Plant-Derived Micronutrients Suppress Monocyte Adhesion to Cultured Human Aortic Endothelial Cell Layer by Modulating Its Extracellular Matrix Composition

Vadim Ivanov, PhD, Svetlana Ivanova, MD, Tatiana Kalinovsky, MS, Aleksandra Niedzwiecki, PhD, and Matthias Rath, MD

Abstract: Monocyte adhesion to endothelium plays an important role in atherosclerosis. We investigated the effects of micronutrients on monocyte-binding properties of extracellular matrix (ECM) produced by human aortic endothelial cells (AoEC). Confluent cultures of AoEC were exposed to ascorbic acid, quercetin, gotu kola extract (10% asiatic acid), green tea extract (40% epigallocatechin gallate), or a mixture of these micronutrients for 48 hours. AoEC-produced ECM was exposed by differential treatment. U937 monocyte adhesion was assayed by fluorescence. ECM composition was assayed immunochemically and with radiolabeled metabolic precursors. AoEC exposure to micronutrients reduced ECM capacity to bind monocytes in a dose-dependent manner. This effect was accompanied by profound changes in the ECM composition. Correlation analysis revealed that changes in monocyte adhesion to ECM had the strongest positive correlation with ECM content for laminin (CC = 0.9681, $P < 0.01$), followed by fibronectin, collagens type III, I, and IV, biglycan, heparan sulfate, and elastin. The strongest negative correlation was with chondroitin sulfate (CC = −0.9623, $P < 0.01$), followed by perlecan and versican. Individual micronutrients had diverse effects on ECM composition and binding properties, and their mixture was the most effective treatment. In conclusion, micronutrient-dependent reduction of monocyte adhesion to endothelium is partly mediated through specific modulation of ECM composition and properties.

Key Words: micronutrients, monocytes, endothelium, extracellular matrix

(*J Cardiovasc Pharmacol*™ 2008;52:55–65)

INTRODUCTION

Cardiovascular disease (CVD) remains the most frequent cause of morbidity and mortality in modern societies. Atherosclerotic modifications of arterial walls are the underlying driving force for the majority of clinical manifestations of CVD.[1] Understanding complex molecular and cellular mechanisms involved in initiation and progression of atherosclerotic lesions should eventually lead to the development of successful prevention and treatment strategies.

The extracellular matrix (ECM) plays an important structural and functional role in maintaining proper contractility and integrity of arterial walls. It was demonstrated that the composition and structure of ECM undergoes significant changes during the atherosclerotic process as a result of compromised regulation of the matrix metalloproteinase network and modifications of matrix component synthesis and deposition.[2,3] Resident arterial wall cells, endothelial cells (EC) and smooth muscle cells (SMC), are responsible for ECM production and deposition. Earlier, we described modulation of ECM cell growth regulating properties produced by aortic SMC in response to supplementation with ascorbic acid and plant-derived polyphenols.[4,5] Changes in ECM activities were accompanied by changes in ECM protein and glycosaminoglycan (GAG) composition.

According to the response-to-injury hypothesis, the atherosclerotic process is initiated by blood leukocyte attachment to sites of mechanically or chemically injured arterial endothelial layers.[6] The ECM plays an important role in this process. Denudation of the arterial wall surface from EC and exposure of the underlying ECM to blood dramatically increases monocyte binding to injured sites and their arterial wall invasion.[7] Leukocyte adhesion to ECM components is mediated by cell surface–expressed integrins with different specificity and affinity.[8–14] However, it is not clear whether alteration of EC-formed ECM determines the degree of leukocyte attachment. Among influences that could affect ECM composition and properties, naturally occurring nutrients play an important part.[15] Such substances as vitamin C (ascorbic acid), epigallocatechin gallate (EGCG), quercetin, and asiatic acid have been shown to affect ECM properties and composition in different tissues.[16–19]

Several rationales support use of a mixture of biologically active micronutrients in preference to megadoses of a single compound. Individual natural compounds display different spectra of specificity and activity toward complex biological processes in accordance with differences in chemical structure. When several compounds target a molecular mechanism, they act in combined efficiency. However, when different compounds affect different molecular mechanisms of a complex biological process, an additive or even synergistic effect can be produced. In addition, there is also a possibility of direct interaction between effective compounds.

Received for publication February 28, 2008; accepted April 30, 2008.
From the Dr. Rath Research Institute, Santa Clara, California.
The authors state that they have no financial interest in the products mentioned within this article.
Reprints: Aleksandra Niedzwiecki, PhD, 1260 Memorex Drive, Santa Clara, CA 95050 (e-mail: author@drrath.com).

The goal of this study was to investigate whether plant-derived micronutrients could modulate a process of monocyte adhesion to endothelium-derived ECM when used individually or as a mixture.

MATERIALS AND METHODS

Reagents

All reagents were from Sigma-Aldrich (St. Louis, MO) except when indicated. The micronutrient mixture (NM) was prepared as a dry powder by VitaTech (Hayward, CA) and stored at 4°C under desiccation. Green tea leaf extract (GTE, 40% EGCG) and gotu kola extract (GKE, 10% asiatic acid) were supplied by VitaTech. For experiments, a stock solution of 2 mg/mL NM was prepared daily by dissolving in serum-free cell culture medium and sterilizing by passing through a 0.2-μm filter. When diluted appropriately, the 100 μg/mL NM solution contained 16 μg/mL GTE (14 μM EGCG), 11 μg/mL GKE (2 μM asiatic acid), 27 μM quercetin, 110 μM L-ascorbic acid, 110 μM L-lysine HCl, 140 μM L-proline, 22 μM L-methionine, 27 μM L-cysteine HCl, 6 μM choline bitartrate, 1 μM copper glycinate, 0.24 μM cyanocobalamin, 0.04 μM folic acid, and 0.8 μM pyrodoxine HCl. When tested individually, solutions of all components were prepared similarly except for quercetin, EGCG, and asiatic acid, which were added to cell culture medium from stock solutions prepared in ethanol. Final ethanol concentration in cell culture media was either 0.075% or 0.135%, with the exception of 1 experiment in which it was 0.375%. No significant alterations of cellular responses to variations in applied ethanol concentration were noticed.

Cell Cultures

Human U937 monocytes were supplied by ATCC (Manassas, VA) and propagated in suspended culture in RPMI-1640 medium (ATCC) containing antibiotics and 10% fetal bovine serum (FBS, ATCC). Human aortic endothelial cells (AoEC) were purchased from Cambrix (East Rutherford, NJ) and maintained in EGM-2 medium (containing 2% FBS) as specified and supplied by Cambrix. All cell cultures were maintained at 37°C and 5% CO_2 atmosphere. Cell viability was monitored with MTT assay. None of the experimental conditions used resulted in statistically significant cell death (data not shown).

