

Anti-Atherogenic Effects of a Mixture of Ascorbic Acid, Lysine, Proline, Arginine, Cysteine, and Green Tea Phenolics in Human Aortic Smooth Muscle Cells

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Abstract: Certain drastic behavioral modifications by arterial wall smooth muscle cells (SMC) have been considered key steps in the formation of atherosclerotic lesions: massive migration of SMC from the media to the intima layer of the vessel, dedifferentiation of SMC to proliferating phenotype, and increased secretion of inflammatory cytokines as a response to inflammatory stimuli. We investigated the anti-atherogenic effects of naturally occurring compounds (ascorbic acid, green tea extract, lysine, proline, arginine, and N-acetyl cysteine) using the model of cultured aortic SMC. Cell growth was measured by DNA synthesis, cell invasiveness was measured through Matrigel, matrix metalloproteinase-2 (MMP-2) secretion was measured by zymography, and SMC secretion of monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6) was measured by immunochemistry. Fetal bovine serum-stimulated SMC growth was inhibited by the nutrient mixture (NM) with 85% inhibition at 100 $\mu\text{g}/\text{mL}$. A corresponding concentration of epigallocatechin gallate (EGCG; 15 μM), the most active tea phenolic, produced a significant effect but one lower than NM. NM inhibited aortic SMC Matrigel invasion in a dose-dependent manner and significantly decreased MMP-2 expression. Stimulation of SMC with tumor necrosis factor- α significantly increased production and secretion of such mediators of inflammation as IL-6 and MCP-1; addition of 100 $\mu\text{g}/\text{mL}$ NM inhibited secretion of MCP-1 and IL-6 by 65% and 47%, respectively. These data suggest that the NM of ascorbic acid, tea phenolics, and selected amino acids has potential in blocking the development of atherosclerotic lesions by inhibiting atherogenic responses of vascular SMC to pathologic stimuli and warrants in vivo studies.

Key Words: atherosclerosis, human aortic smooth muscle cell, cytokines, matrix metalloproteinases

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INTRODUCTION

Atherosclerosis and its associated vascular complications are the principal causes of cardiovascular and cerebrovascular diseases leading to myocardial infarction and stroke,

respectively. Every year more than 12 million people worldwide die from the results of atherosclerosis, heart infarctions, and strokes. According to the American Heart Association's 2004 *Heart and Stroke Statistical Update*, more than 79 million people worldwide suffer from cardiovascular disease, which has been the leading cause of death in the United States for decades.¹

The formation of an atherosclerotic lesion is associated with drastic behavioral modifications by arterial wall smooth muscle cells (SMC), including massive migration of SMC from the vascular medial to the intimal layer, dedifferentiation of SMC to proliferating phenotype, and increased secretion of inflammatory mediating cytokines as an autocrine response to inflammatory stimuli. These events trigger vessel wall thickening and monocyte recruitment from blood and lead to progression of the atherogenic cascade. Pathogenic changes of the blood vessel wall in atherosclerosis are accompanied by neointimal thickening resulting from the increased deposition of extracellular matrix (ECM) proteins by SMC that migrate and proliferate in the affected blood vessel areas.² Various pathophysiologic events can promote this process, such as inflammation involving local secretion of inflammatory mediators (cytokines), oxidative processes accompanying low-density lipoprotein and lipoprotein(a) deposition, and intracellular membrane-mediated events such as changes in protein kinase C activity.^{3,4} Various matrix components also affect cellular proliferation, differentiation, and expression of specific genes.⁵

The ECM serves as a reservoir to which various growth factors may be bound affecting vascular cell growth, differentiation status, and ECM production. Thus, factors affecting matrix components may influence various metabolic processes that accompany initiation and progression of atherosclerosis.

