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ORIGINAL CONTRIBUTION

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Effect of vitamin E and vitamin C on the DNA synthesis of human umbilical arterial endothelial cells

Summary *Background* Endothelial cell growth and reendothealization after vascular injury protect the vessel wall against endothelial dysfunction which is believed to play a major role in the pathogenesis of atherosclerosis. *Aim of the study* To investigate a possible protective role of antioxidant vitamins in the present study, the effect of vitamin E (α-tocopherol) alone and in combination with vitamin C on the DNA synthesis of human umbilical arterial endothelial cells (HUAEC) was examined. Furthermore, because oxidized low-density lipoprotein (ox-LDL) is thought to be involved in atherogenesis, the combined effect of vitamin E and vitamin C with ox-LDL and the influence of vitamin-pretreated LDL on HUAEC proliferation were investigated. *Methods* DNA-synthesis was determined by measurement of [^{3}H]thymidine incorporation into the cell DNA. *Results* Vitamin E alone and in combination with vitamin C resulted in an increase in [^{3}H]thymidine incorporation into cell DNA, especially in the presence of basic fibroblast growth factor (bFGF). All vitamin-pretreated LDL samples and ox-LDL led to a nearly complete inhibition of endothelial DNA-synthesis. The ox-LDL-induced effect could not be prevented by vitamin E alone nor in combination with vitamin C. *Conclusions* It seems that once LDL oxidation is in process, vitamin E alone and in combination with vitamin C is ineffective to exert its antioxidative capacity under the conditions used. Thus, vitamin E alone and combined with vitamin C may act as antiatherogens by inducing endothelial cell growth.

Key words Vitamin E – Vitamin C – DNA synthesis – Human endothelial cells

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Introduction

The endothelium is involved in several physiological processes such as hemostasis and regulation of vascular tone and cell growth. As an interface between the blood and the vessel wall, an intact endothelium acts as a selective barrier. Dysfunctional changes in the endothelium may result in alterations in permeability, adhesiveness, growth regulation, etc. Endothelial cell injury disturbes the integrity of the endothelium and may lead to endothelial dysfunction which is believed to play a central role in the pathogenesis of atherosclerosis [1, 2]. If vascular injury occurs, the lesions have to be covered immediately to prevent progression of atherosclerosis. Endothelial cell proliferation during the physiologic replacement of endothelial cells and reendothelialization ensure the integrity of the endothelium. Several studies have described that antioxidants may act as antiatherogens [3, 4]. Besides its antioxidative activity, α-tocopherol possesses additional properties such as stabilizing membranes [5] and inhibiting cell growth of smooth muscle cells [6–9]. In the present study, we investigated the effect of vitamin E on the DNA synthesis of human umbilical arterial endothelial cells (HUAEC). A synergistic antioxidant effect between vitamin C and vitamin E has been observed [10]. Therefore, we also examined the combined effect of vitamin E and vitamin C on HUAEC growth. Because oxidized low-density lipoprotein (ox-LDL) has been implicated in atherogenesis [1, 11] we investigated 1) the effect of ox-LDL alone and combined with the antioxidant vitamins and 2) the in-

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fluence of vitamin-pretreated LDL on HUAEC proliferation.

Material and methods

Materials

Medium 199, HAM's F-10, penicillin/streptomycin and human fibronectin were obtained from Gibco-BRL (Eggenstein, Germany). Collagenase I, collagen and vitamin C (L-ascorbic acid) were from Sigma Chemical (Deisenhofen, Germany). The recombinant basic fibroblast growth factor (bFGF), Dispase II and fetal calf serum (FCS) were obtained from Boehringer Mannheim (Mannheim, Germany). [Methyl–^{3}H]thymidine was obtained from Amersham Buchler (Braunschweig, Germany) and vitamin E (RRR-α-tocopheryl acetate) from Hoffmann-La Roche (Basel, Switzerland).

Methods

Culture of human umbilical arterial endothelial cells (HUAEC)

HUAEC were isolated and cultured as described previously [12]. Isolation was carried out enzymatically from human umbilical cord arteries with a Dispase II (0.5 U/mg)/ collagenase I (260 U/mg) solution according to a modified method of Jaffe et al. [13]. Cells were cultured over several passages (up to 5) at 37 °C on collagen coated 75 cm^2 flasks in Medium 199 supplemented with 20 % FCS, penicillin 100 IU/ml, streptomycin 100 µg/m, 10 µg/ml heparin and 30 µg/ml crude ECGF/ml according to Hinsbergh et al. [14] in a humified atmosphere of 5 % CO_2 and 95 % air.

