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Anti-angiogenic effect of curcumin, curcumin ethylenediamine derivative and curcumin ethylenediamine manganese complex

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We investigated the anti-angiogenic effect of curcumin, curcumin ethylenediamine derivative (curcumin ED) and curcumin ethylenediamine manganese complex (curcumin EDMn) through the inhibition of the formation of tube-like structures by human umbilical vascular endothelial cells (HUVEC). Curcumin, curcumin ED, curcumin EDMn did not show cytotoxicity to HUVEC at concentrations equal and lower than 10 μM. At the concentration of 10 μM, curcumin, curcumin ED and curcumin EDMn inhibited the tube formation by approximately 94%, 40% and 65%, respectively. These results suggest that curcumin ED and curcumin EDMn might be useful as anti-angiogenic drugs in addition to their anti-lipid peroxidase and superoxide dismutase activities as described in our previous studies.

Key words angiogenesis, cancer, tube formation, curcumin, human umbilical vascular endothelial cell.

Abbreviations HUVEC, human umbilical vascular endothelial cells; curcumin ED, curcumin ethylenediamine; curcumin EDMn, curcumin ethylenediamine manganese complex; FBS, fetal bovine serum; PBS, phosphate buffer saline; SOD, superoxide dismutase.

Introduction

Tumeric powder from the rhizome of *Curcuma longa* L. has been used for flavoring and as a colorant in many kinds of foods.[1] Traditionally, it has been utilized for treatment of inflammatory disorders and other diseases.[2] Phenolic substances isolated from tumeric are known as curcuminoids, which exhibit anti-bacterial and anti-helmintic activities and reduce cholesterol level. Many studies showed that curcumin possesses neutralizing effect on carcinogenic free radicals, anti-oxidant and anti-angiogenic activities.[3] Curcumin also shows inhibitory effects on several signal transduction pathways such as the protein kinase C, the transcription factor NF-κB, phospholipase A_2 bioactivity, arachidonic acid metabolism and EGF receptor auto-phosphorylation.[4-8] In addition, curcumin inhibits tumor initiation induced by benzo(a)pyrene and 7,12-dimethylbenzanthracene and tumor promotion induced by phorbol esters.[8-10] Natural resources including tree bark, fungi, mushroom, shark muscle and cartilage, sea coral, green tea, ginseng, squalamine, curcumin and garlic showed inhibitory effects on angiogenic pathways.[11] The *in vivo* anti-angiogenic activity of curcumin was recently demonstrated in the peritoneal angiogenesis assay and chorioallantoic membrane assay.[12,13] Thus it is interesting to investigate the anti-angiogenic activity of curcumin derivatives or complexes.

Anti-angiogenesis is an approach of interest for treating cancer patients. Angiogenesis is the formation of new blood vessel within a tumor, which is essential in the pathogenesis of tumor metastasis. The survival of tumors depends on the supply of oxygen and nutrients and the elimination of metabolic decomposition products from these vessels.[14] Angiogenesis process involves differentiation, acquisition of migrative and proliferative abilities with high matrix-degrading activity, and tube formation of endothelial cells.[15] Inhibition of angiogenesis can be an alternative for cancer therapy. Anti-angiogenetic agents are subdivided as 1) vasculostatic agents, which interfere in the new blood vessel formation and 2) vasculotoxins comprise that use elements of newly formed blood vessels to target toxic principles.[16]

The curcumin derivatives and complexes including diacetylcurcumin (AcylCp), curcumin manganese complex (CpCpX), diacetylcurcumin manganese complex (AcylCpCpX), curcumin ED and curcumin EDMn were previously synthesized and investigated for their *in vitro* anti-lipid peroxidation and superoxide dismutase (SOD) activities.[17] The manganese complexes showed a great capacity to protect brain lipid against peroxidation with IC_{50} of 6.3-26.3 μM. All manganese complexes showed much greater SOD activity than their corresponding anti-oxidant ligands as well as trolox with IC_{50} of 8.9-29.9 μM.

The present study focuses on the inhibitory effect of curcumin ED and curcumin EDMn on the formation of tube-like structures by HUVEC. The findings indicate the potential anti-angiogenic activity of these compounds, apart from their anti-oxidant effect.

Materials and Methods

Endothelial cell. HUVEC were seeded in 75-cm^2 flask (Corning Inc., NY, USA). These cells were maintained as

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monolayer culture in HuMedia EG2 supplemented with 10% heat-inactivated FBS.

Chemicals. Curcumin was separated from the commercial curcuminoids or tumeric extract using silica gel column chromatography (eluent: chloroform/methanol/acetic acid, 98:5:2).[17] The synthesis of curcumin ED and curcumin ED Mn was reported in a previous study[17] and their chemical structures are shown in Fig.1. For the *in vitro* experiments each compound was dissolved in dimethylsulfoxide (Wako Pure Chemical Ind., Osaka, Japan) to obtain the concentration of 1 mg/ml and was further diluted to obtain the concentration of 0.01-100 μM.

