Extracellular Matrix-Mediated Control of Aortic Smooth Muscle Cell Growth and Migration by a Combination of Ascorbic Acid, Lysine, Proline, and Catechins

Vadim Ivanov, PhD, Svetlana Ivanova, MD, M. Waheed Roomi, PhD, Tatiana Kalinovsky, MS, Aleksandra Niedzwiecki, PhD, and Matthias Rath, MD

Abstract: Extracellular matrix (ECM) function and structure are severely compromised at atherosclerotic lesion sites, contributing to initiation and progression of the disease. This study investigated whether ECM biological properties would be beneficially affected by exposure to nutrients essential for collagen synthesis and post-translational modification. Confluent layers of human aortic smooth muscle cells (SMC) grown on collagen substrate were cultured in the presence of the tested compounds for 7 to 10 days. Pretreated cells were removed from the ECM surface by differential treatment and replaced with secondary innocent SMC cultures. Secondary SMC growth rate and invasiveness were assayed in standard growth medium. ECM protein composition was assayed immunochemically. ECM produced in the presence of ascorbic acid reduced SMC proliferation in a dose-dependent manner. Plant-derived phenolic extracts expressed different degrees of SMC growth inhibition when present during ECM production. A combination of selected nutrients had a greater effect than did individual components. The ECM deposited by SMC in the presence of ascorbate, lysine, proline, and green tea catechins inhibited SMC migration rate up to 70%. The ECM produced under conditions of chronic essential nutrient deficiency can support proatherosclerotic SMC behavior. A combination of selected nutrients can counteract these adverse effects stronger than individual components.

Key Words: aortic smooth muscle cell, extracellular matrix, ascorbic acid, lysine, proline, catechins

(*J Cardiovasc Pharmacol*™ 2007;50:541–547)

Received for publication March 20, 2007; accepted June 16, 2007.
From the Dr. Rath Research Institute, Santa Clara, California.
This research study was funded by Dr. Rath Health Foundation (Plantation, FL), a nonprofit organization.
The authors disclose the following competing interests: (1) 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, Wahid M. (Sunnyvale, CA); Niedzwiecki, Aleksandra (San Jose, CA); Rath, Matthias (Almelo, NL). (2) 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).
Reprints: Aleksandra Niedzwiecki, PhD, 1260 Memorex Drive Santa Clara, CA. 95050 (email: a.niedz@drrath.com).

INTRODUCTION

High consumption of fruit and vegetables has been associated with the reduced risk of cardiovascular disease (CVD); however, exact mechanisms involved are still under investigation.[1,2] Earlier publications by Rath and Pauling[1,2] focused on the critical roles of vitamin C and other micronutrients important in extracellular matrix (ECM) formation in triggering a series of cellular pathological events leading to atherosclerosis. Accordingly, chronic suboptimal intake of vitamin C and consequently weakening structure of the arterial wall leads to compensatory arterial wall thickening due to increase in ECM and smooth muscle cell (SMC) growth and migration,[3] characteristic of vascular atherosclerosis. In addition to ascorbate, other dietary components can affect composition and structure of the ECM. Maritime pine bark extract containing proanthocyandins has been used historically to prevent the development of scurvy, mimicking to some extent the effects of vitamin C. Other plant-derived nutrients also have been shown to affect ECM composition in vitro.[4]

Much recent attention has been paid to the relationship between the functional and structural behavior of tissue residential cells and surrounding ECM. Increased matrix synthesis is accompanied by a change in the relative proportions of connective tissue proteins, such as collagen II, IV, and I.[5,6] Immunochemical analysis by Katsuda et al[7] demonstrated increased distribution of types I and III collagens in the thickened intima of all stages of atherosclerotic lesions. Collagens types I and III, which are missing in normal intima, play a critical role in formation and progress of atherosclerosis. Though collagen IV, a basement membrane collagen present in normal intima, showed some increase with progression of atherosclerosis, the relative amount of collagen IV to collagen I decreased significantly in atherosclerotic tissues.

In addition to collagens, the arterial subendothelial matrix also contains 3 major classes of glycosaminoglycans: heparan sulfate, chondroitin sulfate, and dermatan sulfate. Several studies have shown that the composition and content of glycosaminoglycans change during lesion development.[8–11] Heparan sulfate and dermatan sulfate, but not chondroitin sulfates, have been shown to bind plasma low-density lipoproteins with high affinity and specificity. It appears that such binding increases proportionally to an increase of glycosaminoglycan index of sulfation. Several studies have shown that sulfation index of glycosaminoglycans increases during atherosclerotic lesion development. In addition,

heparan sulfate has been shown to specifically bind and retain several growth factors implicated in contributing to progress of the atherosclerotic process.[12] Thus modulation of ECM structure and composition could beneficially interfere with the development of atherosclerotic lesions.