LDL isolation and oxidation

LDL (d = 1.019–1.063 g/ml) was isolated from fresh human plasma of apparently healthy volunteers by ultracentrifugation according to Redgrave et al. [15]. LDL was untreated or treated with antioxidants. Untreated LDL was dialyzed against 0.15 M NaCl/1 mM EDTA, pH 7.4, for 20 h. LDL prepared by this method is referred to as native LDL (n-LDL). For the preparation of oxidized LDL (ox-LDL) dialysis was performed against 0.15 M NaCl, pH 7.4, for 20 h. Then, LDL was oxidized by incubation with 5 µM $CuSO_4$ at 37 °C for 6 h. To stop further oxidation 1 mM EDTA was added, followed by dialysis against 0.15 M NaCl/1 mM EDTA, pH 7.4, for 20 h. Treatment of LDL with antioxidants was performed by addition of 30 µM vitamin E (vitE) or 30 µM vitamin E + 60 µM vitamin C (vitC) to freshly isolated LDL. The dialysis was carried out against 0.15 M NaCl, pH 7,4, for 20 h. The LDL pretreated by this method is referred to as p-LDL/vitE and p-LDL/vitE+vitC, respectively. One part of these LDL preparations was incubated with 5 µM $CuSO_4$ at 37 °C for 6 h. Then, 1 mM EDTA was added and dialysis was performed against 0.15 M NaCl/1 mM EDTA, pH 7.4, for 20 h (ox-LDL/vitE, ox-LDL/vitE+vitC). All LDL preparations were sterilized by passing them through a 0.22 µm filter (Millex, Millipore, Eschborn, Germany) and were kept in the dark at 4 °C until use within 4 weeks. Quantification of LDL was performed by determination of the protein component according to the method of Bradford [16].

Measurement of lipid peroxidation

The degree of LDL oxidation was performed by measurement of absorption at 234 nm, which is the absorption maximum of conjugated dienes that are formed during LDL oxidation. If LDL is oxidized, the absorption increases in comparison to n-LDL, indicating an increase in conjugated diene formation of fatty acids [17]. Comparison of measurements at 234 nm performed with 10 µg and 50 µg of all LDL samples/ml PBS revealed that a 5-fold higher LDL concentration also gave about 5-fold higher absorption values. For 10 µg and 50 µg n-LDL/ml PBS, the absorption values were 0.027 and 0.165, respectively; for 10 µg and 50 µg ox-LDL/ml PBS the values were 0.061 and 0.350, respectively. Therefore, to economize the different LDL preparations for determination of conjugated diene formation, 10 µg LDL/ml PBS were used. Each LDL sample was measured in duplicate.

Measurement of [^{3}H]thymidine incorporation

As a well-established method for determination of DNA synthesis the incorporation of [^{3}H]thymidine into the cell DNA was measured. The effect of the different LDL preparations, vitamins and bFGF on [^{3}H]thymidine incorporation was measured by a slightly modified method according to Nemecek et al. [18]. Vitamin E was dissolved in dimethylsulfoxide (DMSO, final concentration 0.005 %). Therefore, in addition the effect of DMSO at the same concentration on HUAEC growth was examined. Endothelial cells were seeded in 24-well culture plates and cultured in Medium 199, supplemented with 20 % FCS, 10 µg/ml heparin, 30 µg/ml crude ECGF, penicillin 100 IU/ml and streptomycin 100 µg/ml at 37 °C in a humified atmosphere of 95 % air and 5 % CO_2. When cells reached subconfluence, the medium was replaced by serum-free quiescent medium consisting of Medium 199 and HAM's F-10 (1:1, v:v). Endothelial cells were incubated in this medium for 4 hours. Then, cultures were exposed to n-LDL, ox-LDL, p-LDL/vitE, p-LDL/vitE+vitC, ox-LDL/vitE, ox-LDL/vitE+vitC, vitamin E (vitE), vitamin E + vitamin C (vitE+vitC) in the presence or absence of bFGF for 20 hours prior to adding 2 µCi/ml

[^{3}H]thymidine to the quiescent medium. Four hours after [^{3}H]thymidine addition experiments were terminated by aspirating the medium and subjecting the cultures to sequential washes with PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% trichloroacetic acid and ethanol/ether (1:1, v/v). Phase-contrast microscopy was used to inspect the dishes for evidence of cell detachment or changes in cell morphology. Acid-insoluble [^{3}H]thymidine was extracted into 250 µl/dish 0.5 M NaOH and 100 µl of this solution was mixed with 5 ml scintillator solution and quantified by using a Packard Instrument liquid scintillation counter (Beckmann, Düsseldorf, Germany). Of the residual solution, 100 µl was prepared for the determination of protein using the Bio-Rad protein assay according to the method of Bradford [16].