ECM Production by EC

For experiments, AoEC at seventh and ninth passages were seeded on collagen type I covered plastic plates (Becton-Dickinson, collagen I isolated from rat tail tendon) at a density of 25,000/cm^2 and grown to confluence for 5 to 7 days. Tested compounds were added to cells at indicated concentrations for 48 hours. AoEC-produced ECM was prepared by the procedure detailed by Bashkin et al[20] with slight modifications.[5] Briefly, cell layers were removed from the ECM surface by sequential treatment with 0.5% Triton X100 and 20 mM ammonium sulfate in phosphate-buffered saline (PBS, Life Technologies) for 3 minutes each at room temperature (RT). After 4 washes with PBS, ECM layers were treated with 1% bovine serum albumin (BSA) in PBS for 2 hours at 37°C and used in experiments immediately or stored in 1% BSA/PBS: glycerol (1:1) at –20°C until use within 4 weeks. Alternatively, cell layers were washed 3 times with PBS and fixed with 1% formaldehyde in PBS at 4°C for 1 hour. Fixed cell layers were washed 4 times with PBS and incubated with 1% BSA/PBS for 2 hours at 37°C before monocyte attachment experiments.

Monocyte Attachment Assay

The monocyte attachment assay was done as described by others[21] with slight modifications. Briefly, U937 monocytes were washed from FBS by serial sedimentation in serum-free medium and resuspended in serum-free RPMI-1640 at a density of 4,000,000/mL. Fluorescent dye 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was added at a final concentration of 10 μM for 1 hour at 37°C followed by 3 washes with RPMI-1640. Cells were resuspended in RPMI-1640 and added to 48-well plates with AoEC-produced ECM layers or fixed AoEC (prepared as above) in 250 μL (250,000 cells) per well. Cell cultures were supplemented with 10 μg/mL bacterial lipopolysaccharide (LPS) and incubated for 30 min at 37°C. In some experiments (Figure 1C), the micronutrient mixture was added to the monocyte suspension during its incubation with ECM. Wells were washed twice with RPMI-1640, and cell-trapped dye was released by treatment with 300 μL of lysing buffer (20 mM Tris-HCl, pH 8.0, 1% Triton X-100). Retained fluorescence was measured at 485/530 nm with CytoFluor 4000 fluorescent plate reader (PerSeptive Biosystems). The number of attached cells was calculated by referring to stock-labeled U937 suspension and expressed as percentage of U937 monocyte binding of control AoEC samples prepared under treatment with unsupplemented EGM-2 medium.

ECM Composition by Immunoassay

AoEC-produced ECM layers were prepared in 96-well plates covered with collagen type I as described above. An appropriate number of wells were assigned for immunoassay for each individual ECM component. Sandwich-type immunoasssays were done by sequential incubation with 2 or 3 (when appropriate) antibodies diluted in 1% BSA/PBS for 2 hours (first antibodies only) or 1 hour at RT separated each by 4 washes with 0.1% BSA/PBS. Retained peroxidase activity was measured after the last washing cycle using TMB peroxidase substrate reagent (Rockland) as specified by manufacturer. Optical density was read with SpectraMax 190 plate reader (Molecular Devices) at 450 nm and expressed as a percentage of the control AoEC samples incubated in unsupplemented EGM-2 medium. Antibody sets for individual ECM components were used in appropriate dilutions as follows (first, second, and third where appropriate). For collagen types I and III, elastin, chondroitin sulfate, heparan sulfate and perlecan, mouse monoclonal antibodies (mMABs, all from Sigma, except for heparan sulfate and perlecan mMABs, which were from Chemicon and R&D Systems, respectively) and rabbit anti-mouse IgG-horse radish peroxidase (IgG-HRP, Rockland) were used. For collagen type IV, fibronectin, and laminin, rabbit polyclonal antibodies (PABs) and anti-rabbit IgG-HRP (all from Rockland) were used. For biglycan and decorin, goat PABs and rabbit anti-goat IgG-HRP (all from

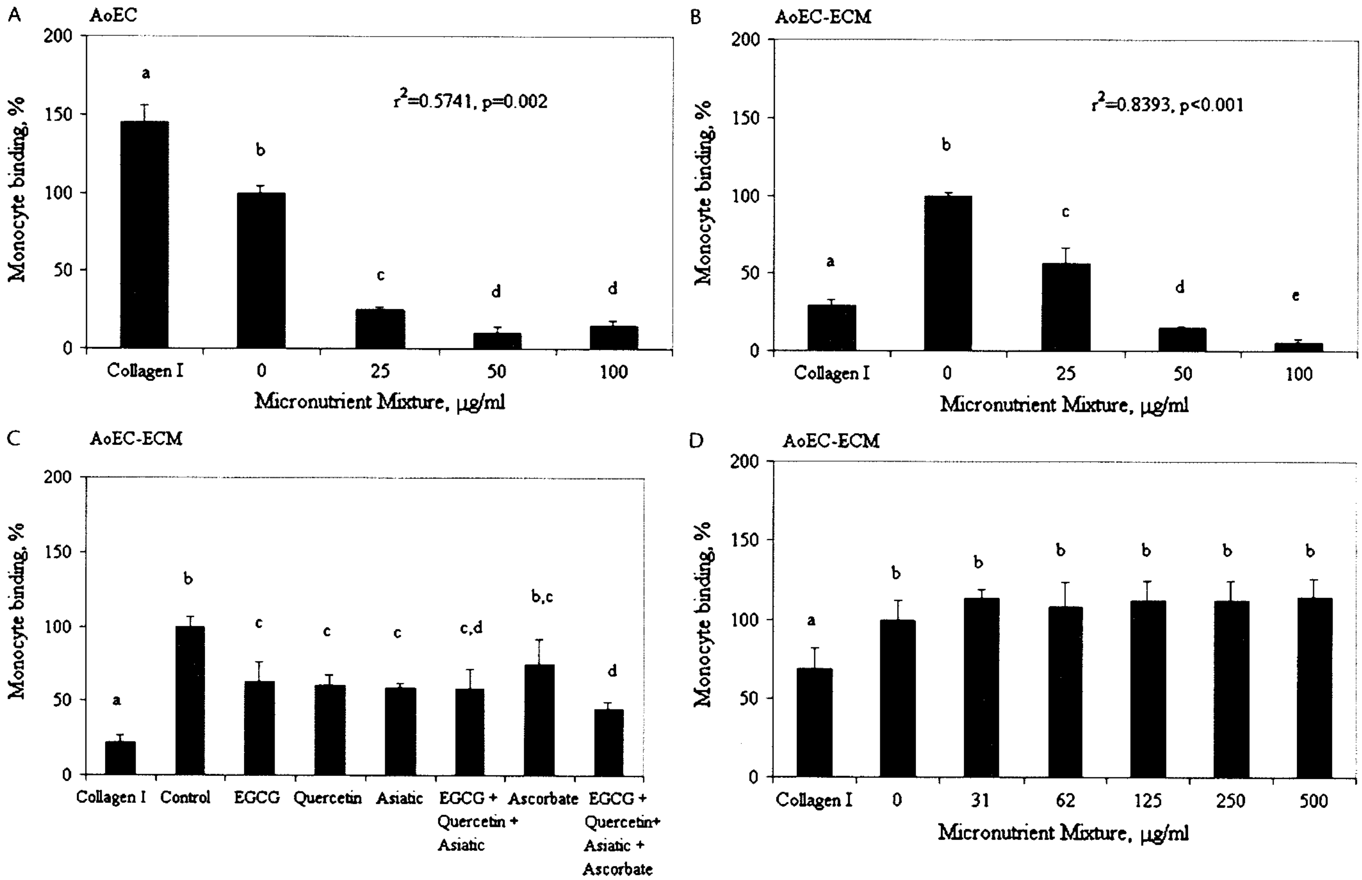

FIGURE 1. The micronutrient mixture reduces monocyte-binding properties of confluent culture of human aortic endothelial cells (AoEC). (A and B) Confluent layers of AoEC cultured on collagen type I-covered plastic were exposed to increased concentrations of the micronutrient mixture. After 48 hours of incubation, cells were either fixed with formaldehyde (A) or were removed to expose the underlying ECM layers (B). Monocyte attachment to resulting layers was assayed in supplementation-free medium by plastic-bound fluorescence and expressed as a percentage of unsupplemented AoEC control. (C) AoEC cultures were incubated for 48 hours with 200 µM ascorbate, individual polyphenols at 15 µM each or a mixture of 3 polyphenols at 5 µM each with or without 200 µM ascorbate. ECM layers were exposed and monocyte attachment was assayed as in A and B. (D) AoEC cultures were incubated in supplementation-free growth medium. ECM layers were exposed and monocyte attachment was assayed in the presence of increased concentrations of the micronutrient mixture. Unmatched lower case letters indicate a statistically significant difference between samples. For detailed experimental conditions, refer to the Material and Methods section.