In a previous study, we demonstrated that a mixture of ascorbic acid, tea phenolics, and selected amino acids inhibited atherogenic responses of vascular SMC to pathological stimuli by decreasing aortic SMC proliferation, secretion of matrix degrading metalloproteinases, and invasion and migration through ECM.[13] Earlier, we observed a dose-dependent decreased proliferation of vascular SMC from guinea pig aortas in the presence of 0.5 to 2.0 mM ascorbate through direct and matrix-mediated effects.[14] In this study, we investigated individual and combined effects of some nutrients essential for collagen synthesis and posttranslational modification on ECM composition and biological properties, including SMC proliferation, migration, and invasion and ECM composition.

MATERIALS AND METHODS

Cell Culture and ECM Preparation

Human aortic SMC (obtained from Clonetics) were cultured in DMEM (Dulbecco modified Eagle medium) supplemented with 10% fetal bovine serum, penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2 and were split 1:3 to 1:5 upon reaching confluence. SMC at passages 5 to 8 were used in the experiments. The ECM was produced by aortic SMC and exposed for analysis as described previously[14] with a few modifications. Aortic SMC were plated in collagen type I covered 24- or 96-well plates (Beckton-Dickinson) at the density of 25,000 cells per cm^2 in DMEM/5% FBS. Upon reaching 90% cell layer confluence, the medium was replaced with fresh medium supplemented with 2% FBS and with other additions, as specified in the protocols. Cells were grown for 7 days; the medium was replaced every other day. Supplement stock solutions were prepared immediately before addition. The ECM was exposed by removing cells by incubation at room temperature with 0.5% Triton X-100 (w/v) in Dulbecco formulated Ca^{2+} and Mg^{2+}-free phosphate buffered saline (PBS, Gibco) for 3 minutes. This process was followed by 3 min of incubation with 20 mM NH_4OH in PBS and 4 subsequent washings with PBS. The matrix was examined by phase-contrast microscopy and either used immediately in experiments or covered with 1% BSA/PBS mixed with glycerol (1:1; v:v) and stored at −20°C until use within the next 10 days.

Cell Proliferation Assay on Pre-made Matrix Substratum

A new cell culture (hereafter referred to as a secondary cell culture) was plated in matrix covered 24-well plates at the density of 510,000 cells/cm^2 in DMEM/5% FBS, allowed to attach for 2 hours, washed with PBS to remove nonattached cells, supplied with fresh medium containing 2% FBS, and incubated for 72 hours. The rate of cellular DNA synthesis was determined by addition of 0.5 μCi/mL [3H] thymidine to the cell culture medium for the last 4 hours of incubation. Cellular DNA-associated radioactivity was determined as described previously.[14]

Enzyme-linked Immunoassay of ECM Components

Primary specific antibodies were rabbit polyclonal (collagen types I, II, and IV, fibronectin, and laminin supplied by Rockland Immunochemicals) or mouse monoclonal (elastin and chondroitin sulfate supplied by Sigma; heparan sulfate supplied by Chemicon International). Optimal dilutions for primary and corresponding secondary horseradish peroxidase-conjugated (goat anti-rabbit or rabbit anti-mouse supplied by Rockland) antibodies were determined in preliminary experiments. Ninety-six-well plates covered with SMC-produced matrices were pre-incubated with 1% BSA/PBS for 2 hours at room temperature, followed step-wise by 2 h incubation with secondary antibody diluted in 1% BSA/PBS, three times washing with 0.1% BSA/PBS, 1 hour of incubation with primary antibody diluted in 1% BSA/PBS, and three times washing with 0.1% BSA/PBS. Content of individual matrix components was determined by exposing to peroxidase substrate solution (TMB substrate solution supplied by Rockland) for 20 minutes and measuring optical density at 450 nm on microplate reader (Molecular Devices). Results are means ± SD of at least 3 repetitions and expressed as percentage to unsupplemented control.

Cell Invasion Assay

Aortic SMC were plated on top of Transwell inserts (3-μm pores, Corning Inc.) pre-covered with Collagen type I and placed in 24-well cell culture plates. Cells were cultured to produce ECM under exposure to nutrient mixture (NM) as described above, except SMC had not been removed from the ECM surface. Stock SMC culture grown in a 75-cm^2 flask was metabolically prelabeled by incubation with 0.5 μCi/mL [3H] Thymidine for 24 hours in 5% FBS/DMEM, suspended in 0.1% BSA/DMEM, and seeded on top of primary SMC-ECM layer formed in the inserts. The bottom part of the well was supplemented with 10 ng/mL basic fibroblast growth factor (Gibco) in 0.1% BSA/DMEM. After incubation for 48 hours, inserts were removed from the plate and washed with PBS. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. Insert membranes were cut off and placed in scintillation vials filled with scintillation liquid. The number of prelabeled SMC that migrated to the lower surface of the membrane was estimated by radioactivity that remained associated with the inserts.