Cytotoxicity assay. Cytotoxicity of compounds to HUVEC was assessed using a WST-1 Cell Counting Kit (Wako Pure Chemical Ind., Osaka, Japan). Briefly, HUVEC (1 x 10^4) in HuMediaEG2 (50 μl) containing 0.2% FBS were seeded in each well of a 96-well culture plate (Becton Dickinson Labware, NJ, USA). After 12 h of incubation, various concentrations (0.01-100 μM) of curcumin, curcumin ED or curcumin EDMn in 50 μl of medium were added to the wells and the culture was incubated for a further 24 h. WST-1 solution (100 μl) was added to each well and incubated at 37℃ for 2 h. The absorbance at 450 nm was measured in an immunoreader (Immuno Mini NH-2300, Nippon Inter-Med. KK, Tokyo, Japan).

Tube formation assay. HUVEC (5 x 10^5 cell/well) were seeded in a 6-well plate (Becton Dickinson Labware, NJ, USA) and, under a sub-confluent condition, the medium (HuMedia EG2 supplemented with 10% FBS) of each well was replaced with a fresh medium (2 ml/well) supplemented with 0.2% FBS and containing a given concentration of curcumin, curcumin ED, curcumin EDMn or doxorubicin, as specified in Results. After 24 h of incubation, the cells were washed once with phosphate buffered saline (PBS), starved with Trypsin-EDTA and centrifuged at 2,000 rpm for 5 min. The cell suspensions (3x10^4 cells) were then prepared in 0.2% FBS-supplemented HuMediaEG-2 medium (250 μl) and seeded on a 48-well plate (Becton Dickinson Labware, NJ, USA) that had been pre-coated with 120 μl of Matrigel (10 mg/ml). The formation of tube-like structures was monitored every 1 h for 4 h. After incubation, the cells were photographed (three fields per well at 40x magnification) and the length of the tubes was measured using a digital calibermeter (Uchida Yoko Co., Ltd., Japan). Briefly, a connecting branch between two discrete endothelial cells was measured as a tube-like structure. The extent of inhibition of the tube-like formation in the presence of curcumin, its derivative and manganese complex was estimated by comparing the length of the tube-like structures formed in the control treatments.

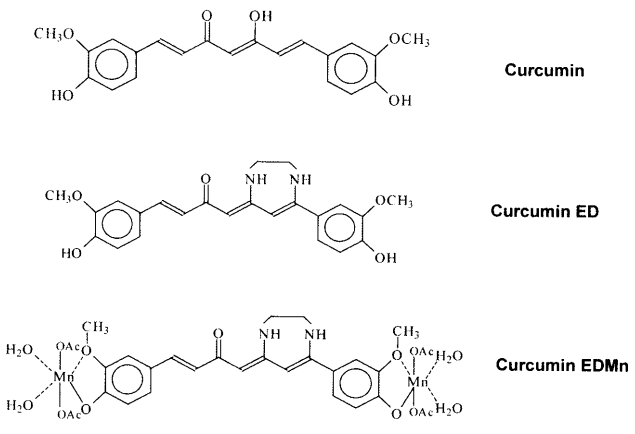

Fig. 1 Chemical structures of curcumin, curcumin ethylenediamine derivative (curcumin ED) and curcumin ethylenediamine manganese complex (curcumin EDMn).

Results

Cytotoxic effect of curcumin, curcumin ED and curcumin EDMn against HUVEC cells *in vitro*. In the present study, the cytotoxic effect of curcumin, curcumin ED and curcumin EDMn on HUVEC cells was investigated at the concentrations ranging from 0 to 100 μM (Fig. 2). Results show that all compounds were non-toxic up to the concentration of 10 μM.

Effect of curcumin, curcumin ED and curcumin EDMn on the tube formation by HUVEC cells *in vitro*. Since HUVEC cells have the specialized function of forming capillary tubes, which is considered to be a key process during angiogenesis, we investigated the inhibitory effect of curcumin, curcumin ED and curcumin EDMn on the tube formation by these cells. HUVEC were seeded in Matrigel-coated wells and monitored for the formation of capillary tubes over 4 h. Figs. 3, 4 and 5 show the inhibitory effect of curcumin, curcumin ED and curcumin EDMn on the tube formation by HUVEC cells, respectively. Figs. 3A, 4A and 5A indicate the well-branching network with formation of long capillary tubes after the culture on Matrigel. The addi-