R&D Systems) were used. For versican, rat MAB, goat anti-rat PAB, and rabbit anti-goat IgG-HRP (all from R&D Systems) were used. For hyaluronic acid assay, a set of biotylated hyaluronic acid-binding proteins (US Biological) and streptavidin-HRP (Rockland) was used. Some anti-ECM antibodies showed a noticeable cross-reactivity with control cell-free collagen type I-covered plastic after its supplementation with EGM-2 cell growth medium for 48 hours; therefore, the results are presented after subtracting HRP-conjugated antibody only blank sample values.

ECM Component Radiolabeling Experiments

AoEC were seeded into 24-well plates covered with collagen type I and treated as above. Additions of 10 µCi/mL [3H] L-leucine or 10 µCi/mL [3H] glucosamine individually or in combination with 20 µCi/mL $Na_2[35S]O_4$ to cell cultures were made in 500 µL/well of EGM-2 along with additions of tested compounds for 48-hour incubation. All radiochemicals were from MP Biomedicals (Solon, OH). To assess soluble GAGs, conditioned cell culture media were collected and divided on 2 200-µL aliquotes. One was treated with 200 µL of 30 U/mL hyaluronidase (from Streptomyces Hyalurolyticus) in 1% BSA/PBS for 3 hours at 37°C; controls received BSA/PBS solution. The enzymatic reaction was terminated by addition of 400 µL of ice-cold 20% TCA for 30 minutes at 4°C. The protein fraction was sedimented by centrifugation for 10 minutes at 13,000 × *g*, protein precipitate was washed twice by suspension in 1 mL of ice-cold ethanol and sedimentation by centrifugation. The final precipitate was dissolved in 500 µL of 0.2 N NaOH/0.2% sodium dodecyl sulfate for 2 hours at 50°C. The resulting solution was adjusted to neutral pH by addition of 50 µL of 2 N HCl and mixed with 7 mL of scintillation fluid (Beta Blend, MP Biomedicals). Radioactivity for [3H] and [35S] was measured as depositions per minute with double isotope program on LS800 scintillation counter (Beckman Coulter). To assess ECM-incorporated GAGs by

AoEC, ECM layers were prepared as above and washed with deionized water, followed by washing with 70% ethanol and drying on air. ECM layers were incubated with 500 μL of 30 U/mL hyaluronidase in 1% BSA/PBS for 3 hours at 37°C. Controls received BSA/PBS. Supernatants were collected for radioactivity count after mixing with scintillation fluid. ECM layers were further dissolved by incubation with 500 μL of 0.2 N NaOH/0.2% SDS for 2 hours at 50°C. Solutions were neutralized with 2 N HCl and mixed with scintillation fluid for radioactivity counts. Total ECM protein synthesis and deposition by AoEC was assayed by ECM-associated [3H] L-leucine as above, except enzymatic treatment was omitted.

ECM Treatment with Enzymes

The ECM was produced by AoEC cultures in the presence or absence of the micronutrient mixture as above and exposed to treatment with 1.5 U/mL Chondroitinase AC (MP Biomedics) or Chondroitinase B in Dulbecco Modified Eagle's Medium (ATCC) supplemented with 1% BSA for 3 hours at 37°C. Resulting ECM layers were washed 3 times with PBS and ECM chondroitin sulfate content and ECM U937 monocyte binding were assayed as described above.

Statistical Analysis

Results in figures are means ± SD from 3 or more repetitions of the most representative of at least 2 independent experiments. Results in Table 1 are means ± SD combined from 3 independent experiments. Differences between samples were estimated with a 2-tailed Student t test using Microsoft Excel and accepted as significant at P levels less than 0.05. Correlation analysis of relationship between changes in monocyte attachment and ECM composition (presented as correlation coefficients at P level of significance) and linear regression analysis (by ANOVA) of relationship between NM concentration and changes in ECM monocyte binding properties or content for individual components (presented as regression coefficient r^2 and P level of significance) were done with MedCalc software (Mariakerke, Belgium). Assessed relationships were considered significant at P levels less than 0.05.

RESULTS

Monocyte Attachment to EC and ECM Produced by EC

Human aortic EC plated and cultured to confluence significantly reduced (by 31%) monocyte binding properties of collagen type I–covered plastic (Figure 1A). Supplementation of AoEC with the micronutrient mixture further reduced the capacity of AoEC layer to immobilize fluorescently labeled monocytes in a dose-dependent manner. The reduction reached statistical significance at the lowest tested NM concentration (25 μg/mL) with 75% inhibition ($P < 0.001$). Inhibitory effect of NM reached saturation at 50 μg/mL with 90% inhibition.

ECM produced and deposited by AoEC cultures grown in plain EGM-2 medium significantly increased (by 3.5-fold) monocyte retention of original collagen type I matrices (Figure 1B). AoEC supplementation with NM significantly reduced ECM monocyte binding in a dose-dependent manner, with reduction of 56% at 25 μg/mL NM, compared to control values, and 94% at 100 μg/mL NM ($r^2 = 0.8393$ at $P < 0.001$ for a trend). When assessed as a modulation of monocyte-binding properties of original collagen type I matrices by

TABLE 1. Effects of Individual Components of the Micronutrient Mixture on the Composition of the ECM Produced by Human Aortic Endothelial Cells (AoEC) and Its Capacity to Retain Human U937 Monocytes