Plant-Derived Phenolic Extracts and Composition of the Nutrient Mixture

Phenolic extracts were provided as follows: Pycnogenol French Maritime Pine Bark Extract was supplied by Natural Health Science (Hillside, NJ) with phenolic content 70%; grape seed extract ActiVin GSE-2000-S was supplied by Dry Creek Nutrition Inc. (Fresno, CA) with phenolic content 80%; and green tea extract derived from green tea leaves was obtained form US Pharma Lab (Somerset, NJ). According to

the manufacturer's specification, it contained total polyphenols 80%, catechins 60%, epigallocatechin gallate (EGCG) 35%, and caffeine 1%. The nutrient mixture solution at the concentration of 100 μg/mL contained 100 μM ascorbic acid, 100 μM lysine, 100 μM proline, 50 μM arginine, 25 μM N-acetyl cysteine, and 20 μg/mL green tea extract (corresponding to 15 μM EGCG) as a surplus to the basic DMEM composition.

Statistical Analysis

All experiments were performed at least twice in triplicate. The results for each representative study are expressed as mean ± SD for the groups. Data was analyzed by independent sample 2-tailed *t* test.

RESULTS

Effect of ECM Developed by Exposure of SMC to Individual Nutrients and the Nutrient Mixture on Secondary SMC Proliferation

Plant-derived phenolic extracts expressed different degrees of SMC growth inhibition when present during ECM production, as shown in Figure 1. Green tea extract had the most potent inhibitory effect (reduction of 33% compared to the control; $P = 0.003$), followed by grape seed extract (29%; $P = 0.005$), and pycnogenol (12%, $P = 0.1$). The ECM produced in the presence of 100 μM ascorbic acid inhibited SMC proliferation by 16% (Figure 2). N-acetyl cysteine and arginine also produced some cell growth inhibition. However, the combined mixture of nutrients tested had a greater effect than did individual components. NM inhibited secondary SMC growth by 34% ($P = 0.99$) compared with the control (Figure 2). Results did not reach statistical significance. Secondary SMC growth was inhibited by NM greater than by ascorbic acid in a dose-dependent manner, as shown in Figure 3, with 30% ($P = 0.18$) reduction in DNA synthesis compared to the control in the presence of 300 μM ascorbic acid and

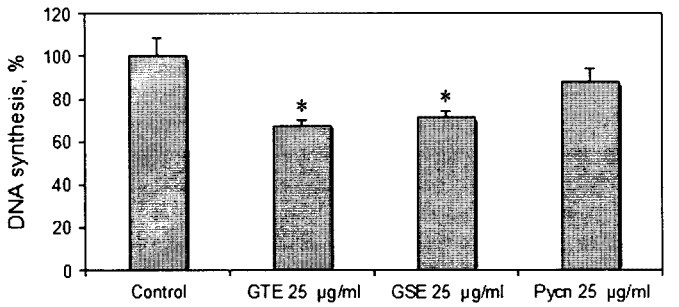

FIGURE 1. ECM-mediated effects of plant extracts (25 μg/mL phenolics) on SMC growth. GTE, green tea extract; GSE, grape seed extract; Pycn, pycnogenol. ECM production for 7 days, aortic SMC growth 72 h. Cell growth rate was evaluated by incorporation of [3H]-thymidine into cellular DNA during last the 4 hours of the experiment. Plant-derived phenolic extracts expressed different degrees of SMC growth inhibition when present during ECM production: green tea extract had the most potent inhibitory effect (reduction of 33% compared to the control; $P = 0.003$) followed by GSE (29%; $P = 0.005$), and pycnogenol (12%, $P = 0.1$).

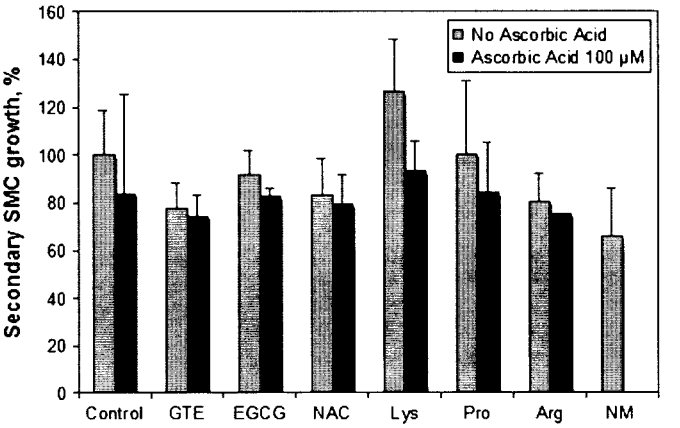

FIGURE 2. Effect of nutrient mixture (NM) components on ECM-mediated regulation of SMC growth. GTE, green tea extract (22 μg/mL); EGCG, epigallocatechin gallate (15 μg/mL); NAC, N-acetyl cysteine (22 μM); Lys, lysine (100 μM); Pro, proline (100 μM); Arg, arginine (50 μM); NM, nutrient mixture (100 μg/mL). The ECM produced in the presence of 100 μM ascorbic acid inhibited SMC proliferation by 16%. N-acetyl cysteine and arginine also produced some cell growth inhibition. However, the combined mixture of nutrients tested had a greater effect than did individual components. NM inhibited secondary SMC growth by 34% ($P = 0.99$) compared to the control. Results did not reach statistical significance.