Rath et al proposed that chronic subclinical vitamin C deficiency is a primary cause of atherosclerotic plaque formation because it leads to deposition of lipoprotein(a) and fibrinogen/fibrin in the vascular wall.⁶ Ascorbic acid is essential to the synthesis and maintenance of collagen, which maintains blood vessel wall stability. Prolonged deficiency of ascorbic acid, a nutrient not produced in the body, hinders the enzymatic hydroxylation of proline and lysine residues in collagen molecules, thereby weakening the stability of the vascular wall. Thus, combined supplementation with ascorbic acid and lysine has been proposed as a preventive measure to atherosclerosis development.^{6,7} A previous study of the direct

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and matrix-mediated effects of ascorbate on the proliferation rate of vascular smooth muscle cells (VSMC) isolated from guinea-pig aorta revealed diminished cell proliferation in the presence of 0.5–2.0 mM ascorbate, in a dose-dependent manner without cytotoxic effect.⁸ Ascorbate has been shown to induce SMC differentiation,⁹ which is characterized by reduction in cell growth. Additionally, a number of studies show cardioprotective effects, through potent antioxidant activity, from chronic tea consumption.¹⁰

Naturally occurring compounds demonstrate a wider spectrum of biological activity and fewer side effects than synthetic drugs, and a mixture of natural compounds often produces synergistically enhanced therapeutic actions. This reasoning prompted us to investigate whether a mixture of nutrients (NM), including ascorbic acid, lysine, proline, arginine, N-acetyl cysteine, and epigallocatechin gallate (EGCG; from green tea extract), would modulate the atherogenic effects of pathogenic stimuli, such as tumor necrosis factor- α (TNF- α), using the model of cultured VSMC.

MATERIALS AND METHODS

Materials

Tissue culture plastics were obtained from Becton Dickinson (USA). Tissue culture supplies (growth media, antibiotics, and trypsin-ethylenediamine tetraacetic acid) were obtained from Life Technologies (USA). Fetal bovine serum (FBS) was from BioWhittaker (Walkersville, Maryland, USA). Scintillation fluid BetaBlend and [methyl-³H] Thymidine (25 Ci/mole) were from ICN Biomedicals (Costa Mesa, California, USA). L-ascorbic acid, bovine serum albumin (BSA; fraction V), and other chemicals were from Sigma-Aldrich (USA).

Cell Culture

Human aortic smooth muscle cells (obtained from Clonetics) were cultured in DMEM (Dulbecco's modified Eagle's medium), supplemented with 10% FBS, penicillin (100 μ g/mL), and streptomycin (100 μ g/mL) at 37°C in a humidified atmosphere containing 5% CO₂, and were split 1:3 to 1:5 on reaching the confluence. SMC at passages 5–8 were used in experiments.

Cell Proliferation Assay

SMC proliferation was assayed by [³H]-thymidine incorporation into cellular genetic material. Cells were plated in 24-well plates at a density of 10,000 cells per cm² in 0.5 mL of DMEM supplemented with 2% FBS. The attached cells were supplied every 24 hours with fresh growth medium plus additions, as specified in the protocols. Test agents included the NM and individual components. A stock solution of the NM was prepared daily immediately before addition to cell cultures by dissolving in DMEM to a concentration of 10 mg/mL, vigorously vortexed for 1 minute, and filtered through a 0.2 μ m sterile filter. Cell proliferation was measured 3 days later by the addition of 1 μ Ci/mL [³H]-thymidine to the cell culture for the last 24 hours of the experiment. Cells were washed 3 times with cold phosphate buffered saline (PBS), pH 7.2, incubated with 10% trichloroacetic acid for 15 minutes at 4°C, washed with cold ethanol, air-dried, solubilized in 0.5 N

sodium hydroxide, and then neutralized with hydrochloric acid. Samples were mixed with scintillation fluid and counted using a liquid scintillation counter (model 6500 LS, Beckman Instruments, USA). Cellular DNA-incorporated radioactivity was expressed as d/min per well.

Cell Invasion Assay

Invasion studies were conducted using Matrigel (Becton Dickinson) inserts in 24-well plates. Suspended in medium, human aortic SMC were supplemented with nutrients, as specified in the design of the experiment, and seeded on the insert in the well. Thus both the medium on the insert and in the well contained the same supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO₂ for 24 hours. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel membrane and migrated onto the lower surface of the Matrigel were stained with hematoxylin and eosin and visually counted under the microscope.