	Control	NM 100 (μg/mL)	GTE 16 (μg/mL)	GKE 11 (μg/mL)	AA 110 (μM)	Quercetin 27 (μM)
Collagen type I	100.0 ± 6.8	55.6 ± 8.9*	47.0 ± 2.8*	89.2 ± 11.2	98.6 ± 16.8	92.7 ± 11.7
Collagen type III	100.0 ± 6.2	57.9 ± 8.1*	50.1 ± 6.7*	105.6 ± 9.4	101.2 ± 5.5	94.1 ± 2.1
Collagen type IV	100.0 ± 5.3	65.5 ± 24.2*	39.1 ± 23.8*	93.9 ± 6.5*	100.0 ± 7.7	84.0 ± 4.9*
Laminin	100.0 ± 1.4	85.2 ± 5.6*	82.2 ± 8.7*	98.0 ± 5.9	94.7 ± 6.7	102.8 ± 2.5
Fibronectin	100.0 ± 3.3	84.4 ± 7.0*	78.7 ± 4.2*	96.6 ± 5.4	96.9 ± 2.8*	101.5 ± 2.8
Elastin	100.0 ± 4.4	66.3 ± 18.9*	51.0 ± 2.4*	96.9 ± 3.6	92.2 ± 5.6*	75.9 ± 2.6*
Biglycan	100.0 ± 3.6	95.3 ± 7.2	97.9 ± 6.3	100.1 ± 5.4	100.4 ± 4.4	99.0 ± 4.8
Decorin	100.0 ± 9.1	85.4 ± 11.1*	107.4 ± 5.2*	93.1 ± 18.3	94.2 ± 7.1	94.2 ± 27.2
Perlecan	100.0 ± 4.0	100.8 ± 11.3	98.7 ± 18.2	96.0 ± 9.3	96.0 ± 6.2	90.9 ± 5.0*
Versican	100.0 ± 8.2	122.7 ± 19.7*	110.4 ± 14.2	116.3 ± 31.6	107.6 ± 27.0	106.8 ± 12.6
Heparan Sulfate	100.0 ± 5.2	90.1 ± 15.0*	72.1 ± 8.4*	98.1 ± 17.5	99.8 ± 14.8	98.9 ± 11.2
Chondroitin Sulfate	100.0 ± 5.3	127.5 ± 36.6*	133.2 ± 29.0*	104.3 ± 15.9	97.5 ± 10.1	91.7 ± 7.4*
Hyaluronic Acid	100.0 ± 5.0	78.4 ± 14.0*	83.0 ± 13.3*	85.6 ± 3.3*	101.4 ± 7.0	70.3 ± 4.4*
U937 attachment	100.0 ± 10.9	56.8 ± 35.3*	58.8 ± 23.6*	104.6 ± 24.6	97.0 ± 17.4	108.7 ± 32.3

Data are means ± SD combined from 3 independent experiments and expressed as percentage of unsupplemented control.

NM, micronutrient mixture; GTE, green tea extract; GKE, gotu kola extract; AA, ascorbic acid.

Confluent layers of AoEC were incubated for 48 hours with indicated additions. Extracellular matrices were exposed by differential treatment. ECM hyaluronic acid content was assayed with byotinelated hyaluronate-binding protein, and other ECM components were assayed with corresponding immunoassay. Collagen type I (no cell control) values were 17.1 ± 6.2 for biglycan, 74.9 ± 29.0 for decorin, 2.1 ± 0.9 for perlecan, 77.2 ± 26.4 for versican, and 51.8 ± 11.8 for hyaluronic acid. Monocyte attachment to ECM was measured by fluorescence assay (see Materials and Methods section for details). NM components other than mentioned in Table 1 did not cause any significant effects when used individually at concentrations corresponding to 100 μg/mL NM (data not shown).

*$P < 0.05$.

AoEC under 100 μg/mL NM supplementation, inhibition of monocyte attachment was 81%.

NM polyphenol components, when supplied individually to AoEC cultures at 15 μM, significantly reduced monocyte-binding properties of AoEC-produced ECM (Figure 1C). There were no significant differences between tested individual polyphenols or their equimolar mixture. Ascorbic acid at 200 μM caused a 25% reduction in monocyte binding to ECM (nonsignificantly) when supplied to AoEC both in plain EGM-2 medium and in combination with polyphenols.

NM added to the incubation medium simultaneously with monocytes at concentrations up to 500 μg/mL did not influence monocyte attachment to AoEC-produced ECM (Figure 1D).

Composition of ECM Produced and Deposited by EC

AoEC supplementation with NM caused a dose-dependent decrease in relative ECM content for collagen types I, III, and IV, elastin, fibronectin, and laminin (Figure 2). The most pronounced inhibition was observed for collagen type IV, reaching 15% of unsupplemented control values at 200 μg/mL NM (Figure 2C). The least pronounced inhibitory effect of NM was observed for laminin, reaching 81.5% of unsupplemented control values at 200 μg/mL NM (Figure 2F). However, dependence of ECM laminin content on increasing NM concentration was relatively high and statistically significant ($r^2 = 0.7556$, $P < 0.001$). All other ECM proteins tested in this experiment demonstrated highly significant dependence on NM concentration.

The micronutrient mixture had a statistically significant inhibitory effect on heparan sulfate ECM incorporation ($r^2 = 0.3772$, $P = 0.007$), reaching 37% inhibition at 200 μg/mL NM (Figure 3). In contrast, NM increased ECM content of chondroitin sulfate in a dose-dependent manner ($r^2 = 0.9531$, $P < 0.001$), reaching 218% of control values at 200 μg/mL NM. Effects of individual NM components on ECM chondroitin sulfate content varied. EGCG at 15 μM increased ECM chondroitin sulfate by 255% of control, followed by asiatic acid at 139%. Effects of other components were not significant.

Incorporation of hyaluronic acid into the ECM was significantly reduced by AoEC supplementation with NM in a dose-dependent manner (Figure 3D), reaching 71% of the unsupplemented control at 100 μg/mL NM. In contrast, levels of hyaluronic acid synthesized and secreted by AoEC into cell culture media under NM supplementation gradually increased with increased NM concentrations, reaching 237% of control at 100 μg/mL NM. The soluble fraction of hyaluronic acid secreted by AoEC under control conditions exceeded that of ECM-associated hyaluronic acid by 16.1-fold in contrast to 7.1-fold prevalence of media-secreted total GAGs onto ECM-associated GAGs. The hyaluronic acid portion of total cell-conditioned media GAGs was 34.8%, whereas that of ECM-associated GAGs was only 15.4%. These tendencies were further emphasized by AoEC supplementation with NM. Accordingly, the hyaluronic acid part of total media GAGs was increased to 59.2%, and that of ECM-associated GAGs was reduced to 8.3% at 100 μg/mL NM supplementation.

As presented in Figure 3D, total incorporation of GAGs into the ECM by AoEC treated with 100 μg/mL NM was significantly increased both in polysaccharide content (up by 35% with [3H] glucosamine incorporation) and sulfate content (up by 70% with $[35S]O_4^{2-}$ incorporation). Polysaccharide content of total GAGs secreted to the conditioned media by AoEC under NM supplementation was significantly increased by 39%, whereas their sulfate content was not significantly changed. These changes were reflected in the changes of GAG sulfation index, defined as the relative change in the ratio of $[35S]O_4^{2-}$ incorporation to [3H] glucosamine incorporation normalized to unsupplemented control. Accordingly, NM-supplemented AoEC resulted in increased sulfation index for ECM-incorporated GAGs to 1.26 from 1.0 of unsupplemented control, and decreased sulfation index for media-secreted GAGs to 0.86 from 1.0 of unsupplemented control.

Changes in ECM proteoglycan incorporation by AoEC supplemented with NM, analyzed by immunoenzymatic assay, are presented in Table 1. ECM content for decorin was reduced by 100 μg/mL NM (14% reduction, $P < 0.05$), whereas versican content was significantly increased (23% increase, $P < 0.05$). Levels of biglycan and perlecan were not affected by NM. Total ECM protein deposition by AoEC, assayed with [3H] leucine incorporation, was increased to 129.7 ± 10.4% of unsupplemented control ($P < 0.05$) by 100 μg/mL NM supplementation (data not shown).