60% ($P = 0.01$) reduction in DNA synthesis in the presence of 300 μg/mL NM. The rate of DNA synthesis by secondary SMC on ECM grown in NM or in control media decreased with time, as shown in Figure 4. Furthermore, secondary SMC growth on ECM produced in the presence of NM was significantly lower than under control conditions at each time interval: 15% ($P = 0.08$) at 24 hours, 20% ($P = 0.019$) at 48 hours, 36% ($P = 0.006$) at 72 hours.

Effect of ECM Developed by Exposure of SMC to NM on Secondary SMC Invasion and Migration

The ECM deposited by SMC in the presence of the combination of nutrients containing ascorbate, lysine, proline, and green tea catechins significantly inhibited penetration and migration of secondary aortic SMC by 70% ($P = 0.0005$) compared to the control, as shown in Figure 5.

Effect of Exposure of SMC to NM on Collagen Synthesis and Composition

ECM composition was altered with exposure of SMC to NM. When compared to the control, NM exposure resulted in decreased collagen type I (by 50%, $P = 0.004$), collagen type III (by 12%, $P = 0.06$) and decreased collagen type IV (by 39%, $P = 0.0001$), as shown in Figure 6A. NM exposure resulted in increased collagen type IV to I ratio to 1.24 (Figure 6B). In contrast, AA exposure resulted in increased collagen types I, III, and IV production.

NM exposure resulted in increased chondroitin sulfate (121%, $P = 0.008$) and decreased heparan sulfate (66%,

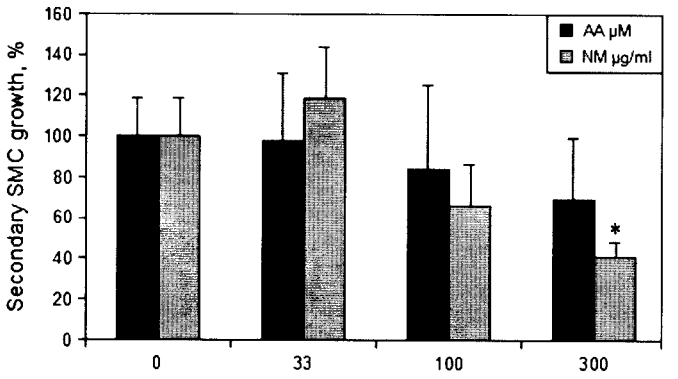

FIGURE 3. ECM-mediated effects of the nutrient mixture (NM) and ascorbic acid (AA) on aortic SMC growth. ECM production for 7 days, aortic SMC growth 72 hours. Cell growth rate was evaluated by incorporation of [3H]-thymidine into cellular DNA during the last 4 hours of the experiment. Secondary SMC growth was inhibited by NM greater than by ascorbic acid in a dose-dependent manner with 30% (P = 0.18) reduction in DNA synthesis compared to the control in the presence of 300 μM ascorbic acid and 60% (P = 0.01) reduction in DNA synthesis in the presence of 300 μg/mL NM.

P = 0.0002), as shown in Figure 7A. The resultant chondroitin sulfate to heparan sulfate ratio with NM exposure was 3.52 (Figure 7B). Ascorbate had slight insignificant effect on chondroitin sulfate and heparan sulfate incorporation into the ECM.

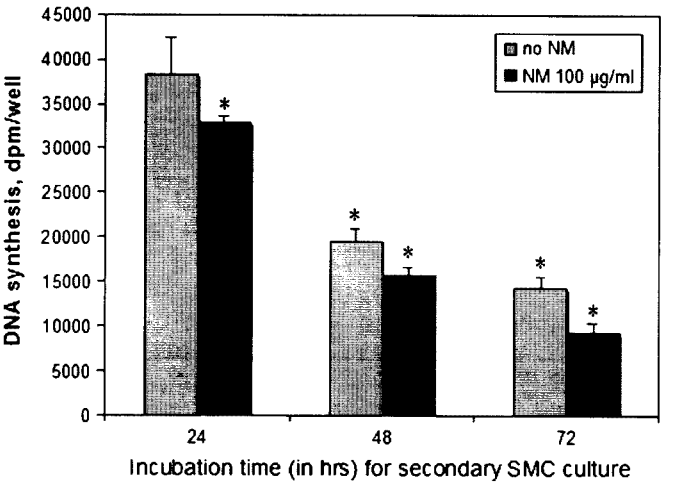

FIGURE 4. Nutrient mixture (NM) time-course inhibition of SMC growth mediated through alteration of ECM properties. ECM production for 7 days, aortic SMC growth 24, 48, and 72 hours. Cell growth rate was evaluated by incorporation of [3H]-thymidine into cellular DNA. The rate of DNA synthesis by secondary SMC on ECM grown in NM or in control media decreased with time: by 50% (P = 0.001) at 48 hours and by 63% (P = 0.006) at 72 hours. Furthermore, secondary SMC growth on ECM produced in the presence of NM was significantly lower than under control conditions at each time interval: 15% (P = 0.08) at 24 hours, 20% (P = 0.019) at 48 hours, 36% (P = 0.006) at 72 hours.