Gelatinase Zymography

MMP activity in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast SDS-polyacrylamide gel (SDS-PAGE; Invitrogen Corporation) in the presence of 0.1% gelatin in nonreduced conditions. Culture media (20 μ L) mixed with sample buffer were loaded and SDS-PAGE was performed with tris glycine SDS buffer as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM CaCl₂ at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and destained. Protein standards were run concurrently, and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

Cytokine Expression

Aortic SMC were plated into 24-well plastic plates at 50,000 cells per well and grown to confluence. Cell culture medium was replaced with 0.5 mL serum-free DMEM supplemented with 0.1% bovine serum protein and indicated amounts of the nutrient mixture. After incubation for 24 hours, media were replaced with fresh DMEM/BSA media containing the same amounts of nutrient mixture and 10 ng/mL of TNF- α or no stimulator as control. Following 24 hours of incubation, conditioned media were collected and frozen at -80°C individually for cytokine assay. Cell protein was measured by BCA protein micromethod (Pearce) after cell layer washing with PBS and dissolving in 0.1N NaOH for 2 hours at 37°C. Test samples of cell protein content per well did not differ significantly from control (un-supplemented) samples, indicating unimpaired cell viability. The cytokine level in cell-conditioned media was assayed with enzyme-linked immunosorbent assay (ELISA) kits (Quantikine, R&D

Systems) according to manufacturer's protocol. All experiments were performed at least twice in triplicates.

Morphology Study

Human aortic SMC were grown to confluence and then exposed to various concentrations of the nutrient mixture for 24 hours. They were stained with hematoxylin and eosin (H&E), and cell morphology was examined by microscopy and photographed.

Composition of the Nutrient Mixture

Green tea extract derived from green tea leaves was obtained from US Pharma Lab (Somerset, New Jersey). According to manufacturer's specification it contained total polyphenol 80%, catechins 60%; NM solution as a surplus to the basic DMEM composition contained 100 μ M ascorbic acid, 100 μ M lysine, 100 μ M proline, 50 μ M arginine, 25 μ M N-acetyl cysteine, and 15 μ M EGCG from green tea extract.

Statistical Analysis

The results for each representative study are expressed as mean \pm standard deviation for the groups. Data were analyzed by independent sample 2-tailed *t* test. Correlation coefficients were determined for dose-dependent studies using MecaCalc Software (Mariakerke, Belgium).

RESULTS

Effect of Individual Nutrients and the Nutrient Mixture on Human Aortic SMC Proliferation

FBS-stimulated growth of aortic SMC was inhibited by addition of the nutrient mixture in a dose-dependent manner (correlation coefficient $r = -0.9606$; $P = 0.0023$). The inhibitory effect of the nutrient mixture reached 85% of control at 100 μ g/mL NM ($P < 0.0001$) (Fig. 1).

In testing a corresponding concentration of the most active tea phenolic in the NM, EGCG (15 μ M), aortic SMC proliferation was inhibited by 59% over control ($P = 0.002$), in contrast to 85% ($P = 0.009$) when tested at the same concentration combined with other nutrients. The 26% difference

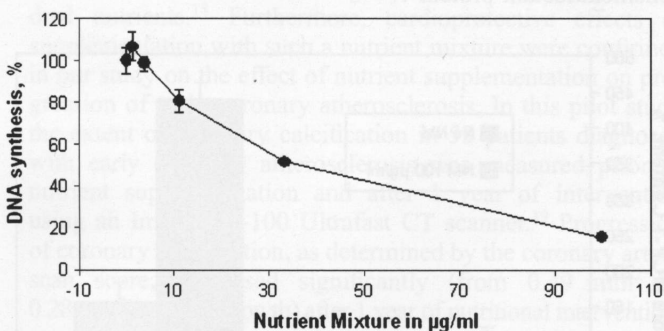


FIGURE 1. Nutrient mixture inhibits FBS-stimulated growth of human aortic SMC. Proliferating SMC cultures were incubated with indicated amounts of the nutrient mixture for 72 hours. Cell growth rate was evaluated by incorporation of [³H]-thymidine into cellular DNA during the last 24 hours of the experiment.