Effects of Individual Components of the Micronutrient Mixture

Effects of individual NM components on ECM composition and monocyte-binding properties were compared to the effects of NM at matched concentrations, as presented in Table 1. ECM capacity to retain monocytes was reduced by AoEC supplementation with green tea extract comparable to that of NM (down to 57% and 59% of unsupplemented control, respectively), whereas other components had no significant effect at the concentrations tested. ECM composition of AoEC supplemented with green tea extract followed the pattern of NM supplementation except for decorin content, which was increased by green tea and decreased by NM. AoEC supplementation with gotu kola extract decreased ECM hyaluronic acid and collagen type IV content, whereas other components were not affected significantly. Ascorbate effects were within 10% of unsupplemented controls for all ECM components. Quercetin was more effective in reducing ECM hyaluronic acid content (reduction by 30%) than any other tested micronutrient, including NM. It also effectively reduced elastin and collagen type IV content (by 24% and 16%, respectively). Effects of quercetin supplementation on chondroitin sulfate content of AoEC-produced ECM (reduction by 8%, $P < 0.05$) was opposite to that of NM and green tea extract, which produced statistically significant increases by 28% and 33%, respectively.

Correlation Analysis

As shown in Table 2, changes in the laminin content of the ECM had the strongest positive association with changes in monocyte attachment to ECM (CC = 0.9681, $P = 0.0015$). Other ECM components that had positive statistically

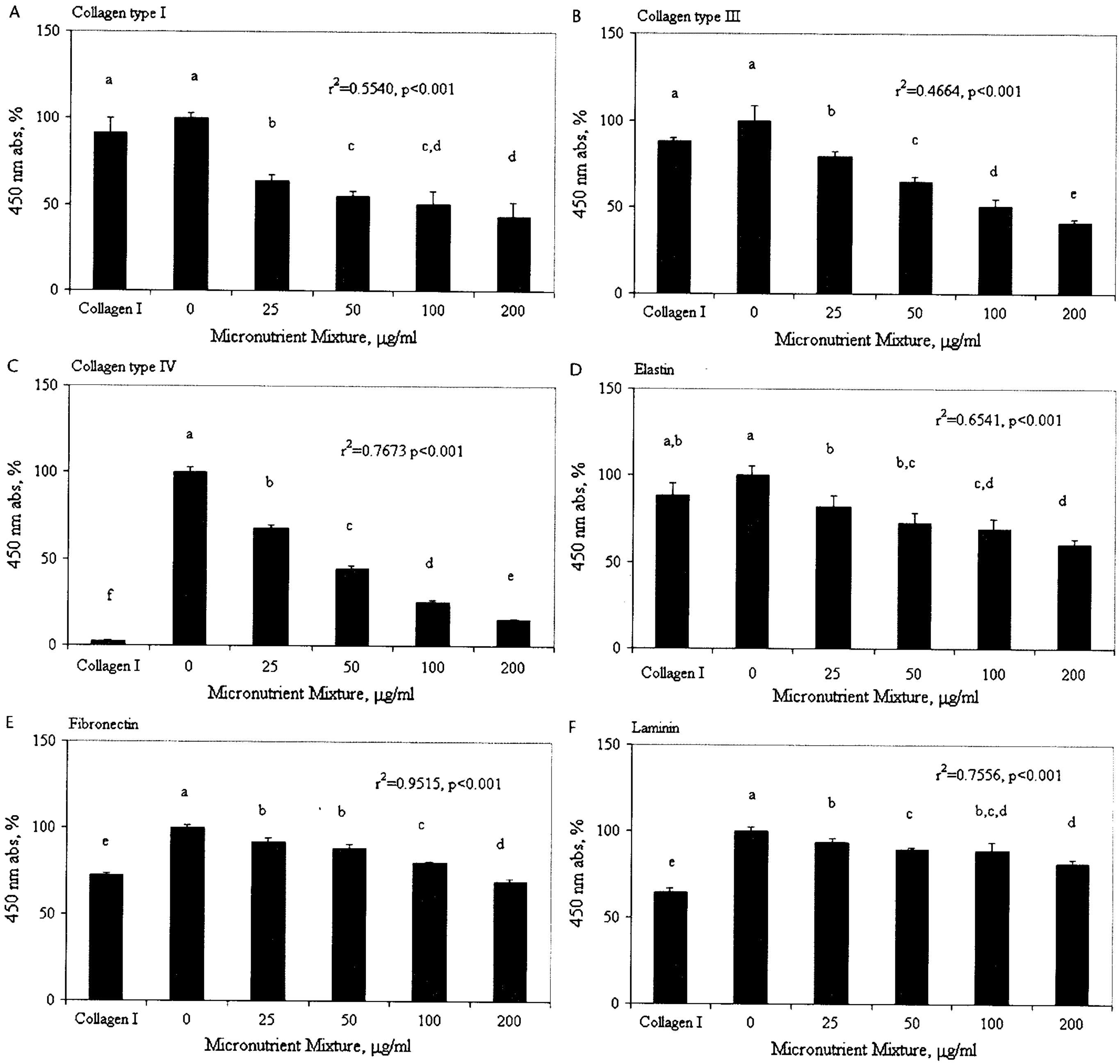

FIGURE 2. The micronutrient mixture modulates the deposition pattern of ECM proteins by aortic endothelial cells. ECM layers produced by confluent AoEC cultured on collagen type I-covered plastic in the presence of different doses of the micronutrient mixture were exposed by differential treatment. Relative changes in ECM composition were assayed by immunoassays specific for collagens type I (A), type III (B), and type IV (C), elastin (D), fibronectin (E), and laminin (F) as described in detail in the Materials and Methods section. Unmatched lower case letters indicate a statistically significant difference between samples (n = 4).

significant correlation with monocyte binding were, in descending order, fibronectin, collagens type III, I, and IV, biglycan and heparan sulfate. Changes in ECM elastin positively correlated with ECM monocyte binding properties (CC = 0.8002), but results did not reach statistical significance. Changes in chondroitin sulfate content of the ECM had the strongest negative correlation with changes in monocyte attachment (CC = −0.9623, P = 0.0021). Large proteoglycans, versican and perlecan, also had negative correlations with monocyte binding (CC = −0.6684 and −0.5476, respectively), but results did not reach statistical significance. Hyaluronic acid and decorin content of ECM were not significantly associated with monocyte binding.