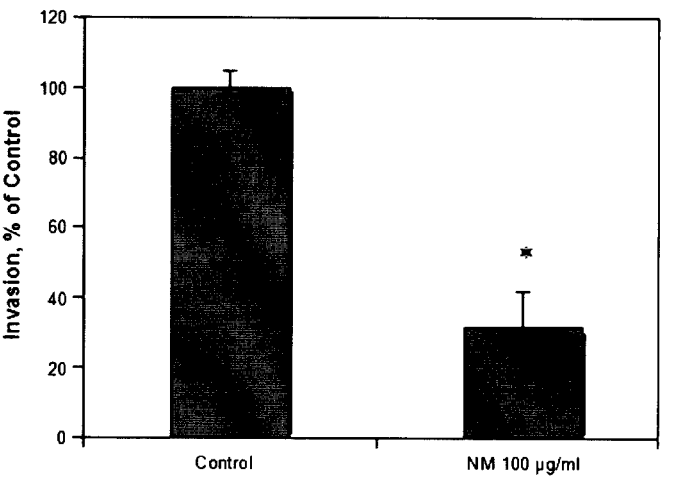

FIGURE 5. Aortic SMC invasion through SMC-produced ECM exposed to NM. Cell invasion was estimated in 24 hours by counting cells migrated to the other side of the membrane and expressed as percent of unsupplemented control. The ECM deposited by SMC in the presence of the combination of nutrients containing ascorbate, lysine, proline, and green tea catechins significantly inhibited penetration and migration of secondary aortic SMC by 70% (P = 0.0005) compared to the control.

In SMC treated with NM, incorporation of elastin into the ECM decreased significantly (27%, P = 0.058), and laminin and fibronectin incorporation into ECM increased slightly, 5% and 7%, respectively (Figure 8). Ascorbic acid had no effect on fibronectin and laminin, but it resulted in a lower elastin level (21%, P = 0.136).

DISCUSSION

As mentioned earlier, a hallmark of atherosclerosis is increased matrix synthesis characterized by increased proportions of collagens I and III and decreased relative proportion of collagen IV. The composition of the ECM has been shown to affect atherogenesis and thus is a potential target for blocking atherosclerosis. Our results demonstrated reduction in collagen production by culture SMC with NM exposure.

Collagen type I is not only upregulated in atherosclerotic plaques,[15] but it has been found to affect the degree of vascular calcification, a frequent component of atherosclerosis.[16] Matrix produced by rapidly mineralizing cells was found to contain 3 times the amount of collagen I and fibronectin but 70% less collagen IV than nonmineralizing clones. Furthermore, slowly mineralizing cells cultured on purified collagen I or fibronectin exhibited increased mineralization parameters in contrast to culturing these cells on purified collagen IV, which inhibited mineralization parameters. The results from our study demonstrated increased strength and integrity of the ECM produced by SMC exposed to the mixture of nutrients in contrast to the control. The resultant ECM was characterized by increased collagen type IV to I ratio and profound inhibitory effects on aortic SMC migration and connective tissue invasion. Increased ECM structural stability under treatment with nutrients is further supported by NM-mediated