Statistical analysis

Data are given as mean±SD of representative experiments. Statistical analysis was performed using the Mann-Whitney U-test [19]. A value of $p < 0.05$ is considered as significant. Each experiment was carried out independently at least three times and an individual experiment was performed in triplicate.

Results

In order to investigate the effect of vitamins on DNA synthesis, HUAEC were stimulated with vitamin E (30 µM) alone or in combination with vitamin C (60 µM). The DMSO solvent for the lipophilic vitamin E in the administered concentration had no significant effect on [^{3}H]thymidine incorporation into the DNA of HUAEC (Fig. 1). Single application of vitamin E increased [^{3}H]thymidine incorporation from 39±7 (basal level) to 54±5 cpm/µg protein. Simultaneous addition of vitamin E and vitamin C tripled DNA synthesis (126±18 cpm/µg protein) compared to the basal level (Fig. 1). Besides vitamin E and C, n-LDL (30 µg/ml) also increased [^{3}H]thymidine incorporation into the DNA of HUAEC, here from 39±7 to 71±6 cpm/µg protein. In contrast, ox-LDL (30 µg/ml) led to a nearly complete inhibition of DNA synthesis (5±0 cpm/µg protein) (Fig. 1).

To investigate the effect of the vitamins under a second condition, HUAEC were stimulated with the above agents in combination with the growth factor bFGF. The results are given in Fig. 2. bFGF (50 ng/ml) increased [^{3}H]thymidine incorporation by about seven times from 39±7 to 286±52 cpm/µg protein. Coincubation of bFGF with vitamin E or vitamin E + vitamin C further enhanced [^{3}H]thymidine incorporation up to 416±12 (10 times) and 720±99 cpm/µg protein (18 times), respectively. Thus, under two conditions, vitamin E alone or in combination with vitamin C increased DNA synthesis of HUAEC.

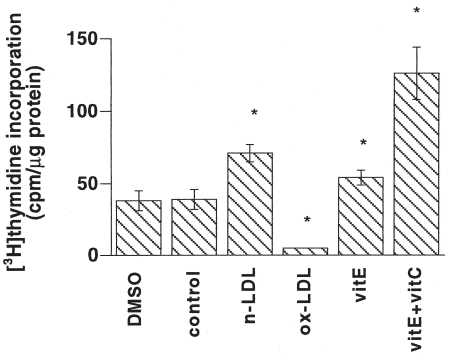

Fig. 1 Effect of 30 µg/ml n-LDL, 30 µg/ml ox-LDL, 30 µM vitamin E (vitE) and 30 µM vitamin E + 60 µM vitamin C (vitE+vitC) on [^{3}H]thymidine incorporation into the DNA of HUAEC. Data are expressed as mean±SD, n=3. * $p < 0.05$.

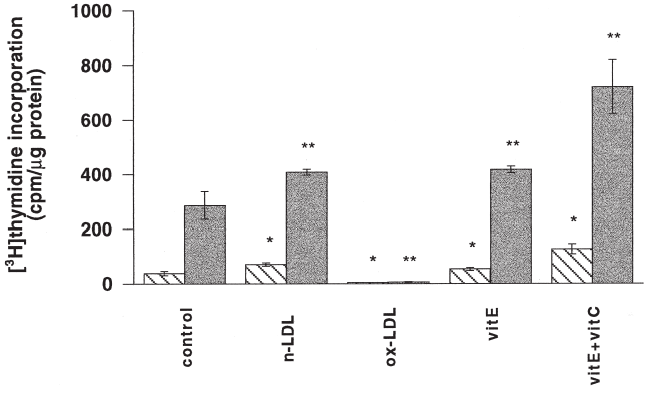

Fig. 2 Effect of 30 µg/ml n-LDL, 30 µg/ml ox-LDL, 30 µM vitamin E (vitE) and 30 µM vitamin E + 60 µM vitamin C (vitE+vitC) on [^{3}H]thymidine incorporation into the DNA of HUAEC in the absence (▨) and presence of bFGF (■). Data are expressed as mean±SD, n=3. * $p < 0.05$ for n-LDL, ox-LDL, vitE and vitE+vitC versus basal value. ** $p < 0.05$ for n-LDL, ox-LDL, vitE and vitE+vitC in the presence of bFGF versus bFGF.

