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Research Article

Fat-Soluble Vitamins Affect Composition of Extracellular Matrix Deposited by Human Aortic Smooth Muscle and Endothelial Cells *In Vitro*

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Atherosclerotic cardiovascular disease is accompanied by changes in arterial connective tissue. We evaluated the effects of fat-soluble vitamins A, D, and E individually and in combinations on the composition of extracellular matrix produced and deposited by arterial wall cells, human aortic smooth muscle cells, and endothelial cells. Individually, vitamins D and E stimulated collagen type I extracellular matrix deposition in human aortic smooth muscle cell cultures. However, vitamins A, D, and E reduced collagen type IV deposition by human aortic smooth muscle cell, counteracting the stimulatory effects of vitamin C. The extracellular matrix deposition of heparan sulfate by human aortic smooth muscle cells increased by vitamin C and its combination (C+D+E). β-carotene + D + C induced the extracellular matrix deposition of collagen I by endothelial cells. Vitamin E with other vitamins resulted in either induction (E+C+A) or inhibition (E+D). The extracellular matrix deposition of type IV collagen and elastin by human aortic endothelial cells was not affected by test vitamins, except the extracellular matrix type IV collagen decrease by combinations (A+E), (A+D+E), and (C+D+E). The extracellular matrix deposition of all tested glycosaminoglycans was reduced by vitamin A and its combination (A+C+D+E). Therefore, the fat-soluble vitamins applied individually or in combination—both with each other or with ascorbic acid—can affect extracellular matrix deposition of type I and IV collagens, and key glycosaminoglycans by cultured human aortic arterial wall cells.

Keywords: Aortic smooth muscle cells, Atherosclerosis, Collagen, Extracellular matrix, Fat-soluble vitamins

Abbreviations Used: Cardiovascular disease, CVD; Chondroitin sulfate, ChS; DMEM, Dulbecco's modified Eagle medium; EBM-2 medium, Endothelial cell growth basal medium-2; Extracellular matrix, ECM; Fetal bovine serum, FBS; Glycosaminoglycans, GAGs; Heparan sulfate, HS; Horse radish peroxidase, HRP; Human aortic endothelial cells, HAEC; Human aortic smooth muscle cell, HASMC; Hyaluronic acid, HA; Phosphate-buffered saline, PBS; Smooth muscle cells, SMC; 3,3′,5,5′-Tetramethylbenzidine, TMB

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INTRODUCTION

CVD remains a leading cause of mortality and morbidity in the modern world. An estimated 92.1 million US adults have at least one type of CVD. It is projected that by 2030, 43.9% of the US adult population will have some form of CVD. There is no significant difference in gender distribution of CVD (Benjamin et al., 2017; GBD, 2015). Based on epidemiological data, an imbalanced diet is being considered as an independent risk factor for CVD development (Casas et al., 2018). The prevalence of processed food in the average diet leads to the development of chronic deficiency of vitamins and other essential nutrients (Lacroix et al., 2017). Specifically, targeted epidemiological studies evidence the benefits of supplementation with specific vitamins, such as vitamin C (Shi et al., 2018), vitamin E (Mathur et al., 2015), vitamin D (Wang et al., 2008), and vitamin A (Wood et al., 2014; Wang et al., 2014) among others, in protecting against CVD.

Vitamins are essential nutrients for maintaining normal body metabolism and sustaining life. Generally, they are divided into two groups: water-soluble (vitamin C and B-group vitamins) and fat-soluble (vitamins A, D, and E). Different vitamin metabolic functions include the regulation of numerous biochemical pathways through the activation of specific enzymes, cellular receptors, as well as antioxidant action and many others (Ames, 2018). Micronutrient-related changes in these metabolic pathways have been implicated in the development of various pathologies, including CVD.

Atherosclerosis is one of the main underlying pathological mechanisms in CVD development (Tabas, 2017; Ross, 1999). The