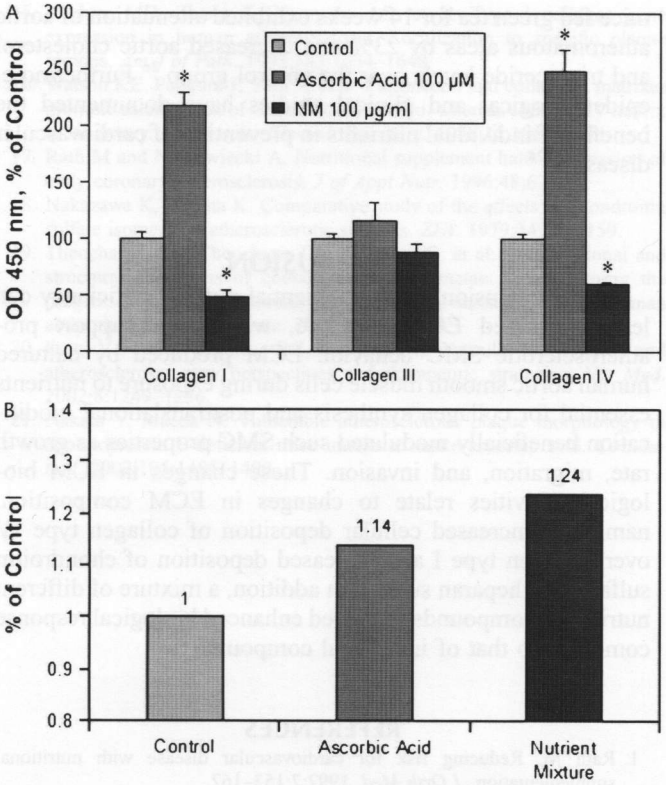

FIGURE 6. A, Incorporation of collagens type I, III, and IV into ECM by SMC treated with the nutrient mixture (NM) or ascorbate; B, Collagen type IV:I ratio. ECM composition was altered with exposure of SMC to NM and AA. When compared to the control, NM exposure resulted in decreased collagen type I (by 50%, $P = 0.004$), collagen type III (by 12%, $P = 0.06$) and decreased collagen type IV (by 39%, $P = 0.0001$). NM exposure resulted in increased collagen type IV to I ratio to 1.24. In contrast, AA exposure resulted in increased collagen types I, III, and IV production.

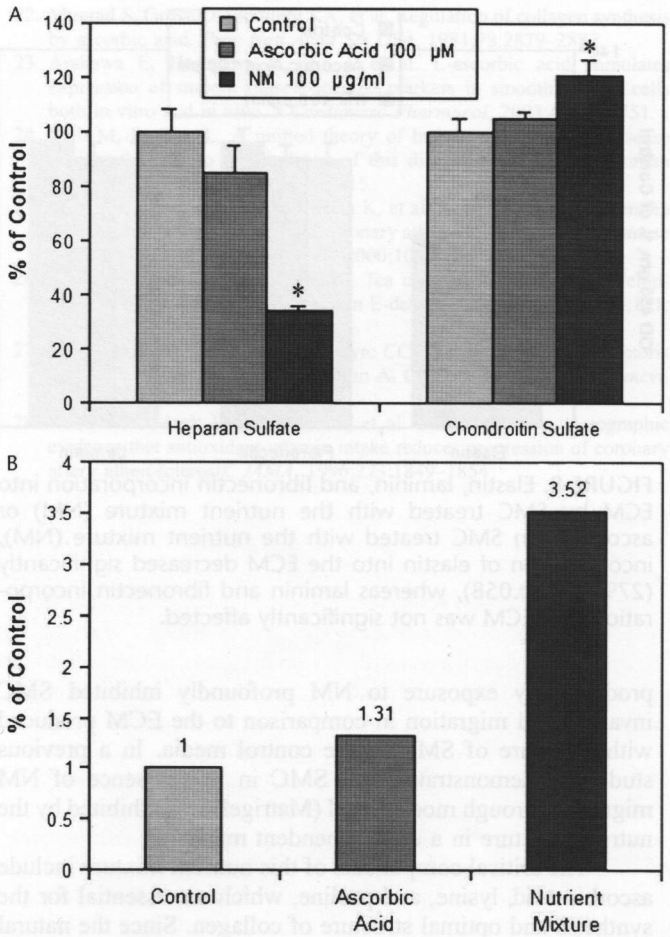

FIGURE 7. A, Chondroitin sulfate and heparan sulfate incorporation into ECM by SMC treated with the nutrient mixture or ascorbate; B, Chondroitin sulfate:heparan sulfate ratio. NM exposure resulted in increased chondroitin sulfate (121%, $P = 0.008$) and decreased heparan sulfate (66%, $P = 0.0002$). The resultant chondroitin sulfate to heparan sulfate ratio with NM exposure was 3.52. Ascorbate had a slight insignificant effect on chondroitin sulfate and heparan sulfate incorporation into the ECM.

interference with ECM remodeling by inhibition of production and secretion of matrix metalloproteinases-2 by cultured SMC as reported previously.[13]

Furthermore, the cardioprotective effects of micronutrient supplementation on progression of early coronary atherosclerosis were confirmed in a previous clinical study. In this pilot study, the extent of coronary calcification in 55 patients diagnosed with early coronary atherosclerosis was measured before micronutrient supplementation and after 1 year of intervention by using an Imatron C-100 Ultrafast CT scanner.[17] Progression of coronary calcification, as determined by the CAS score, decreased significantly (from 0.49 mm^2 to 0.28 mm^2 monthly growth) after 1 year of nutritional intervention; in some cases, a complete reversal of early atherosclerosis was observed.[17]

The literature contains contradictory reports regarding the role of matrix glycosaminoglycans in atherosclerosis. Some studies report increased chondroitin sulfate and reduced heparan sulfate concentrations with atherosclerosis. However, clinical treatment of atherosclerotic patients with isomers of chondroitin sulfate resulted in decreased serum cholesterol and reduced thrombus formation.[18] Furthermore, the concentration of chondroitin sulfate was reported to decrease during progression of atherosclerosis and aneurysmal dilatation of the human abdominal aorta, while the concentration of dermatan sulfate remained relatively constant.[19] We found an increased chondroitin sulfate to heparan sulfate ratio in the ECM produced with SMC exposure to NM.