Effects of Differential ECM Treatment with Digestive Enzymes on Monocyte Attachment

To evaluate direct involvement of increased ECM chondroitin sulfate on monocyte attachment, ECM layers

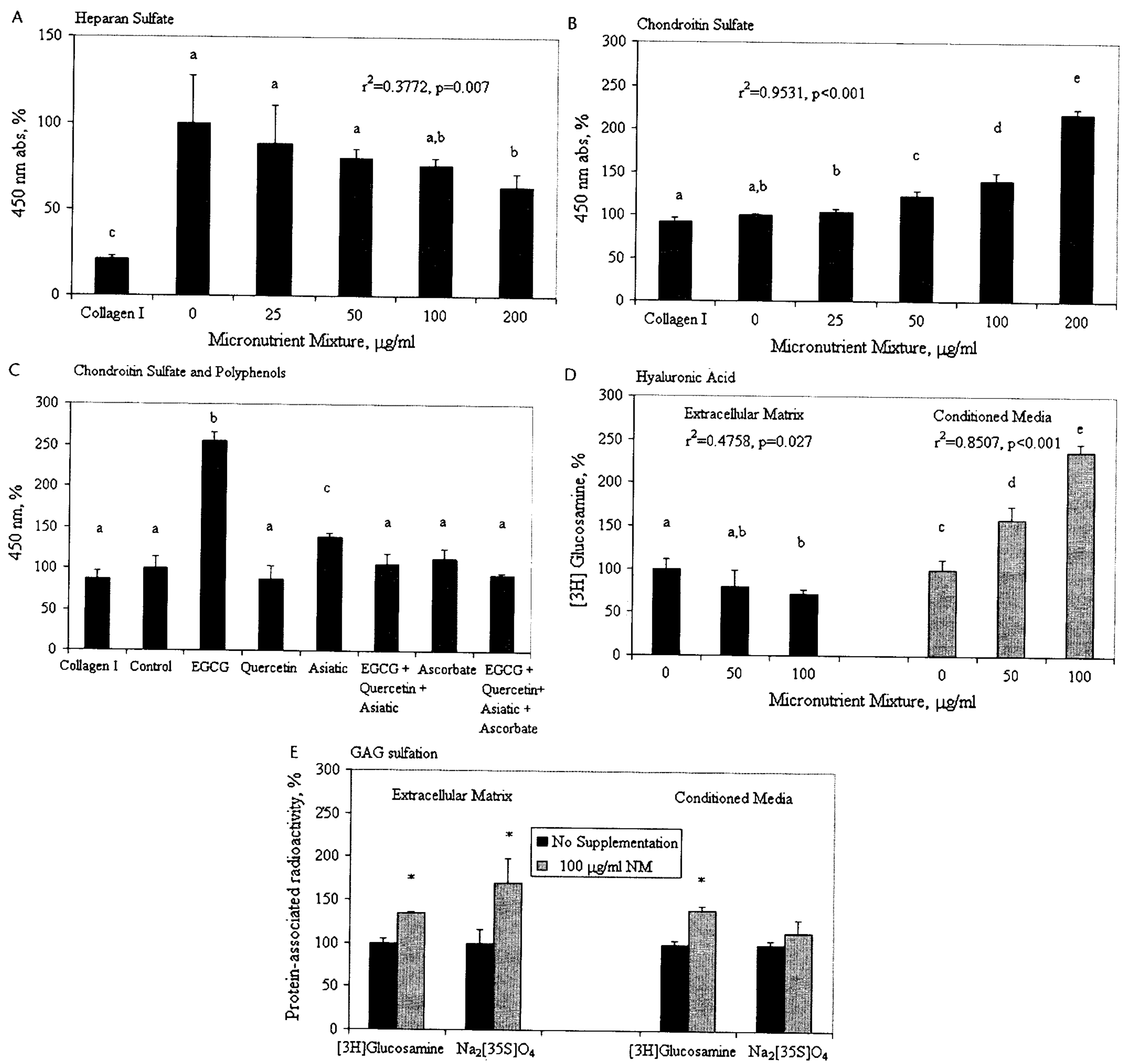

FIGURE 3. The micronutrient mixture modulates the deposition pattern of ECM-associated glycosaminoglycans (GAGs) by AoEC. The ECM was produced by AoEC under treatment with the micronutrient mixture (A, B, D, and E) or polyphenols (15 µM final concentration) with or without 200 µM ascorbate (C) as in Figures 1 and 2. ECM-associated GAGs were assayed by immunoenzymatic assay for heparan sulfate (A) and chondroitin sulfate (B, C) or by metabolic labeling with [3H] glucosamine and [35S] SO_4^{2-} for hyaluronic acid (D) and total GAGs (E) assays. Hyaluronic acid incorporation into ECM (C, black bars) and secretion to cell cultured media (D, grey bars) were estimated as hyaluronidase-sensitive release of radioactivity. Unsupplemented control values for hyaluronic acid were as follows: conditioned media: 17,238 ± 1970 dpm/well (34.8% of total media GAGs); ECM: 1071 ± 124 dpm/well (15.4% of total ECM-associated GAGs). Ratio of media hyaluronic acid release to ECM-associated hyaluronic acid was 16.1 to 1. Ratio of total media GAGs to ECM-associated GAGs was 7.1 to 1. Sulfation index in NM-supplemented samples (ratio of [35S] SO_4^{2-} to [3H] glucosamine GAG incorporation normalized by values for unsupplemented controls) was 1.26 for ECM-incorporated GAGs (E, left panel) and 0.86 for GAGs secreted to media (E, right panel). Unmatched lower case letters indicate a statistically significant difference between samples. *$P < 0.05$ as compared with unsupplemented controls.

produced by NM-supplemented AoEC and under control conditions were treated with chondroitinase AC or B. Both treatments significantly reduced levels of ECM-associated chondroitin sulfate (Figure 4). In NM-dependent ECM, 60.5% and 39% of total chondroitin sulfate were sensitive to chondroitinase AC and B treatment, respectively. Whereas,

TABLE 2. Correlation Analysis of Changes in the ECM Composition in Relation to ECM Capacity to Retain U937 Monocytes

Rank	ECM Component	Correlation Coefficient	*P*	95% Confidence Interval
1	Laminin	0.9681	0.0015	0.7300 to 0.9966
2	Fibronectin	0.9587	0.0025	0.6627 to 0.9956
3	Collagen type III	0.9548	0.0030	0.6366 to 0.9952
4	Collagen type I	0.9391	0.0054	0.5363 to 0.9935
5	Collagen type IV	0.8438	0.0347	0.1021 to 0.9825
6	Biglycan	0.8351	0.0386	0.0729 to 0.9815
7	Heparan Sulfate	0.8184	0.0465	0.0204 to 0.9794
8	Elastin	0.8002	0.0559	−0.0324 to 0.9772
9	Hyaluronic Acid	0.2098	0.6899	−0.7252 to 0.8728
10	Decorin	−0.0650	0.9026	−0.8327 to 0.7881
11	Versican	−0.5476	0.2607	−0.9410 to 0.4751
12	Perlecan	−0.6684	0.1467	−0.9595 to 0.3129
13	Chondroitin Sulfate	−0.9623	0.0021	−0.9960 to -0.6882

Analysis was based on experimental data from Table 1.

in control ECM, 48.7% and 32.4% of total chondroitin sulfate were sensitive to chondroitinase AC and B treatment, respectively. Both enzyme treatments resulted in significant reduction of consequent monocyte binding to control ECM prepared by unsupplemented AoEC, whereas chondroitin sulfate removal from NM-dependent ECM did not significantly affect consequent monocyte attachment.