arterial wall consists of several morphologically distinct layers characterized by specific structures and functions. The intima layer, the innermost layer of arterial wall, forms a selective barrier between blood and tissue. It is formed by an endothelial cell monolayer resting on the so-called basal membrane, a dense cell free connective tissue produced and deposited by endothelial cells. The media layer of the arterial wall is built of connective tissue and resident smooth muscle cells, which produce ECM. The media is responsible for the mechanical stability of the arterial wall. The contractile function of smooth muscle cells allows the arterial wall to withstand pulsing blood pressure from the beating heart. The adventitia layer is the outermost part of the arterial wall, separating blood vessels from surrounding tissues. It is formed by fibroblasts and produced by connective tissue. During the atherosclerotic process, destabilization of the ECM affects the vascular environment leading to detachment of endothelial cells in the intima, which facilitates an uncontrolled influx of blood cells and blood factors to inner wall regions through newly exposed basal membrane. In response, vascular smooth muscle cells (SMC) migrate from the media to the intima, overpopulating under basal membrane regions. There, SMC lose their contractile properties, start to proliferate, and produce and deposit abnormal quantities of the ECM to stabilize the vascular wall. All these events contribute to local inflammation and further destabilization of the arterial wall's structural integrity and function. The ECM of the arterial wall plays an essential role in maintaining vascular mechanical stability and proper functionality. Its components are produced and deposited by the resident arterial wall cells, smooth muscle, and endothelial cells, and form a complex three-dimensional (3D) structure. The ECM consists of structural proteins, such as different tissue-specific types of collagen, elastin, fibronectin, and proteoglycans. The latter are a specific class of proteins covalently bound to sulfated polysaccharide chains, GAGs, which are predominantly represented by HS and ChS molecules. Another important nonprotein bound glycosaminoglycan component, hyaluronic acid (HA), is responsible for regulation of tissue water retention and osmotic properties of the ECM (Wagenseil, 2009). The basic 3D structure of the ECM in the arterial wall is determined by the proper formation and structure of elongated collagen fibrils. The predominant collagen species in the media layer is type I collagen, produced and deposited by SMCs and vascular fibroblasts, whereas the basal membrane is enriched with collagen type IV, produced and deposited by endothelial cells. The composition of ECM undergoes dramatic changes during the atherosclerotic process (Shekhonin et al., 1987). The synthesis and post-translational modifications of collagen molecules depend on sufficient ascorbic acid (vitamin C) which functions as a coenzyme for several limiting enzymes in collagen synthesis and directly regulates collagen gene expression (Cameron et al., 1979; Kim et al., 2013). An acute vitamin C deficiency leads to the development of clinical scurvy within a few months, characterized by a weakened arterial wall structure incapable of maintaining blood within the vascular system. In contrast, chronic decades long subclinical deficiency in vitamin C supplementation, a typical condition of the modern human diet, can weaken the arterial wall, triggering a cascade of compensatory mechanisms that result in the formation of atherosclerotic vascular plaques and other pathological changes, which are eventually manifested as clinical CVD (Rath et al., 1992). Previously we reported on the numerous health benefits of ascorbic acid (vitamin C) including its involvement in regulating the quantities and qualities of the ECM produced and deposited by cultured arterial smooth muscle and endothelial cells, as well as preventing triggering events in atherosclerosis (Ivanov et al., 2007, 2008, 2016; Cha et al., 2015).

Although vitamins are widely used in the form of supplements, their effects on ECM production and composition in the arterial wall remain largely unknown. In recent years, several studies have investigated the role of vitamins in CVD, including vitamin C and fat-soluble vitamins A, D, and E (Tsugawa, 2015; Galli et al., 2017; Miller et al., 2020). However, these studies focused on different metabolic aspects than detail characteristics of vascular ECM investigated in our study (addressed in detail in the section on "Discussion"). As such, the effects of fat-soluble vitamins on ECM production and composition in the arterial wall remain largely unknown. This information is also important considering that fat-soluble vitamins are widely used as nutritional supplements and taken either individually or in combinations for various health aspects, including cardiovascular health.

Therefore, in this study we investigated the effects of fat-soluble vitamins – tested individually or in various combinations, as well as with vitamin C added – on the ECM produced in vitro by residential arterial wall cells such as HASMC and HAEC. The results of our study add novel information and contribute to better understanding of the involvement of fat-soluble vitamins in regulating the structural stability and function of the arterial wall.

MATERIALS AND METHODS

Reagents

All reagents were from Sigma-Aldrich (St. Louis, MO) except when indicated differently.

Cell Cultures

Human aortic smooth muscle cells and human aortic endothelial cells were purchased from Cambrix (East Rutherford, NJ). HASMC stock cultures were maintained in DMEM medium (ATCC) containing antibiotics and 5% fetal bovine serum (FBS, ATCC). HAEC were cultured in EBM-2 medium (Cambrix). Control growth media did not contain any vitamins A, C, D, or E. 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 resulted in statistically significant cell death (data not shown).

Deposition of ECM Proteins and Glycosaminoglycans by Human Vascular Cells

For the experiments, HAEC or HASMC, at 5th to 8th 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 5–7 days in corresponding growth media. Supplementation with tested compounds in experiments was done in 2% FBS/DMEM and in EBM-2 medium for HASMC and HAEC, respectively. All tested compounds were added to the cells at indicated concentrations for 72 h. Cell-produced ECM was exposed by sequential treatment with 0.5% Triton X-100 and 20 mM ammonium sulfate in phosphate-buffered saline (PBS, Life

Technologies) for 3 min each at room temperature as described previously (Ivanov et al., 2007). After four washes with PBS, ECM layers were treated with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature and immediately used in experiments.

Immunoassays for types I and IV collagen, elastin, heparan sulfate, chondroitin sulfate, and hyaluronic acid were done as described previously by sequential incubation with corresponding primary monoclonal antibodies (Sigma-Aldrich, except for anti-heparan sulfate supplied by the US Biologics) in 1% BSA/PBS for 2 h followed by 1-h incubation with secondary goat antimouse IgG antibodies labeled with HRP (Ivanov et al., 2008). Retained HRP activity was measured after the last washing cycle (three times with 0.1% BSA/PBS) using 3,3′,5,5′-Tetramethylbenzidine peroxidase substrate reagent. Optical density at 450 nm was evaluated by plate reader (Molecular Devices) and expressed as a percentage of control cell samples cultured in nonsupplemented media. In each experiment immunoassays were performed simultaneously and identically in HAEC and HASMC plates, allowing the expression of individual ECM components in two different cell types to be directly compared.