Control of excessive SMC growth is a major strategic goal in the development of atherosclerosis treatment.[20] In the present study, we demonstrated that the altered ECM produced by exposure of SMC to NM, including ascorbic acid and amino acids lysine, proline, and arginine, resulted in inhibition of secondary SMC proliferation. We previously demonstrated direct inhibition of SMC DNA synthesis when cultured in medium containing this nutrient mixture.[13]

Another aspect of atherosclerotic process, migration of arterial wall SMC, was also addressed in this study. The ECM

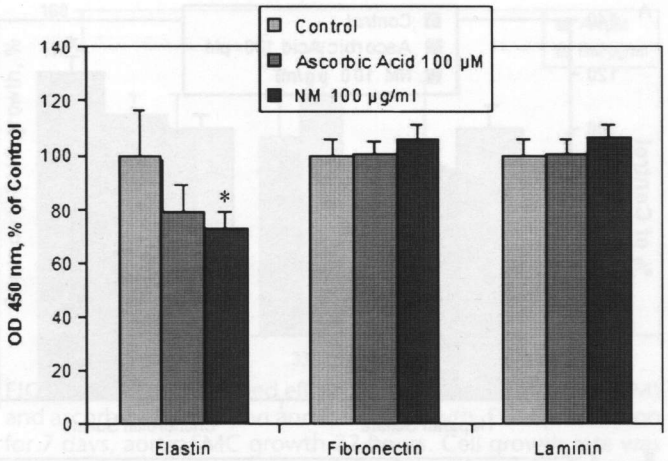

FIGURE 8. Elastin, laminin, and fibronectin incorporation into ECM by SMC treated with the nutrient mixture (NM) or ascorbate. In SMC treated with the nutrient mixture (NM), incorporation of elastin into the ECM decreased significantly (27%, $P = 0.058$), whereas laminin and fibronectin incorporation into ECM was not significantly affected.

produced by exposure to NM profoundly inhibited SMC invasion and migration in comparison to the ECM produced with exposure of SMC to the control media. In a previous study, we demonstrated that SMC in the presence of NM migration through model ECM (Matrigel) was inhibited by the nutrient mixture in a dose-dependent manner.[13]

The critical components of this nutrient mixture include ascorbic acid, lysine, and proline, which are essential for the synthesis and optimal structure of collagen. Since the natural occurrence of atherosclerosis is limited to humans, primates, and guinea pigs (species not producing vitamin C) and usually manifests in mechanistically stressed areas of the coronary arteries, Rath and Pauling proposed that chronic subclinical vitamin C deficiency has a destabilizing effect on vascular wall structure and function, leading to deposition of lipoprotein(a) and fibrinogen/fibrin in the vascular wall and triggering other physiological changes characteristic of atherosclerosis.[1] Ascorbic acid plays a critical role in vascular wall stability as a cofactor in the hydroxylation of proline and lysine residues in collagen fibers, important for enhanced stability and strength of the connective tissue.[1,3] Lysine, the most abundant amino acid in collagen, is a natural inhibitor of plasmin-induced proteolysis, which triggers MMP activation cascade and ECM degradation process.[21]

Nakata and Maeda[22] showed that a loss of vitamin C production in mice, which normally synthesize vitamin C, resulted in structural changes in the coronary arteries resembling early atherosclerosis. In addition, ascorbate has been shown to induce SMC differentiation,[23] which results in a reduction in cell growth, important in curbing atherosclerotic plaque development. In addition to ascorbate, several other nutrients are essential in optimizing vascular connective tissue structure and function, such as lysine, proline, copper, manganese, and others.[24] Additionally, antiatherogenic effects of green tea extract have been reported both in clinical[25] and experimental studies.[26] 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.[26] Furthermore, epidemiological and clinical studies have documented the benefits of individual nutrients in prevention of cardiovascular disease.[27,28]

CONCLUSION

In conclusion, chronic essential nutrient deficiency can lead to altered ECM structure, which can support pro-atherosclerotic SMC behavior. ECM produced by cultured human aortic smooth muscle cells during exposure to nutrients essential for collagen synthesis and posttranslational modification beneficially modulated such SMC properties as growth rate, migration, and invasion. These changes in ECM biological activities relate to changes in ECM composition, namely to increased cellular deposition of collagen type IV over collagen type I and increased deposition of chondroitin sulfate over heparan sulfate. In addition, a mixture of different nutritional compounds produced enhanced biological response compared to that of individual compounds.