DISCUSSION

Numerous epidemiological studies clearly indicate beneficial health effects of diets based on increased consumption of fresh fruits and vegetables. In particular, risk for the development of cardiovascular disease, especially heart attacks and strokes, is reduced.[22–24] This prompted a vigorous research effort directed toward testing the possible molecular mechanisms involved.[25,26]

Antioxidant activities of plant-derived vitamins and bioflavonoids were attributed to curbing oxidative stress-related modifications of blood plasma low-density lipoprotein and foam cell formation in atherosclerotic lesions.[27] Antioxidant micronutrients were also shown to beneficially affect vascular cell function by modulating redox-dependent signaling pathways.[28] In addition, recent studies demonstrated participation of certain micronutrients in regulation of gene expression, particularly genes involved in the inflammatory response, through interaction with nuclear factor kappa beta (NF-κB)–dependent signaling.[29]

Blood leukocyte adhesion to arterial EC is regulated and mediated by differential expression of specialized adhesive glycoproteins belonging to several families. These include selectins, cadherins, vascular cell adhesion molecules, intercellular adhesion molecules (ICAM), and others.[30] Different antioxidants, natural and chemically synthesized, demonstrated reduction in cell adhesion molecular expression by EC.[31] Frei's group reported effective blocking of tumor necrosis factor alpha (TNFα)–induced overexpression of adhesion molecules in human aortic EC by α-lipoic acid and dietary flavonoids, but not by ascorbic acid.[32,33] In contrast, Mo et al found ascorbate to be an effective inhibitor of ICAM-1 expression in a similar cell model along with similar effects from brown algae-derived alginate and garlic-derived allicin.[34] In addition, Mamputu and Renier reported inhibitory effects of α-tocopherol and ascorbate on ICAM-1 expression in bovine retinal EC stimulated by advanced glycation end products. They also demonstrated that this effect was oxidative stress-sensitive and involved protein kinase C and NF-κB–dependent signaling pathways.[35]

ECM components such as laminin, fibronectin, collagens, and heparan-sulfate proteoglycans were reported to mediate leukocyte adhesion through interaction with integrins expressed on leukocyte surface.[8–14] However, the information on possible regulation of leukocyte adhesion to EC–derived ECM by modulation of its composition and properties is largely missing. Here we report the effects of human AoEC supplementation with ascorbic acid, green tea catechins, asiatic acid, and quercetin on monocyte-binding properties and composition of AoEC-produced and deposed ECM.

L-ascorbic acid is an essential cofactor of three deoxygenases that play critical functions in procollagen post-translational modifications and proper collagen fibril formation.[36] Discovery of ascorbic acid deficiency as a molecular basis for scurvy, which is characterized by compromised structural integrity and function of connective tissue in general and that of blood vessels in particular, has attributed to it a vitamin status (Vitamin C). It was further hypothesized that life-long chronic subclinical shortage in ascorbate supplementation could be a cause for arterial atherosclerosis in humans who share an inherited lack in internal ascorbate synthesis with just a few other species.[37] This hypothesis has received experimental support from studies demonstrating development of spontaneous atherosclerotic lesions in animals, such as guinea pigs and genetically modified mice that lack internal ascorbate synthesis when kept on low ascorbate supply.[38,39] We previously reported ascorbic acid-dependent modification of cell growth controlling properties of ECM produced by vascular smooth muscle cells.[4]

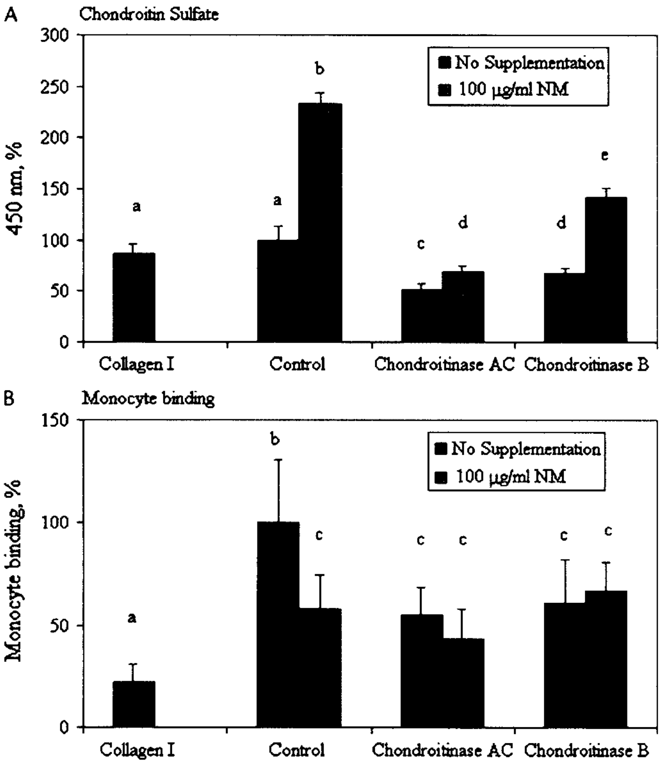

FIGURE 4. Effects of chondroitin sulfate removal by differential enzymatic treatment on U937 monocyte attachment to AoEC-produced ECM. The ECM was produced by AoEC cultures in the presence (grey bars) or absence (black bars) of 100 μg/mL micronutrient mixture. Exposed ECM was treated with chondroitinase AC or B for 3 hours at 37°C. Changes in chondroitin sulfate ECM composition (A) and U937 monocyte attachment to resultant ECM (B) were followed with immunoassay and monocyte attachment assay, respectively. Unmatched lower case letters indicate a statistically significant difference between samples. For detailed experimental conditions, refer to the Materials and Methods section.

In this study, we tested a mixture of micronutrients that have been previously shown to modulate ECM composition. Supplementation of cultured confluent AoEC with NM for 2 days resulted in a dramatic dose-dependent inhibition of the AoEC-deposed ECM to bind human monocytes. This inhibition was less pronounced when compared directly to NM inhibitory effects on monocyte adhesion to the entire EC layer, indicating that the ECM-specific effect was contributing to, but did not completely determine, a reduction in monocyte attachment. Interestingly, AoEC layers cultured under standard experimental conditions without supplementation bound monocytes significantly less than the original collagen type I matrix, which was used as a substrate for AoEC culture plating and growth. When the collagen layer was modified with ECM components produced and deposited by the AoEC while cultured without supplementation, its ability to bind monocytes significantly increased. This indicates an involvement of ECM components, other than collagen type I, in the monocyte adhesion process. Indeed, analysis of ECM composition by immunochemical assay demonstrated that cultured confluent layers of AoEC significantly increased content of such ECM proteins as collagen type IV, fibronectin, and laminin. Immunochemical detection of collagens type I and III and elastin did not significantly differ between AoEC-deposed ECM and original collagen type I cell growth substrate preincubated in EGM-2 cell growth medium.

The pattern of ECM protein deposition dramatically changed when AoEC were cultured under micronutrient supplementation. The relative content of all tested ECM proteins was decreased by NM supplementation in a dose-dependent manner with the exception of versican, which was increased, and biglycan and perlecan, which were not significantly affected. Decrease in ECM collagen content was probably not the result of increased activity of matrix metalloproteinases (MMPs). As we have shown previously, MMP activity was significantly reduced in cultured endothelial and other cells treated with NM.[40–42] Correlation analysis revealed a strong positive relationship between changes in monocyte adhesion and modulations of ECM content, in decreasing order, for laminin, fibronectin, and collagens type III, I, and IV, and it was less pronounced for elastin. Decrease in relative collagen types I, III, and IV, laminin, fibronectin, and elastin content can be a contributory factor to molecular mechanisms responsible for the observed decline in monocyte adhesion to ECM, as these proteins have been reported to mediate leukocyte adhesion.[8–14] Whether a decline in the content of these ECM proteins under NM supplementation is a causative factor for reduction of monocyte binding remains to be seen. All polyphenols, which have been included in NM, reduced monocyte-binding properties of AoEC-produced ECM, whereas ascorbic acid did not produce a significant inhibitory effect. Polyphenols have been shown to bind proteins,[40] thus ECM-bound NM components could contribute to a reduction of monocyte binding. This possibility was ruled out because there was no direct effect of NM on monocyte attachment to AoEC-produced ECM.

