [2].

Metabolic and nutritional therapy of degenerative diseases

The elucidation of the function of thioretinamide, thioretinaco and cystathionine synthase in the etiology of hyperhomocysteinemia in degenerative diseases led to a proposal for a metabolic and nutritional protocol for treatment and prevention of diseases of aging [99]. The rationale of this proposal is that administration of thioretinamide as a precursor of thioretinaco synthesis from cobalamin will

enhance oxidative metabolism by increased formation of thioretinaco ozonide, decreasing oxidative stress by metabolizing oxygen radical compounds, and decreasing the formation of homocysteine that accumulates in degenerative diseases [2]. Pancreatic enzymes and pro-enzymes are combined with thioretinamide to increase catabolism of homocysteinylated proteins, nucleic acids, and glycosaminoglycans that arise from the excessive formation of homocysteine thiolactone during aging [3]. Another aspect of this protocol is the use of retinol as a precursor of thioretinamide by enhancing the oxidation of retinol to retinoic acid, increasing endogenous synthesis of thioretinamide, thioretinaco and thioretinaco ozonide.

This metabolic and nutritional protocol for the control of degenerative diseases depends upon a combined approach to enhancing endogenous synthesis of thioretinaco ozonide with a metabolic program designed to increase the catabolism of homocysteine released from homocysteinylated proteins, nucleic acids, and glycosaminoglycans by pancreatic enzymes. As a part of the protocol, the vitamins folate, pyridoxal, and cobalamin enhance the activity of cystathionine synthase and methionine synthase, increasing the conversion of homocysteine to cystathionine by trans-sulfuration and conversion of homocysteine to methionine, by remethylation. The coenzyme adenosyl methionine is employed to activate cystathionine synthase and to inhibit methylenetetrahydrofolate reductase by allosteric effects on these enzymes. Essential amino acids, including tryptophan, are employed to enhance synthesis of transthyretin, the plasma carrier of retinol binding protein and retinol, increasing the delivery of retinol to cells for synthesis of thioretinamide and thioretinaco ozonide. Ascorbate with mixed bioflavonoids is employed to provide dehydroascorbate that is required for oxidation of retinol to retinoic acid in the synthesis of thioretinamide by cystathionine synthase. Nitrilosides from plants or supplements, such as amygdalin, are employed to provide cyanide for reaction with hydrogen sulfide that is produced by the action of cystathionine synthase and cystathionase on homocysteine and cystathionine, respectively, to synthesize thiocyanate through intermediate formation of thiosulfate. In diseases exacerbated by a microbial etiology, such as arteriosclerosis and dementia, the use of appropriate antibiotics and medium chain saturated triglycerides is employed to counteract microbial growth and inflammation, thereby facilitating the resolution of pathological lesions by metabolic, nutritional, and enzyme therapy.

Human chronic degenerative diseases that are associated with aging are characterized by oxidative stress produced by an increased number of free radicals that damage cellular constituents and lead to the accumulation of altered proteins within cells and tissues during the disease process [3]. The oxidative stress of these degenerative diseases is produced by the effect of excess metabolic accumulation of homocysteine and homocysteine thiolactone, which interferes with normal oxidative phosphorylation catalyzed by thioretinaco ozonide [2]. This nutritional and metabolic program theoretically ameliorates ineffective metabolic regulation of oxidative stress in human disease by enhancing endogenous synthesis of thioretinamide and thioretinaco within cells and tissues, thereby stimulating cellular oxidative metabolism and reducing the endogenous accumulation of reactive oxygen species. In addition, the program theoretically decreases the overproduction of homocysteine thiolactone from methionine by increasing its conversion to cysteine, metabolites of cysteine, and sulfate, preventing the deleterious homocysteinylation of macromolecules that is characteristic of degenerative diseases of aging.

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Dedication

This review is dedicated to the memory of F. William Sunderman, Jr., M.D. (1931-2011), a cherished mentor, colleague and lifelong friend.

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