Statistical Analyses

Results are presented as mean ± SD from four or more repetitions from six independent experiments. Not all the experiments contained all listed experimental conditions, but each experimental condition was assayed in at least one independent experiment. Control samples cultured in nonsupplemented media were present in each individual experiment and served as 100% reference value within each experiment. Differences between samples were estimated with a two-tailed Student's *t*-test using Microsoft Excel software and accepted as significant at P-values less than 0.05.

RESULTS

In the experiments, we chose the most effective dosages of vitamins, which were determined in a separate preliminary dose-dependent experiment. The dosages of vitamins were within the physiological human blood plasma concentration range for each individual compound (data not shown).

Deposition of Extracellular Matrix Proteins by Human Aortic Smooth Muscle Cells

As expected, the most significant deposition of type I collagen by cultured HASMC was induced by treatment with ascorbic acid (increase by 201%). Also, other vitamins applied individually had smaller, but significant collagen I-stimulating effects compared to controls, such as vitamin D (by 24.6%), vitamin E (by 23.4%), and by vitamin A precursor beta-carotene (by 30.4%) (Fig. 1). The effects of combining the two vitamins varied. The combination of vitamin C with vitamins E and D resulted in increased type I collagen deposition by HASMC by 444% and 402%, respectively, compared to controls. The highest stimulatory effects on collagen type I deposition were observed in a combination of vitamin C plus beta-carotene (400% over control and 200% more than achieved with vitamin C individually). Vitamin A supplementation at 2 μM was not effective when applied individually and its combination

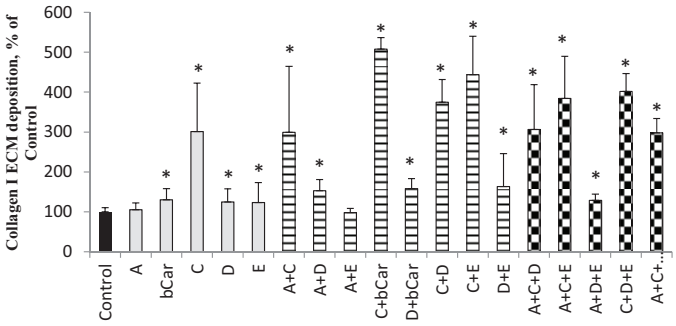

FIGURE 1 | Extracellular matrix (ECM) deposition of type I collagen by human aortic smooth muscle cells. Confluent layers of HASMC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in 2% FBS/DMEM for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for type I collagen content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bars stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

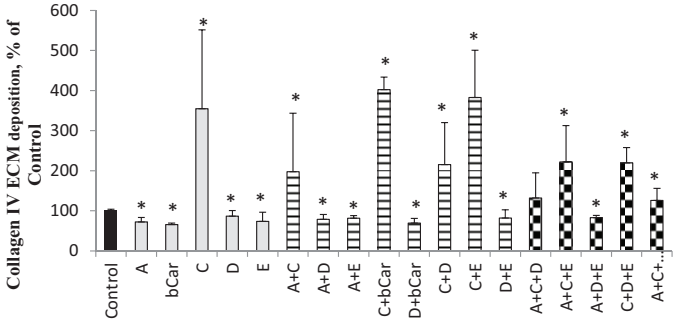

FIGURE 2 | Extracellular matrix deposition of type IV collagen by human aortic smooth muscle cells. Confluent layers of HASMC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in 2% FBS/DMEM for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for type IV collagen content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

with vitamin C did not result in additional collagen I deposition. Three or more vitamin combinations had stimulatory effects on collagen I compared to control, with a modest increase observed in vitamins A+D+E (by 28%) and the highest for vitamins A+C+E (284%) and vitamins C+D+E (301%).

As presented in Figure 2, the ECM deposition of type IV collagen by cultured HASMC increased in the presence of 100 μM vitamin C (by 255%), which was statistically significant. Supplementation with fat-soluble vitamins individually showed inhibitory effects, such as 14% reduction by vitamin D, 26% reduction by vitamin E, 28% reduction by vitamin A, and 34% reduction by beta-carotene compared to control. When combinations of the two vitamins were applied, the stimulatory effect of vitamin C was

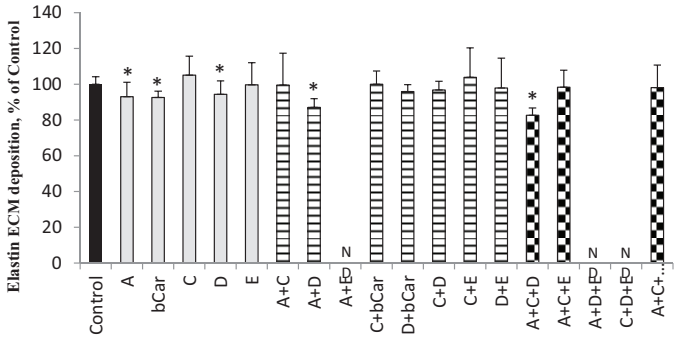

FIGURE 3 | Extracellular matrix deposition of elastin by human aortic smooth muscle cells. Confluent layers of HASMC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in 2% FBS/DMEM for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for elastin content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$). ND: not determined.