The observed decline in the ECM content of most of the tested proteins under NM supplementation does not agree with NM-driven increase in total ECM protein deposition. The modest increase in ECM versican content apparently is not sufficient to explain this discrepancy. A possible explanation is that some proteins other than those assayed were increased in AoEC-deposed ECM. Another possible explanation is the limited capacity of the immunochemical assay to accurately describe quantitative changes in ECM composition, such as limited antibody access to antigens located deep within the complex 3-dimensional ECM mesh.

Total ECM deposition of glycosaminoglycans by AoEC was significantly increased by NM, accompanied by qualitative changes in the structure of GAGs, as their sulfation increased by 26%. It was suggested that decreased sulfation of arterial wall proteoglycans may predispose atherosclerotic lesions to thrombosis by disrupting osmotic regulation and limiting antithrombin activity.[44] Thus, NM-driven increase in ECM-associated GAG sulfation could be regarded as a beneficial antiatherosclerotic effect. The major portion of GAGs synthesized by cultured AoEC was secreted to cell culture medium. This tendency was further emphasized under supplementation with NM. However, in contrast to ECM-associated

GAGs, the sulfation level of media-secreted GAGs was decreased by 14% by micronutrient action. These data are supported by observation of NM-mediated increase in synthesis and release to media of hyaluronic acid, which is not a sulfated GAG species, whereas progressive ECM depletion with hyaluronate provides a possible mechanistic explanation to increased sulfation levels of ECM-associated GAGs.

Qualitative changes in deposition of GAGs to ECM by AoEC were further assessed by analysis of individual GAG species. ECM deposition of heparan sulfate and hyaluronic acid by AoEC was significantly inhibited by NM supplementation. Changes in ECM heparan sulfate content had statistically significant positive association with monocyte binding, and changes in ECM hyaluronic acid content were not associated. In contrast, control AoEC cultures produced little chondroitin sulfate, and NM supplementation caused significant dose-dependent increase in its ECM deposition. These changes were strongly negatively associated with monocyte binding ($r^2 = -0.9623$, $P = 0.0021$). Increase in chondroitin sulfate ECM content under AoEC treatment with NM was due the result of increased chondroitin sulfate subspecies A and C, rather than B (also called dermatan sulfate) as followed from ECM differential enzyme treatment studies. NM-mediated increase in ECM chondroitin sulfate was accompanied by ECM enrichment with large proteoglycan versican, the main glycosaminoglycan portion of which is presented by chondroitin sulfate. NM-dependent reduction in ECM heparan sulfate was not accompanied by significant changes in the major heparan sulfate-bearing large proteoglycan perlecan. Possible explanations to this discrepancy could be either depletion of perlecan molecule from its glycosaminoglycan chains or the presence of other heparan sulfate-bearing proteoglycans.

The reduction in monocyte-binding properties of ECM produced by AoEC under NM supplementation was accompanied by increased ECM content of chondroitin sulfate; therefore, we tested whether chondroitin sulfate directly mediated monocyte binding. Treatment with chondroitinases AC and B resulted in decreased monocyte retention by ECM from unsupplemented AoEC and reduced chondroitin sulfate content, indicating its direct involvement in monocyte binding. In contrast, effective removal of chondroitin sulfates from NM-dependent ECM did not result in significant alteration of monocyte attachment to ECM. This suggests that AoEC supplementation with NM could alter properties of chondroitin sulfate synthesized and deposited by AoEC.

Micronutrient-driven replacement of ECM hyaluronate with chondroitin sulfate may indicate formation of a denser and more robust ECM; hyaluronic acid has been shown to retain water within the ECM, increasing its volume and decreasing its density. Elevated hyaluronic acid content was observed in the subendothelial space in arterial regions affected by atherosclerosis.[45] Increased ECM density could contribute to a reduction in monocyte retention by ECM.

Tested micronutrients had variable effect on monocyte adhesion to ECM and ECM composition as compared to NM, the most effective being green tea extract. However, when effects of purified polyphenols at matched concentrations were directly compared in the monocyte-binding assay, there were no significant differences. Nevertheless, a mixture of all tested flavonoids and ascorbic acid produced the most pronounced inhibitory effect. In this experiment, the effects of AoEC supplementation with individual polyphenols on ECM chondroitin sulfate content differed significantly, and these changes did not correlate with modulations in monocyte attachment to ECM (compare Figures 1C and 3C; CC = 0.1463, $P = 0.6678$), supporting therefore a suggestion of more complex mechanisms involved.

Green tea, gotu kola, and quercetin-enriched fruits and vegetables, such as apples and onions have been considered supportive of good health. Effective dosages of the micronutrients in our model were comparable to concentrations observed in human tissues after oral supplementation,[36,46–48] justifying study of the tested micronutrient mixture in clinical and in vivo studies.

CONCLUSION

A mixture of ascorbic acid, green tea extract, gotu kola extract, and quercetin effectively reduced monocyte-binding properties of ECM produced by cultured human aortic EC. This effect contributed to the general reduction of monocyte adhesion to the EC layer formed under micronutrient supplementation. Reduction in monocyte adhesion was accompanied by profound changes in ECM composition, providing mechanistic explanation for the observed modulations of monocyte adhesion. Individual nutrients had diverse effects demonstrating possible involvement of multiple molecular mechanisms.

ACKNOWLEDGMENTS

The research study was funded by Dr. Rath Health Foundation (Plantation, FL), a nonprofit organization.

REFERENCES

1. International atherosclerosis society harmonized guidelines on prevention of atherosclerotic cardiovascular diseases. April 30, 2003. [IAS web site]. Available at: http://www.athero.org/download/fullreport.pdf. Accessed April 9, 2008.
2. Barnes MJ, Farndale RW. Collagens and atherosclerosis. *Exp Gerontol.* 1999;34:513–525.
3. Heeneman S, Cleutjens JP, Faber BC, et al. The dynamic extracellular matrix: intervention strategies during heart failure and atherosclerosis. *J Pathol.* 2003;200:516–525.
4. Ivanov VO, 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.
5. Ivanov V, Ivanova S, Roomi MW, et al. Extracellular matrix-mediated control of aortic smooth muscle cell growth and migration by a combination of ascorbic acid, lysine, proline, and catechins. *J Cardiovasc Pharmacol.* 2007;50:541–547.
6. Ross R. Atherosclerosis-an inflammatory disease. *N Engl J Med.* 1999; 340:115–126.
7. Roque M, Fallon JT, Badimon JJ, et al. Mouse model of femoral artery denudation injury associated with the rapid accumulation of adhesion molecules on the luminal surface and recruitment of neutrophils. *Arterioscler Thromb Vasc Biol.* 2000;20:335–342.
8. Schapira K, Lutgens E, de Fougerolles A, et al. Genetic deletion or antibody blockade of alpha1beta1 integrin induces a stable plaque phenotype in ApoE−/− mice. *Arterioscler Thromb Vasc Biol.* 2005;25:1917–1924.
9. Boisvert M, Gendron S, Chetoui N, et al. Alpha2 beta1 integrin signaling augments T cell receptor-dependent production of interferon-gamma in human T cells. *Mol Immunol* 2007;44:3732–3740.