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

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**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

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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).
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## 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

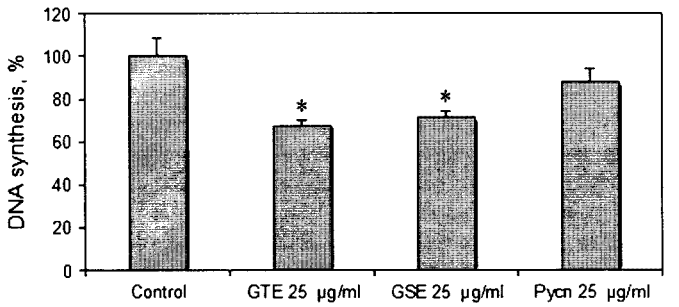


**FIGURE 1.** ECM-mediated effects of plant extracts (25 μg/mL phenolics) on SMC growth. GTE, green tea extract; GSE, grape seed extract; Pycn, pycnogenol. ECM production for 7 days, aortic SMC growth 72 h. Cell growth rate was evaluated by incorporation of [3H]-thymidine into cellular DNA during last 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%, $P = 0.1$).

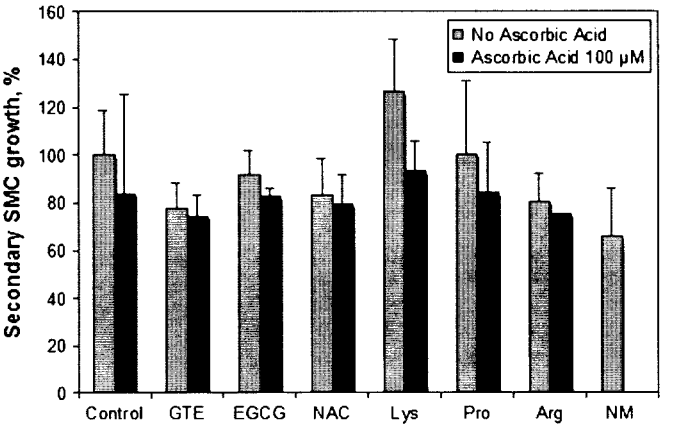


**FIGURE 2.** Effect of nutrient mixture (NM) components on ECM-mediated regulation of SMC growth. GTE, green tea extract (22 μg/mL); EGCG, epigallocatechin gallate (15 μg/mL); NAC, N-acetyl cysteine (22 μM); Lys, lysine (100 μM); Pro, proline (100 μM); Arg, arginine (50 μM); NM, nutrient mixture (100 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%,

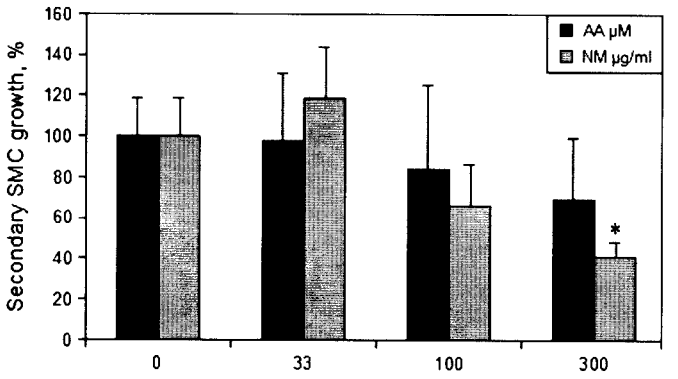


**FIGURE 3.** ECM-mediated effects of the nutrient mixture (NM) and ascorbic acid (AA) on aortic SMC growth. ECM production for 7 days, aortic SMC growth 72 hours. Cell growth rate was evaluated by incorporation of [3H]-thymidine into cellular DNA during the last 4 hours of the experiment. Secondary 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.

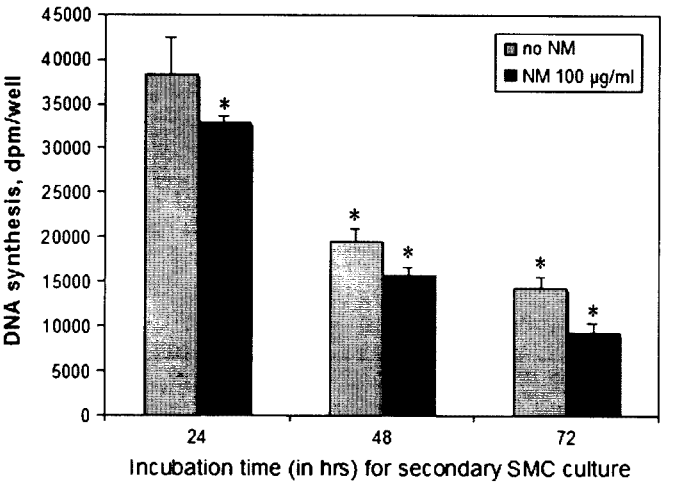


**FIGURE 4.** Nutrient mixture (NM) time-course inhibition of SMC growth mediated through alteration of ECM properties. ECM production for 7 days, aortic SMC growth 24, 48, and 72 hours. Cell growth rate was evaluated by incorporation of [3H]-thymidine into cellular DNA. The rate of DNA synthesis by 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.

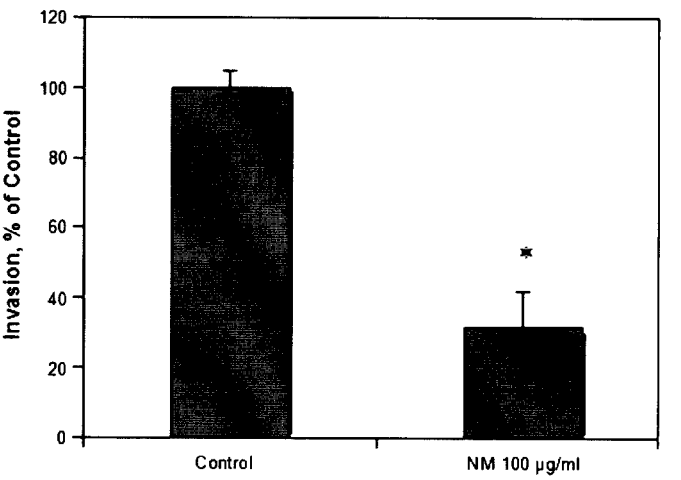


**FIGURE 5.** Aortic SMC invasion through SMC-produced ECM exposed to NM. Cell invasion was estimated in 24 hours by counting cells migrated to the other side of the membrane and expressed as percent of unsupplemented control. The ECM deposited by SMC in the presence of the combination of nutrients containing 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