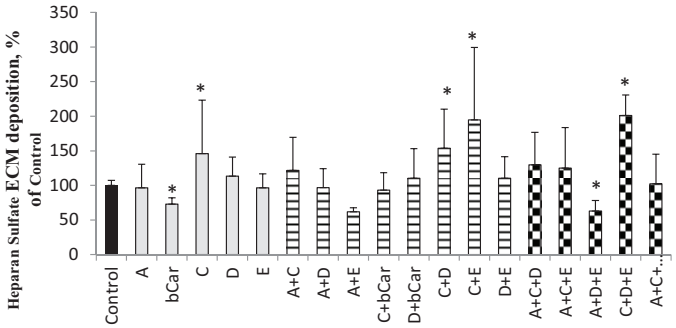

FIGURE 4 | Extracellular matrix deposition of heparan sulfate by human aortic smooth muscle cells. Confluent layers of HASMC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in 2% FBS/DMEM for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for heparan sulfate content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

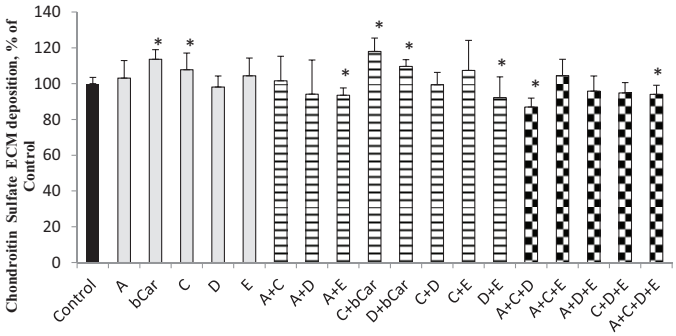

FIGURE 5 | Extracellular matrix deposition of chondroitin sulfate by human aortic smooth muscle cells. Confluent layers of HASMC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in 2% FBS/DMEM for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for chondroitin sulfate content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

significantly reduced by combining it with vitamins A and D (44% and 39% reduction, respectively). However, a combination of vitamin C with beta-carotene increased collagen IV deposition by about 50% compared to vitamin C alone and by 300% over control value. When all the three vitamins, A, D, and E were combined with vitamin C, the stimulatory effects of vitamin C alone on type IV collagen deposition was reduced 10fold from 255% to just 26% over control values (Fig. 2).

Elastin ECM deposition by HASMC was slightly affected by test vitamins with vitamin A and beta-carotene showing a decrease by 8% and vitamin D by 6% compared to control (Fig. 3). Combination of vitamins A and D showed about 12% inhibition, and when these two vitamins were combined with vitamin C, it resulted in a maximum reduction of elastin by 17%.

Sufficient production and maturation of properly assembled collagen type I fibrils by arterial wall resident cells is important for maintaining the tissue integrity, structural stability, and function. Collagen I has an important role in maintaining healthy vascular wall as well as contributing to growth of atherosclerotic plaques. Its content is a net result of a dynamic balance between its synthesis and degradation. In this study, we investigated the contribution of fat-soluble vitamins in the presence and absence of vitamin C on the total collagen type I production by arterial wall cells. Investigation of structural properties of deposited collagen fibrils and its impact on vascular wall properties remained beyond the scope of this study.

Deposition of Glycosaminoglycans by Human Aortic Smooth Muscle Cells

As presented in Fig. 4, the deposition of HS by HASMC was significantly increased by vitamin C (46% increase) and significantly reduced by beta-carotene (27% decrease) compared to control. Other test vitamins did not show any significant effects when used individually. The combination of vitamins A and E resulted in a significant reduction (38%) of HS deposition. Vitamin C combination with vitamin D or E had a stimulatory effect on HS deposition, by 54% and 95%, respectively. Vitamin C together with D and E increased HS deposition by 101% compared to control value.

The results presented in Figure 5 show that none of the tested vitamins and their combinations had significant effects on ChS deposition exceeding 10% with the exception of beta-carotene (13% increase) and a combination of vitamin C and beta-carotene (18% increase). A triple combination of A+C+D decreased ChS deposition by 13%.

HA content in ECM deposited by HASMC (Fig. 6) increased after supplementation with vitamin C (24% increase) and by vitamin C plus beta-carotene (29% increase). HA deposition was slightly decreased by vitamin A and beta-carotene applied individually, by a 12% and 13% decrease, respectively. The most significant

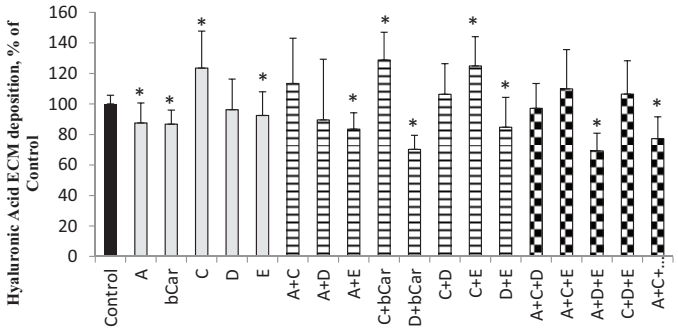

FIGURE 6 | Extracellular matrix deposition of hyaluronic acid by HASMC. Confluent layers of HASMC in collagen type I-covered 96 well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in 2% FBS/DMEM for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for hyaluronic acid content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