REFERENCES

1. Rath M. Reducing risk for cardiovascular disease with nutritional supplementation. *J Orth Med*. 1992;7:153–162.
2. McCarty MF. An expanded concept of "insurance" supplementation-broad spectrum protection from cardiovascular disease. *Med Hyptheses*. 1981;10:1287–1302.
3. Rath M, Pauling L. Solution to the puzzle of human cardiovascular disease: its primary cause is ascorbate deficiency, leading to the deposition of lipoprotein(a) and fibrinogen/fibrin in the vascular wall. *J Ortho Med*. 1991;6:125–134.
4. Tinker D, Rucker RB. Role of selected nutrients in synthesis, accumulation, and chemical modification of connective tissue proteins. *Physiol Rev*. 1985;65:607–657.
5. Geesin JC, Hendricks LJ, Gordon JS, et al. Modulation of collagen synthesis by growth factors: the role of ascorbate-stimulated lipid peroxidation. *Arch Biochem Biophys*. 1991;289:6–11.
6. Murata K, Motayama T, Kotake C. Collagen types in various layer of the human aorta and their changes with the atherosclerotic process. *Atherosclerosis*. 1986;60:251–262.
7. Katsuda S, Okada Y, Minamoto T, et al. Collagens in human atherosclerosis. Immunohistochemical analysis using collagen type-specific antibodies. *Arterioscler Thromb Vasc Biol*. 1992;12:494–502.
8. Ross R, Wright TN, Strandness E, et al. Human atherosclerosis: 1 Cell constitution and characteristics of advanced lesions of the superficial femoral artery. *Am J Path*. 1984;114:79–93.
9. Wagner WD. Proteoglycan structure and function as related to atherosclerosis. *Ann N Y Acad Sci*. 1985;454:52–68.
10. Voelker W, Schmidt A, Oortmann W, et al. Mapping of proteoglycancs in atheorsclerotic lesions. *Eur Heart J*. 1990;11:29–40.
11. Tammi M, Seppala PO, Lhtonen A, et al. Connective tissue components in normal and atherosclerotic human coronary arteries. *Atherosclerosis*. 1978;29:191–194.
12. Pillarisetti S, Paka L, Obunike JC, et al. Subendothelial retention of lipoprotein(a): evidence that reduced heparan sulfate promotes lipoprotein binding to subendothelial matrix. *J Clin Invest*. 1997;100:867–874.
13. Ivanov V, Roomi MW, Kalinovsky T, et al. Antiatherogenic effects of a mixture containing ascorbic acid, lysine, proline, arginine, cysteine and green tea phenolics: in vitro study using human aortic smooth muscle cells. *J Cardiovasc Pharmacol*. 2007;49:140–145.
14. Ivanov V, Ivanova SV, Niedzwiecki A. Ascorbate affects proliferation of guinea-pig vascular smooth muscle cells by direct and extracellular matrix-mediated effects. *J Mol Cell Cardiol*. 1997;29:3293–3303.

15. Rekhter MD, Zhang K, Narayana AS, et al. Type I collagen gene expression in human atherosclerosis. Localization to specific plaque regions. *Am J of Path*. 1993;143:1634–1648.
16. Watson KE, Parhami F, Shin V, et al. Fibronectin and collagen I matrixes promote calcification of vascular cells in vitro, whereas collagen IV matrix is inhibitory. *Arterioscler Thromb Vasc Biol*. 1998;18:1964–1971.
17. Rath M and Niedzwiecki A. Nutritional supplement halts progression of early coronary atherosclerosis. *J of Appl Nutr*. 1996;48:67–78.
18. Nakazawa K, Murata K. Comparative study of the effects of chondroitin sulfate isomers on atherosclerotic subjects. *ZFA*. 1979;34:153–159.
19. Theocharais AD, Theocharis DA, De Luca G, et al. Compositional and structural alterations of chondroitin and dermatan sulfates during the progression of atherosclerosis and aneurismal dilatation of the human abdominal aorta. *Biochimie*. 2002;84:667–674.
20. Dzau VJ, Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat Med*. 2002;8:1249–1256.
21. Nakata Y, Maeda N. Vulnerable atherosclerotic plaque morphology in apolipoprotein E-deficient mice unable to make ascorbic acid. *Circulation*. 2002;105:1485–1490.
22. Munrad S, Grove D, Lindberg KA, et al. Regulation of collagen synthesis by ascorbic acid. *Proc Natl Acad Sci USA*. 1981;78:2879–2882.
23. Arakawa E, Hasegawa K, Irie J, et al. L-ascorbic acid stimulates expression of smooth muscle-specific markers in smooth muscle cells both in vitro and in vivo. *J Cardiovasc Pharmacol*. 2003;42:745–751.
24. Rath M, Pauling L. A unified theory of human cardiovascular disease leading the way to the abolition of this disease as a cause for human mortality. *J Ortho Med*. 1992;7:5–15.
25. Sasazuki, S, Kodama H, Yoshimasu K, et al. Relation between green tea consumption and the severity of coronary atherosclerosis among Japanese men and women. *Ann Epidemiol*. 2000;10:401–408.
26. Miura Y, Chiba T, Tomita I, et al. Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice. *J Nutr*. 2001;131: 27–32.
27. Riemersma RA, Wood DA, Macintyre CC, et al. Risk of angina pectoris and plasma concentrations of vitamin A, C, and E and carotene. *Lancet*. 1991;337:1–5.
28. Hodis HN, Mack WJ, La Bree L, et al. Serial coronary angiographic evidence that antioxidant vitamin intake reduces progression of coronary artery atherosclerosis. *JAMA*. 1996;273:1849–1854.