In addition n-LDL enhanced the bFGF-induced increase of [^{3}H]thymidine incorporation by approximately 10 times up to 408±11 cpm/µg protein. If ox-LDL and bFGF were coincubated, [^{3}H]thymidine incorporation was inhibited (7±1 cpm/µg protein) comparable to the single application of ox-LDL. Thus, although bFGF is a potent stimulator for HUAEC growth, it is unable to compensate the inhibitory effect of ox-LDL.

In view of a possible protective effect of the vitamins against ox-LDL-induced inhibition of [^{3}H]thymidine incorporation, HUAEC were coincubated with ox-LDL (30 µg/ml) and vitamin E (30 µM) or vitamin E (30 µM) + vitamin C (60 µM) in the presence and absence of bFGF (50 ng/ml). Neither vitamin E alone, the combination of vitamin E and vitamin C, nor the simultaneous application of vitamin E or both vitamins and bFGF

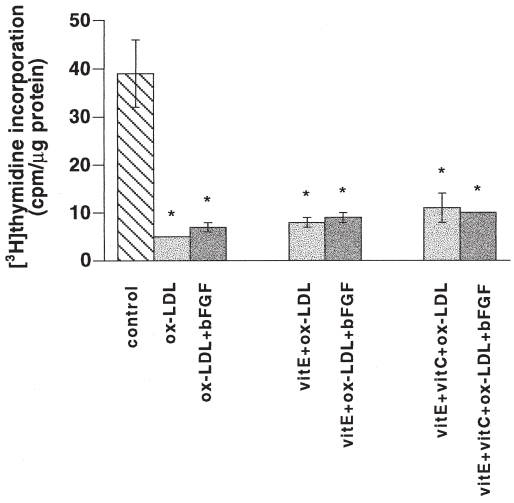

Fig. 3 Effect of 30 µg/ml ox-LDL and 30 µg/ml ox-LDL + 50 ng/ml bFGF in the absence and presence of 30 µM vitamin E (vitE) or 30 µM vitamin E + 60 µM vitamin C (vitE+vitC) on [^{3}H]thymidine incorporation into the DNA of HUAEC. Data are expressed as mean±SD, n=3. * $p < 0.05$.

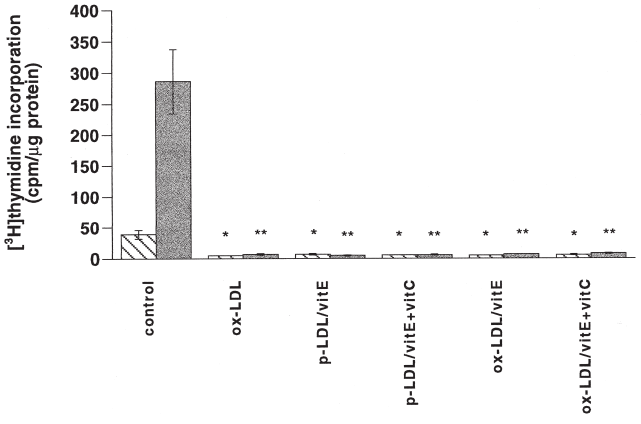

Fig. 4 Effect of ox-LDL and LDL pretreated with different vitamins (detailed description in the methods), each in a concentration of 30 µg/ml, on [^{3}H]thymidine incorporation into the DNA of HUAEC in the absence (▨) and presence of bFGF (■). Data are expressed as mean±SD, n=3. * $p < 0.05$ for ox-LDL and vitamin-pretreated LDL samples versus basal value. ** $p < 0.05$ for ox-LDL and vitamin-pretreated LDL-samples in the presence of bFGF versus bFGF.

could neutralize the inhibitory effect of ox-LDL on [^{3}H]thymidine incorporation (Fig. 3).

Next, the effect of vitamin-pretreated LDL on [^{3}H]thymidine incorporation was investigated. HUAEC were stimulated with p-LDL/vitE, p-LDL/vitE+vitC, ox-LDL/vitE and ox-LDL/vitE+vitC, each in a concentration of 30 µg/ml, in the presence and absence of bFGF. Figure 4 shows that there was no qualitative difference in [^{3}H]thymidine incorporation between the various vitamin-pretreated LDL preparations. All pretreated LDL samples led to a nearly total inhibition of [^{3}H]thymidine incorporation into the cell DNA comparable to ox-LDL, even in the presence of bFGF. A comparison of the conjugated diene values (absorption at 234 nm) of n-LDL, ox-LDL and pretreated LDL revealed that the diene values of the pretreated LDL samples were in the same range (0.049–0.061) compared to those obtained for ox-LDL.