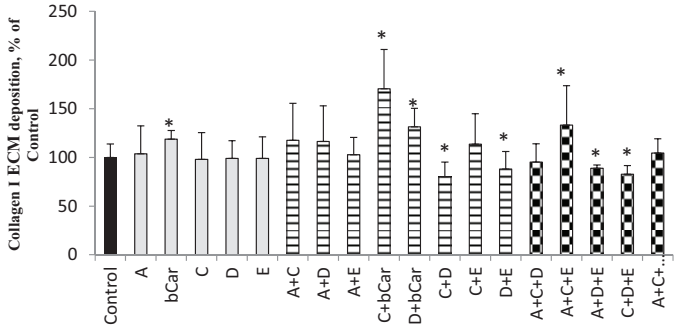

FIGURE 7 | Extracellular matrix deposition of type I collagen by human aortic endothelial cells. Confluent layers of HAEC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in EBM-2 medium for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for type I collagen content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

reduction in HA deposition (Fig. 6) was observed for vitamin D plus beta-carotene (30% reduction) and for a combination of vitamins A+D+E (31% reduction). This combination also reduced the stimulatory effect of vitamin C from a 124% increase to 77% increase as compared to nonsupplemented control.

Deposition of Extracellular Matrix Proteins by Human Aortic Endothelial Cells

As presented in Figure 7, the ECM deposition of type I collagen by HAEC was significantly induced by beta-carotene (18%) and by combining it with vitamins D and C (by 31% and 70%, respectively). Vitamin E applied individually did not affect collagen type I deposition; however, combining it with other vitamins resulted

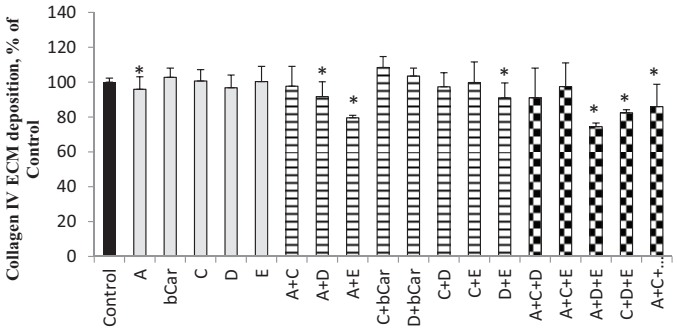

FIGURE 8 | Extracellular matrix deposition of type IV collagen by human aortic endothelial cells. Confluent layers of HAEC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in EBM-2 medium for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for type IV collagen content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

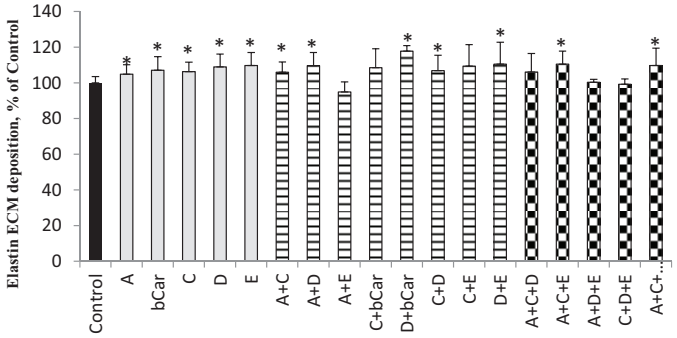

FIGURE 9 | Extracellular matrix deposition of elastin by human aortic endothelial cells. Confluent layers of HAEC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in EBM-2 medium for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for elastin content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

in either induction (33% for combination of vitamins E+C+A) or inhibition (12% for combination of vitamins E+D, 11% for vitamins E+A+D, and 18% for vitamins E+C+D).

The ECM deposition of type IV collagen by HAEC was not affected by any test vitamins applied individually (Fig. 8). However, it was reduced by combinations of vitamins (A+E), vitamins (A+D+E), and vitamins (C+D+E) by 20%, 25%, and 18%, respectively.

Differences in elastin content in the ECM produced by HAEC did not exceed ±10% except for a 17% increase in elastin content in the presence of a combination of vitamin D and beta-carotene (Fig. 9).

Deposition of Glycosaminoglycans by Human Aortic Endothelial Cells

HS deposition by HAEC was significantly reduced by vitamin A (18% reduction) and beta-carotene (29% reduction) as presented in Figure 10. Combinations of vitamins (A+D), (A+C+D), and (A+D+E) caused a decrease in HS content by 25%, 20%, and 43%, respectively.

ChS content in the HAEC-deposited ECM significantly increased by 22% under treatment with vitamins A+C and decreased by 13% and 14% with exposure to vitamin combinations A+E and A+D+E, respectively (Fig. 11).

