

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

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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.

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The authors state that they have no financial interest in the products mentioned within this article.

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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- μ m filter. When diluted appropriately, the 100 μ g/mL NM solution contained 16 μ g/mL GTE (14 μ M EGCG), 11 μ g/mL GKE (2 μ M asiatic acid), 27 μ M quercetin, 110 μ M L-ascorbic acid, 110 μ M L-lysine HCl, 140 μ M L-proline, 22 μ M L-methionine, 27 μ M L-cysteine HCl, 6 μ M choline bitartrate, 1 μ M copper glycinate, 0.24 μ M cyanocobalamin, 0.04 μ M folic acid, and 0.8 μ M pyridoxine 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% CO₂ 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/cm² 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 al²⁰ 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 others²¹ 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 μ M 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 μ L (250,000 cells) per well. Cell cultures were supplemented with 10 μ g/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 μ L 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 immunoassays 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

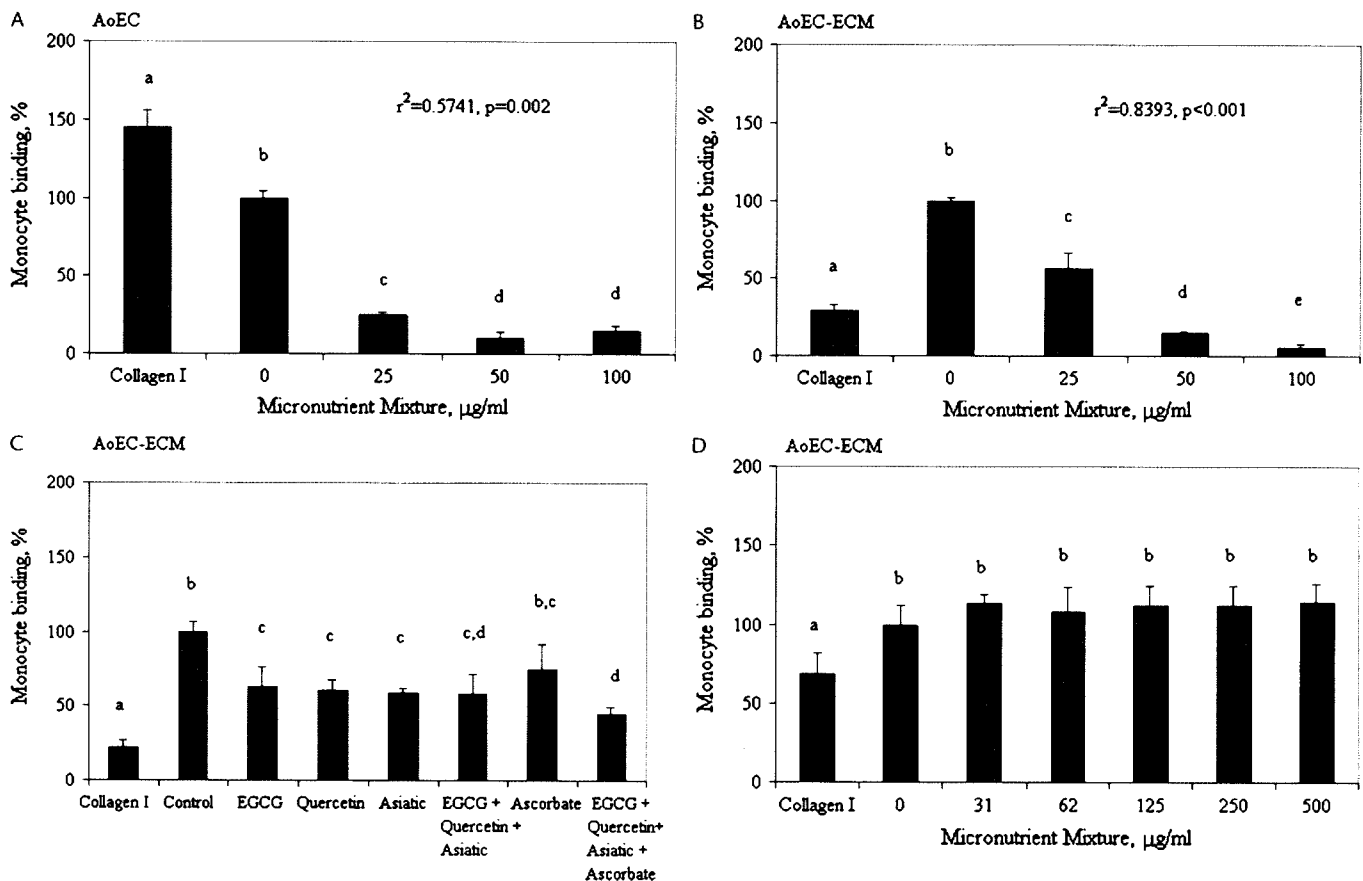


FIGURE 1. The micronutrient mixture reduces monocyte-binding properties of confluent culture of human aortic endothelial cells (AoEC). (A and B) Confluent layers of AoEC cultured on collagen type I-covered plastic were exposed to increased concentrations of the micronutrient mixture. After 48 hours of 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 Na₂[35S]O₄ 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 μ L 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 μ L 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 \pm SD from 3 or more repetitions of the most representative of at least 2 independent experiments. Results in Table 1 are means \pm 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 μ g/mL) with 75% inhibition (*P* < 0.001). Inhibitory effect of NM reached saturation at 50 μ g/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 μ g/mL NM, compared to control values, and 94% at 100 μ g/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 (μ g/mL)	GTE 16 (μ g/mL)	GKE 11 (μ g/mL)	AA 110 (μ M)	Quercetin 27 (μ M)