between the antiproliferative effect of EGCG and NM was significant ($P = 0.0009$). A corresponding concentration of ascorbic acid (100 μ M) had a stimulatory effect on SMC growth (167% of control). The mixture of EGCG and ascorbic acid neutralized the effects of each to yield a growth value near control. Other NM components did not produce any significant effects on SMC growth when used individually (Fig. 2). We had previously shown that ascorbic acid stimulates SMC growth at lower concentrations but inhibits cell growth at higher concentrations.⁸

Invasion Study

The nutrient mixture significantly reduced the penetration and migration of aortic SMC through Matrigel in a dose-dependent fashion, with 35% inhibition at 50 μ g/mL and 94% at 100 μ g/mL NM (correlation coefficient $r = -0.9606$, $P < 0.0001$), as shown in Figure 3.

Gelatinase Zymography Study

Inhibition of SMC invasion by the nutrient mixture was accompanied by a significant decrease in MMP-2 expression with complete inhibition at 1000 μ g/mL NM (Fig. 4).

Cytokine Expression

Stimulation of SMC with TNF- α caused a significant increase (293% of control; $P < 0.0001$) in the autocrine production and secretion of IL-6. Addition of 100 μ g/mL of the NM to the cell culture medium inhibited secretion of IL-6 by 46% ($P = 0.0002$) (Fig. 5). Induced aortic SMC secretion of monocyte attractant protein (MCP-1), which leads to migration of monocytes into the atherosclerotic plaque, was almost completely reversed in the presence of 100 μ g/mL of the nutrient mixture. TNF- α -induced MCP-1 expression increased to 436.7% of the control ($P < 0.0001$). In the presence of NM, SMC MCP-1 expression was inhibited to 153% ($P = 0.0002$) (Fig. 6).

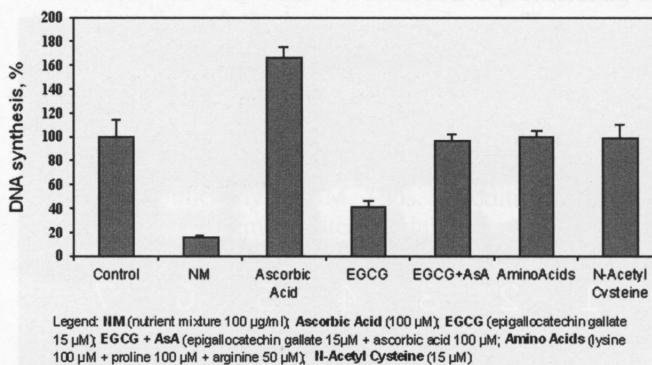


FIGURE 2. Comparison of the effects of individual components of the NM on SMC growth. Compounds were added to proliferating SMC cultures individually or as part of NM (100 μ g/mL): 100 μ M ascorbic acid (AsA); 15 μ M EGCG; 100 μ M lysine, 100 μ M proline and 50 μ M arginine (AminoAcids); and 15 μ M N-acetyl cysteine. Experimental conditions were as described in Figure 1.