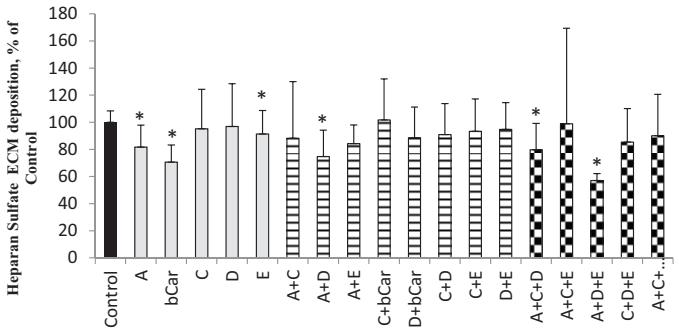

FIGURE 10 | Extracellular matrix deposition of heparan sulfate by human aortic endothelial cells. Confluent layers of HAEC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in EBM-2 medium for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for heparan sulfate content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

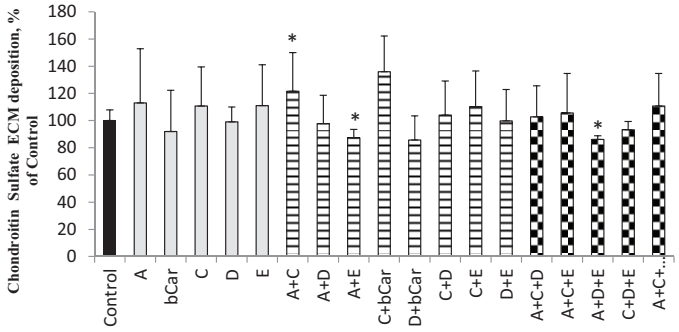

FIGURE 11 | Extracellular matrix deposition of chondroitin sulfate by human aortic endothelial cells. Confluent layers of HAEC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in EBM-2 medium for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for chondroitin sulfate content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

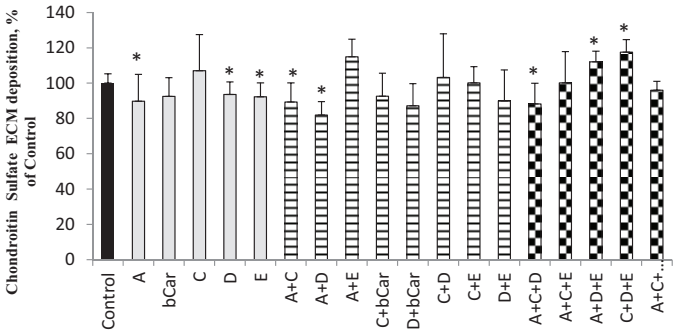

FIGURE 12 | Extracellular matrix deposition of hyaluronic acid by human aortic endothelial cells. Confluent layers of HAEC in collagen type I-covered 96-well plates were supplemented with 2 μM vitamin A (A), 1 μM β-carotene, 100 μM vitamin C (C), 1 μM vitamin D (D), and 100 μM vitamin E (E), applied individually or in combinations, in EBM-2 medium for 72 h. Cell-produced ECM was exposed by differential treatment and analyzed for hyaluronic acid content by immunoassay as described in detail in the "Materials and Methods" section. Results (average ± SD) are expressed as a percentage of nonsupplemented controls (dark solid bar). Light solid bars represent the effects of individual compounds, striped bar stand for combinations of 2 compounds, and chessboard pattern bars stand for combinations of 3 or more compounds. *Indicates statistically significant differences from control values ($P < 0.05$).

As presented in Figure 12, the exposure of HAEC to a combination of vitamins (A+E) and (A+D+E) resulted in increased HA deposition by 15% and 12%, respectively. HA content also increased by 18% in the presence of vitamin combinations (C+D+E). The ECM deposition of HA by HAEC was markedly decreased by vitamin A (10% reduction), and by vitamin A combinations with vitamins C and D (10% and 18% reduction, respectively). A triple vitamin combination (A+C+D) reduced the HA content by 12%.

Direct Comparison of the Deposition of Select Extracellular Matrix Components by Human Aortic Smooth Muscle Cells and Human Aortic Endothelial Cells Cultured in Plain Growth Media

We directly compared the levels of deposition of individual ECM components by two types of resident arterial wall cells, HASMC and HAEC, grown in plain nonsupplemented growth media. The ECM evaluation was conducted by immunoassay within the same experiment in plates with different cell types under identical simultaneous treatment (Table 1). Comparative analysis was based on the values of optical density readings at 450 nm for particular compounds and expressed as a ratio of OD_{450} obtained in HAEC to those in HAMC. As presented in Table 1, the ECM deposition of type I collagen was very similar for HAEC and HASMC cultures with an average ratio of 1:1, whereas the ECM deposition of type IV collagen was 10 fold higher in the HAEC cultures as compared to HASMC. The elastin content in the ECM produced by the HAEC was 1.3-fold higher than in the HASMC cultures. The ECM depositions of HS and HA were respectively 2-fold and 1.4-fold higher in the HAEC culture, whereas the ChS content was 10fold lower in the HAEC cultures as compared to the HASMC.

TABLE 1 | Direct comparison of expression of ECM components in human HAEC and HASMC cultured in plain growth media. ECM content for ECM components was determined by immunoenzymatic assay. Ratios were calculated by dividing average OD 450 nm values for nonsupplemented controls in identically treated plates with cultured HASMC or HAEC within each individual experiment, out of 6 in total. Results are represented as mean ± SD for indicated number of experiments.

ECM components	HAEC to HASMC ratio, Mean ± SD (n)
Collagen type I	1.1 ± 0.4 (6)
Collagen type IV	10.3 ± 3.8 (4)
Elastin	1.3 ± 0.7 (4)
Heparan sulfate	2.0 ± 1.7 (4)
Chondroitin sulfate	0.1 ± 0.0 (5)
Hyaluronic acid	1.4 ± 0.5 (5)

DISCUSSION

Structural integrity and function of the arterial wall is largely determined by the status of the ECM produced by residential arterial wall cells, which largely depend on an optimum supply of vitamin C and other micronutrients. The results of this study demonstrate that in addition to vitamin C, various fat-soluble vitamins can significantly affect ECM composition and therefore affect – or at least partly modulate – vascular wall structure, metabolism, and function.