Collagen type I	100.0 \pm 6.8	55.6 \pm 8.9*	47.0 \pm 2.8*	89.2 \pm 11.2	98.6 \pm 16.8	92.7 \pm 11.7
Collagen type III	100.0 \pm 6.2	57.9 \pm 8.1*	50.1 \pm 6.7*	105.6 \pm 9.4	101.2 \pm 5.5	94.1 \pm 2.1
Collagen type IV	100.0 \pm 5.3	65.5 \pm 24.2*	39.1 \pm 23.8*	93.9 \pm 6.5*	100.0 \pm 7.7	84.0 \pm 4.9*
Laminin	100.0 \pm 1.4	85.2 \pm 5.6*	82.2 \pm 8.7*	98.0 \pm 5.9	94.7 \pm 6.7	102.8 \pm 2.5
Fibronectin	100.0 \pm 3.3	84.4 \pm 7.0*	78.7 \pm 4.2*	96.6 \pm 5.4	96.9 \pm 2.8*	101.5 \pm 2.8
Elastin	100.0 \pm 4.4	66.3 \pm 18.9*	51.0 \pm 2.4*	96.9 \pm 3.6	92.2 \pm 5.6*	75.9 \pm 2.6*
Biglycan	100.0 \pm 3.6	95.3 \pm 7.2	97.9 \pm 6.3	100.1 \pm 5.4	100.4 \pm 4.4	99.0 \pm 4.8
Decorin	100.0 \pm 9.1	85.4 \pm 11.1*	107.4 \pm 5.2*	93.1 \pm 18.3	94.2 \pm 7.1	94.2 \pm 27.2
Perlecan	100.0 \pm 4.0	100.8 \pm 11.3	98.7 \pm 18.2	96.0 \pm 9.3	96.0 \pm 6.2	90.9 \pm 5.0*
Versican	100.0 \pm 8.2	122.7 \pm 19.7*	110.4 \pm 14.2	116.3 \pm 31.6	107.6 \pm 27.0	106.8 \pm 12.6
Heparan Sulfate	100.0 \pm 5.2	90.1 \pm 15.0*	72.1 \pm 8.4*	98.1 \pm 17.5	99.8 \pm 14.8	98.9 \pm 11.2
Chondroitin Sulfate	100.0 \pm 5.3	127.5 \pm 36.6*	133.2 \pm 29.0*	104.3 \pm 15.9	97.5 \pm 10.1	91.7 \pm 7.4*
Hyaluronic Acid	100.0 \pm 5.0	78.4 \pm 14.0*	83.0 \pm 13.3*	85.6 \pm 3.3*	101.4 \pm 7.0	70.3 \pm 4.4*
U937 attachment	100.0 \pm 10.9	56.8 \pm 35.3*	58.8 \pm 23.6*	104.6 \pm 24.6	97.0 \pm 17.4	108.7 \pm 32.3

Data are means \pm 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 biotinylated hyaluronate-binding protein, and other ECM components were assayed with corresponding immunoassay. Collagen type I (no cell control) values were 17.1 \pm 6.2 for biglycan, 74.9 \pm 29.0 for decorin, 2.1 \pm 0.9 for perlecan, 77.2 \pm 26.4 for versican, and 51.8 \pm 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 μ g/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]O₄²⁻ 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]O₄²⁻ 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 $129.7 \pm 10.4\%$ 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