Discussion

An intact endothelium is a prerequiste for its normal function. Endothelial cell injury can lead to endothelial dysfunction which is believed to play a central role in atherogenesis [1, 2]. Endothelial cell proliferation maintains the integrity of the vascular intima during physiologic replacement of endothelial cells and provides reendothelialization following endothelial injury. Vitamin E (30 µM) and vitamin C (60 µM) are considered to be protective against oxidative stress and thereby may reduce the risk for chronic degenerative diseases [20–22]. In our study, we examined the effect of these physiological concentrations of vitamin E and vitamin C on the DNA synthesis as an indicator for proliferation of HUAEC.

Our results show that vitamin E alone and even more so in combination with vitamin C enhances DNA synthesis of HUAEC, especially in the presence of the potent growth factor bFGF, and thus stimulates endothelial cell proliferation. Therefore, these vitamins may play an important role in protecting the vessel wall by maintaining the endothelial integrity and providing repair of endothelial cell microlesions. Kuzuya et al. [23] and Henning et al. [24] also demonstrated a proliferative effect of antioxidants. α-Tocopherol enhances the proliferation of endothelial cells from thoracic aorta of the fetal calf and endothelial cells from porcine pulmonary artery, respectively.

Possibly, effects of vitamin E are dependent on concentration and the cell system. Huang et al. [25] reported that α-tocopherol does not affect the growth of human umbilical vein endothelial cells. The concentration of α-tocopherol in their study was more than 10-fold higher than that used in our experiments. Interestingly, several studies have described an inhibition of rat smooth muscle cell (SMC) proliferation induced by 50 µM or 100 µM α-tocopherol [6–9]. SMC proliferation is involved in the pathogenesis of atherosclerosis [1, 2]. Thus, α-tocopherol may slow the progression of atherosclerosis by its antiproliferative effect on SMC.

The present study shows that n-LDL increases [^{3}H]thymidine incorporation into the DNA of HUAEC and further enhances the growth-promoting effect of bFGF. We demonstrated previously that n-LDL, especially in combination with aFGF, induces HUAEC growth [12]. Here, we compared the effects of n-LDL and

ox-LDL. The strong inhibitory effect of ox-LDL on endothelial DNA-synthesis, seen in our experiments, is probably due to its cytotoxicity. In various studies ox-LDL is shown to be cytotoxic to several cells including endothelial cells [26–28]. Vitamin E alone or in combination with vitamin C without and with bFGF was unable to protect endothelial cells against the ox-LDL-induced inhibition of DNA synthesis. Furthermore, the present results show that all vitamin-pretreated LDL preparations, before and after Cu^{2+}-incubation, have the same inhibitory effect on DNA synthesis of HUAEC like ox-LDL. Comparing the content of conjugated dienes ($\lambda_{234\,nm}$) in the different LDL preparations, this result is not astonishing. The diene content of the pretreated LDL preparations was in the range of ox-LDL. Therefore, we suggest that vitamin E alone and in combination with vitamin C was unable to prevent LDL oxidation under the experimental conditions chosen. Evensen et al. [29] concluded in their study that peroxidation products are formed during fractionation, dialysis and storage of LDL that are cytotoxic to cultured cells. Frei [30] reported that LDL is very suspectible to oxidation after removal from its natural plasma environment. During the isolation process LDL could undergo low autooxidation and under the experimental conditions used here the antioxidative properties of vitamin E alone and in combination with vitamin C were insufficient to protect LDL against further oxidation.

In summary, it seems that once the LDL oxidation process has started vitamin E alone or combined with vitamin C is ineffective to exert its antioxidative capacity under the used conditions. Furthermore, these vitamins are unable to overcome the growth inhibitory (probably cytotoxic) effect of ox-LDL. However, the vitamins used may act as antiatherogens by stimulating endothelial cell proliferation, especially in the presence of the growth factor bFGF, which may ensure the integrity of the endothelium. By this mechanism the vitamins may take part in the prevention of endothelial dysfunction which is thought to play a major role in the pathogenesis of atherosclerosis.

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