While the effects of vitamin C on the expression and deposition of different ECM components by arterial wall cells under normal and pathological conditions have been previously addressed by us and others (Ivanov et al., 2007, 2008, 2016; Cha et al., 2015; Nicosia et al., 1991; Skrivanek et al., 1990; Zern et al., 1985; Barone et al., 1985; Schor et al., 1984), the effects of lipid-soluble vitamins were not investigated and rather limited to individual vitamins A, D, and E.

Vitamin A deficiency has been implemented in the development of CVD in clinical (Wood et al., 2014; Wang et al., 2014) and experimental studies (Relevy et al., 2015). The dietary sources of vitamin A are retinol and mainly retinyl esters from animal origin or provitamin A carotenoids from plant sources comprising numerous isomers of β-carotene (Grune et al., 2010). Vitamin A is involved in the regulation of glucose and lipid metabolism, as well as in the inflammation process involved in atherosclerosis (Rhee et al., 2015). Sparse data is available about its effects on vascular diseases in humans. Axel and coauthors reported that vitamin A was involved in regulating the proliferation, migration, and differentiation of cultured human arterial smooth muscle cells (Axel et al., 2001). They also observed vitamin A-dependent modulation of mRNA-expression of some ECM components, such as a decrease in glycoproteins thrombospondin-1 and fibronectin, and an increase in collagen-1 mRNA expression. Other data using retinol receptor agonists showed stimulation of type X collagen gene expression in chondrogenic ATDC5 cells and type I procollagen protein expression and inhibition of expression of matrix metalloproteinases 3 and 13 in the photoaged skin of experimental mice (Li et al., 2017). Our data support these observations showing that both vitamin A and its plant precursor beta-carotene can stimulate collagen type I deposition in HASMC and reduce collagen type IV deposition. However, in HAEC the deposition of collagen type I was elevated only by beta-carotene, whereas deposition of collagen type IV or elastin was not affected by neither test agent.

Topical retinol treatment was reported to induce HA production in human skin (Li W et al., 2017). However, in another report, hyaluronate synthesis was inhibited by all-trans retinoic acid in an in vitro experimental model of human skin (Deshpande et al., 2014). Tran-Lundmark et al. reported inhibition of SMC proliferation through stimulating the expression of perlecan, an HS-enriched proteoglycan (Tran-Lundmark et al., 2015). In contrast, in our study, beta-carotene reduced HS deposition and induced ChS deposition in HASMC. HA deposition by HASMC was reduced by both agents. In HAEC cultures both vitamin A forms significantly reduced ECM deposition of HS, whereas ChS and HA deposition remained unaffected.

Vitamin D is a fat-soluble vitamin and a prohormone. Its main sources are sunlight, diet, and supplementation. Vitamin D in the skin is present in the form of provitamin D3 (7-dehydrocholesterol) and is photochemically converted to previtamin D3 by ultraviolet-B rays from the sun. Foods rich in vitamin D include fatty fish (e.g., salmon, mackerel), but relative to sun exposure, diet is a poor source of vitamin D. Vitamin D3 is transported to the liver and converted to 25(OH)D3 (calcidiol) through the process of hydroxylation. In the kidneys, 25(OH)D3 goes through a second hydroxylation to 1,25(OH)2D3 (calcitriol) which is the active metabolite (Bikle, 2014; Bartosik-Psujek et al., 2019). The diverse biological actions of vitamin D are mediated by an intracellular vitamin D receptor. These actions are tissue specific and range from highly complex actions essential for the homeostatic control of mineral metabolism to focal actions that control growth, differentiation, and functional activity of numerous cell types, including those of the immune system, skin, pancreas, and bone (Pike et al., 2010). These diverse tissue-specific effects of vitamin D could be an underlying reason for the conflicting reports on its effects on the production of ECM components. Thus, Potter and colleagues observed vitamin D-mediated decrease in α(1) (I) collagen mRNA and protein and the secretion of type I collagen by human liver stellate cells after exposure to TGFβ1 (Potter et al., 2013). Artaza and Norris reported vitamin D-dependent reduction in the expression of collagen and key profibrotic factors by inducing an antifibrotic phenotype in mesenchymal multipotent cells (Artaza et al., 2009). In contrast, studies of osteogenic differentiation of multipotent mesenchymal stromal cells from human adipose tissue revealed activation of gene expression of type I collagen proteins (Logovskaya et al., 2013).

In our study, we observed different responses to vitamin D supplementation in cultured human resident arterial wall cells. Thus, in HASMC, vitamin D stimulated ECM deposition of collagen type I and decreased collagen type IV, therefore widening a difference in their relative presence in the ECM. Notably, vitamin D effects were additive to those of ascorbic acid, which itself acts as a potent modulator of ECM composition in HASMC (Ivanov et al., 2007). In HAEC, vitamin D had minor effect on collagen production. ECM elastin deposition by both cell types was largely unaffected by vitamin D.