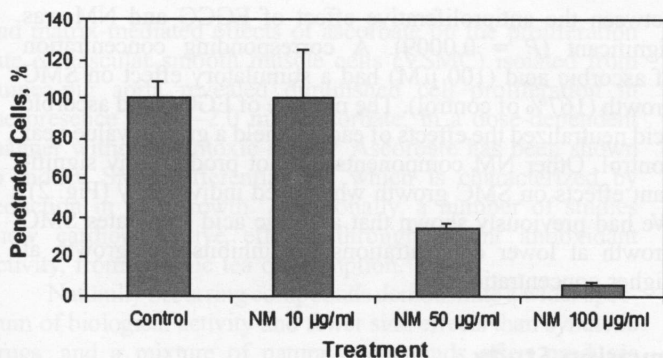


FIGURE 3. Effect of nutrient mixture on human aortic SMC invasion through Matrigel. SMC cultures were plated on top of Matrigel-covered membrane with 3.0 µm pores in serum-free medium containing indicated amounts of the nutrient mixture. Cell migration was initiated by addition of 10% FBS to the lower chamber. Cell invasion was estimated in 24 hours by counting cells migrated to the other side of the membrane and expressed as percentage of unsupplemented control.

Morphology Study (Hematoxylin and Eosin Staining)

The morphology study found no significant morphologic changes even at highest concentration of the NM (Fig. 7A–D).

DISCUSSION

The results from this study demonstrated that the nutrient mixture significantly attenuated the proatherogenic modification of SMC physiologic properties: increased growth rate, invasiveness, and production of inflammatory mediators (IL-6 and MCP-1). The dose-dependent inhibitory effect of the nutrient combination of lysine, proline, arginine, ascorbic acid, and green tea extract on MMP-2 expression of aortic SMC was consistent with its inhibition of matrix invasion, leading to enhanced vascular wall stability. In addition to

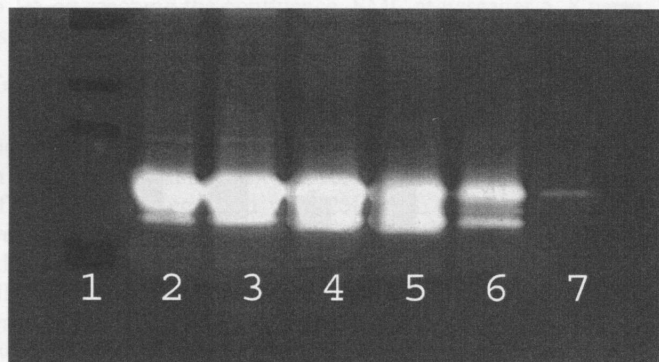


FIGURE 4. Effect of the nutrient mixture on MMP-2 secretion by human aortic SMC. Confluent SMC cultures were supplemented with indicated amounts of nutrient mixture for 24 hours in serum-free medium. MMP-2 activity was assayed by gelatinase zymography of conditioned media. Samples by lanes: 1-Markers, 2-7 0, 10, 50, 100, 500, and 1000 µg/mL NM.

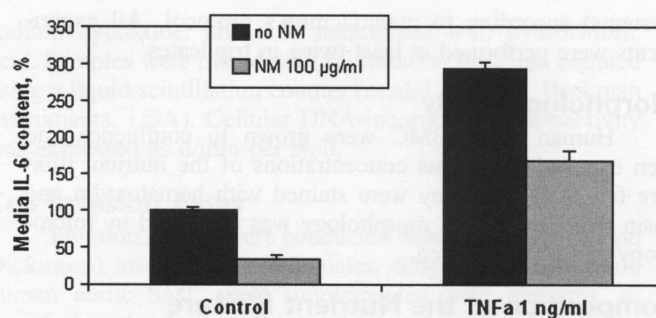


FIGURE 5. Effect of the NM on human aortic SMC secretion of IL-6: Confluent SMC cultures were incubated for 24 hours in serum-free medium supplemented or not with 1 ng/mL TNF-α and 100 µg/mL NM. IL-6 was assayed by ELISA in conditioned culture media.

inhibition of MMP secretion, stability of the vascular wall is dependent on the presence of sufficient quantities of ascorbic acid, lysine, and proline for synthesis of optimal collagen structure. This mixture of nutrients probably enhanced the stability and strength of the connective tissue because optimization of synthesis and structure of collagen fibrils depends on hydroxylation of proline and lysine residues in collagen fiber; ascorbic acid is essential for the hydroxylation of these amino acids. Various studies have shown that restructuring of the vascular matrix is facilitated by ascorbate, pyridoxine, L-lysine, and L-proline.^{11,12}