There is relatively little available data on vitamin D's effects on GAG production. Koh and colleagues reported a dose-dependent inhibition of vitamin D on GAG synthesis by rat aortic SMC (Koh et al., 1990). Vitamin D inhibited GAG synthesis in rabbit craniofacial chondrocytes (Takano-Yamamoto et al., 1992) and hindered synthesis but enhanced degradation of GAG in cultured

osteoblastic cells (Takeuchi et al., 1990). In contrast, long term vitamin D supplementation significantly increased GAG content in the bone tissue of patients with postmenopausal osteoporosis (Paschalis et al., 2017).

Our results demonstrated that vitamin D supplementation reduced heparan sulfate ECM deposition by both HASMC and HAEC. However, deposition of other GAG species – ChS and HA – remained unaffected in both cell types.

Vitamin E is a family of several compounds, of which α-tocopherol is the most widely known and researched. Natural sources of vitamin E are fruits, vegetables, and nuts (Jiang, 2014). Vitamin E metabolism has been linked to cardiovascular health through its antioxidant effects, its inhibition of low-density lipoprotein cholesterol oxidation as well as its anti-inflammatory activity affecting proteins involved in the uptake, transport, and degradation of atherogenic lipids (Mathur et al., 2015). Possible involvement of vitamin E in regulating ECM in relation to arterial wall structure and function has not been addressed systematically. Makpol and colleagues reported that tocopherol stimulated procollagen type I and type III levels in cultured human fibroblasts, implying its role in wound healing and skin remodeling (Makpol et al., 2013). Also, α-tocopherol has been found to stimulate collagen type I synthesis in human gingival fibroblasts (Nizam et al., 2014). These observations were supported by our study showing increased collagen I deposition by vitamin E in HASMC, while collagen type IV deposition was reduced. In HAEC, vitamin E did not affect collagen deposition. In addition, ECM deposition of elastin and GAGs was not significantly affected by vitamin E in either cell type. Various effects of these compounds on cellular mechanisms involved in the complexity of atherosclerotic process could have important implications under physiological conditions.

In this study, we also compared the effects of individual fat-soluble vitamins to their various combinations in the presence and absence of ascorbic acid. In some respects, the vitamins displayed additive effects. Thus, the effect of ascorbic acid on type I collagen ECM deposition by HASMC was further enhanced by beta-carotene (from 301% to 507% of control values), vitamin E (from 301% to 443%), and vitamin D (from 301% to 375%). In addition, a combination of vitamins D and E stimulated collagen type I deposition in HASMC cultures by 63%, which was much higher when these vitamins were applied individually.

However, in other aspects vitamins showed opposite effects. For example, all fat-soluble vitamins A, D, and E and beta-carotene – both individually and in combination – inhibited collagen type IV deposition by HASMC and reduced stimulatory effect of vitamin C.

The stimulatory effects of vitamin C on HS deposition by HASMC were enhanced by vitamins D and E and their combination. However, vitamin A and beta-carotene counteracted the stimulatory effects by vitamins C, D, and E.

Vitamin A also lowered the stimulatory effects of vitamin C on HA deposition by HASMC. A similar inhibitory effect was observed with the addition of vitamin D.

Elastin and ChS ECM deposition by HASMC was not significantly affected by all tested vitamin combinations. Furthermore, we did not observe marked changes in the deposition of ECM components by HAEC when the effects of vitamin combinations were compared to those of individual vitamins. Vitamin-dependent increase in type I collagen deposition by HASMC, demonstrated in our study, could contribute to counteracting the pathologically induced weakness of the arterial wall. A simultaneous vitamin-dependent decrease in ECM deposition of type IV collagen by HASMC could indicate a switch in the use of resources for cellular synthesis from type IV to type I collagen, as the presence of type IV collagen is not essential in the media layer. Type IV collagen predominantly found in the intima layer is a component of basic membrane and is produced and deposited predominantly by arterial endothelial cells (Shekhonin et al., 1987). The accumulation of sulfated GAGs in vascular atherosclerotic lesions contributes to disease development through the retention and consequent oxidative modification of blood plasma low density lipoprotein (Tabas et al., 2007). Therefore, reduced deposition of HS by HAEC and HASMC under vitamin supplementation, as found in our study, could imply their preventive effect on the development of the atherosclerotic process.

Notwithstanding inadequate or conflicting research data, our study evaluated for the first time the effects of fat-soluble vitamins on the deposition of various ECM components into cell-produced ECM. A combined cellular expression of tested ECM components, which includes both cell-associated and soluble ECM components released to the culture medium, was not assessed. In addition, some mechanistic aspects – such as the interaction of vitamins with specific cellular receptors and the regulation of corresponding gene expression – remained outside the scope of this study.

CONCLUSION

We demonstrated that fat-soluble vitamins applied individually or in different combinations – both with each other or with ascorbic acid – can affect ECM deposition of type I and IV collagens, and key glycosaminoglycans, HS, ChS, and HA, by cultured resident arterial wall cells isolated from human aorta. Our data has important implications for the design and use of dietary supplements containing these nutrients. Further studies are required to fully understand the involvement of vitamins in regulating the structural stability and function of the arterial wall, and to apply this knowledge to the development of novel CVD prophylactic and treatment strategies.

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CONFLICT OF INTEREST DECLARATION

The authors have no conflict of interest to declare.

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