In addition, human aortic SMC expression of both inflammatory mediators (IL-6 and MCP-1) studied was enhanced by TNF-α and significantly and dramatically inhibited by the presence of the NM. Cellular adhesion molecules on the surface of endothelial cells cause monocyte adherence to the endothelium and subsequent migration into the arterial wall. Production of these adhesion molecules is directly stimulated by inflammatory cytokines. Several studies suggest that adhesion molecules may be an important target for the prevention and treatment of atherosclerosis and cardiovascular disease because formation of atherosclerotic lesions was found to be significantly decreased in mutant mice that do not express macrophage colony stimulating factors and monocyte chemoattractant protein-1.^{13,14}

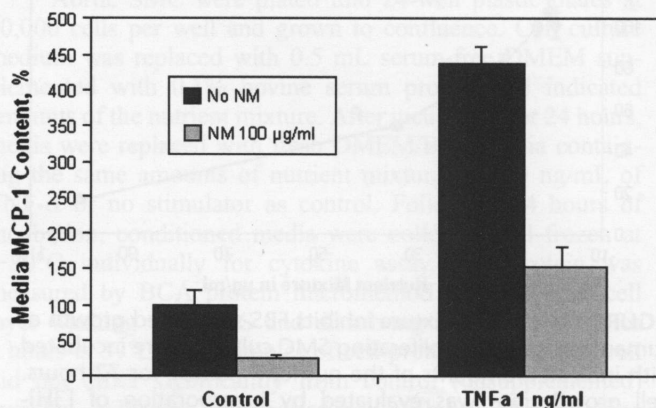


FIGURE 6. Effect of the NM on human aortic SMC secretion of MCP-1. Experimental conditions were as in Figure 5.

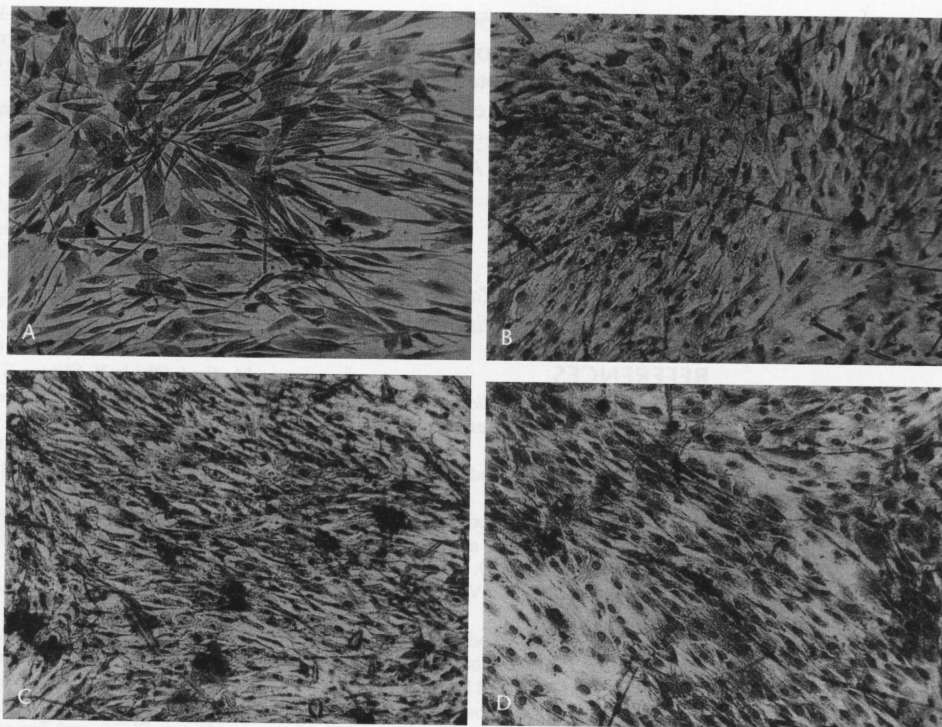


FIGURE 7. Effect of the NM on human aortic SMC morphology. Confluent SMC cultures were supplemented with indicated amounts of the nutrient mixture in serum-free medium for 24 hours. Cells were fixed and stained with hematoxylin and eosin. Cell morphology analysis revealed no signs of cytotoxicity at any NM dosage tested. A, Control; B, NM 100 $\mu\text{g/mL}$; C, NM 500 $\mu\text{g/mL}$; D, NM 1000 $\mu\text{g/mL}$.

The inhibitory effects of individual nutrients composing the mixture have been reported in both clinical and experimental studies. Anti-atherogenic effects of green tea extract have been demonstrated in animal and in vitro studies. For example, apoprotein E-deficient mice fed green tea for 14 weeks exhibited attenuation of aortic atheromatous areas by 23% and decreased aortic cholesterol and triglyceride levels over the control group.¹⁵ Furthermore, epidemiologic and clinical studies have documented the benefits of individual nutrients in prevention of cardiovascular disease.^{16,17}

However, nutrients are less powerful when acting independently than in combination. Our previous studies demonstrated that the anticancer effect of the mixture of ascorbic acid, proline, lysine, and EGCG on several cancer cell lines in tissue culture studies was greater than that of the individual nutrients.¹⁸ Furthermore, cardioprotective effects of supplementation with such a nutrient mixture were confirmed in our study on the effect of nutrient supplementation on progression of early coronary atherosclerosis. In this pilot study the extent of coronary calcification in 55 patients diagnosed with early coronary atherosclerosis was measured prior to nutrient supplementation and after 1 year of intervention using an Imatron C-100 Ultrafast CT scanner.¹⁹ Progression of coronary calcification, as determined by the coronary artery scan score, decreased significantly (from 0.49 mm^2 to 0.28 mm^2 monthly growth) after 1 year of nutritional intervention.

CONCLUSION

Although animal and clinical studies are necessary to better determine the efficacy of the nutrient mixture in atherosclerosis prevention and treatment, the results of this

study suggest that the formulation of ascorbic acid, tea phenolics, and selected amino acids tested has the potential to block the development of atherosclerotic lesions by inhibiting atherogenic responses of vascular SMC to pathological stimuli, such as aortic SMC proliferation, invasion and migration from the vessel medial to intimal layer, and secretion of inflammatory cytokines.

Gelatinase A has been determined to be essential for vascular cells to cross the basement membrane barrier and thus has been considered as a therapeutic target.²⁰ Thus we investigated the effect of NM on MMP-2 and found NM to significantly inhibit MMP-2 secretion. In addition, it has been found that cells must be in an active proliferating state to migrate through the basement membrane. This appears to be a limiting step in plaque formation that is governed by MMPs. Based on these findings, Dzau et al²¹ suggests therapeutic targeting of vascular SMC proliferation. Thus our research investigated aortic SMC proliferation, as well as invasion through Matrigel, and found these targets to be inhibited significantly by NM. Thus, in addition to inhibition of cytokines, we demonstrated modulation of other potential clinical atherogenic targets by the nutrient mixture using the model of human aortic SMC in vitro.

COMPETING INTERESTS

United States Patent Application 20050019429: Nutritional composition and method of inhibiting smooth muscle cell contraction thereof. Inventors: Ivanov, Vadim (Castro Valley, CA); Ivanova, Svetlana (Castro Valley, CA); Roomi, Waheed M (Sunnyvale, CA); Niedzwiecki, Aleksandra (San Jose, CA); Rath, Matthias (Almelo, NL).

United States Patent Application 20050032715: Composition and method for treatment of neoplastic diseases associated with elevated matrix metalloproteinase activities using catechin compounds. Inventors: Netke, Shrirang (San Bruno, CA); Ivanov, Vadim (Castro Valley, CA); Roomi, Waheed M (Sunnyvale, CA); Niedzwiecki, Aleksandra (San Jose, CA); Rath, Matthias (Almelo, NL).

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