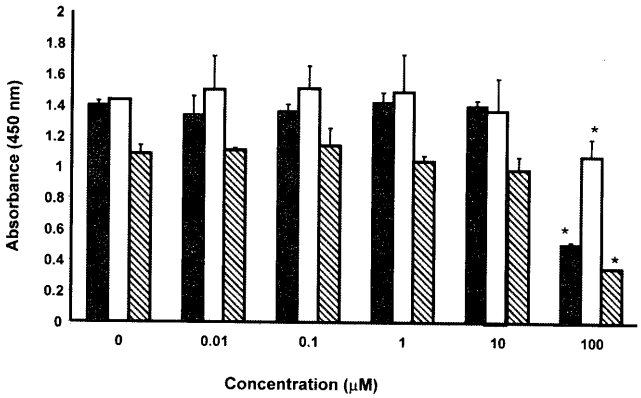

Fig. 2 Cytotoxic effect of curcumin, curcumin ED and curcumin EDMn against HUVEC. Cells (1x10^4 cell/well/ 50 μl) were re-suspended in 50 μl of medium as specified in Material and Methods. After 12 h of pre-incubation, various concentrations (0.01-100 μM) of compounds (curcumin, filled bar; curcumin ED, empty bar; and curcumin EDMn, stripped bar) contained in 50 μl of medium were added to the cultures and incubated for an additional 24 h. For the control (0 μM), 50 μl of medium containing DMSO was added to the cultures. The absorbance at 450 nm was measured in an immunoreader 2 hours after the addition of WST-1 solution (10 μl/ well). The data are expressed as the means ± S.D. of groups of triplicate cultures for each compound. *: $p<0.05$ compared with the untreated controls by the Student's two-tailed t-test.

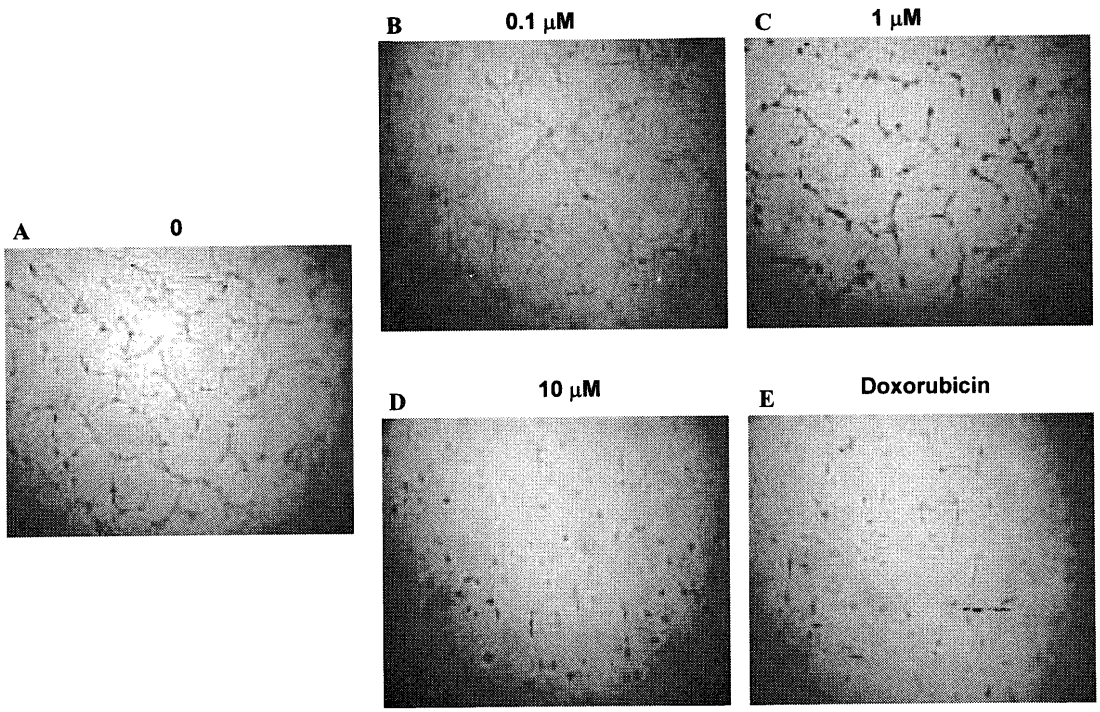

Fig. 3 Effect of curcumin on the formation of tube-like structures by HUVEC. Cells seeded in 6-well plates were pretreated with the indicated concentrations of compounds for 24 h. The cells were washed once with PBS, starved with Trypsin-EDTA and centrifuged at 2,000 rpm for 5 min. Cell suspensions ($3x10^4$ cells/well/500 μl) were then prepared in 0.2% FBS-supplemented HuMediaEG-2 medium (250 μl) and seeded on a 48-well plate, previously coated with 10 mg/ml Matrigel$_{\text{Becton-Dickinson}}$ (120 μl). The formation of tube-like structures was monitored under a microscope every 1 h for 6 h. Photos of the tube formation (6 h) of control (vehicle, Fig. A), those after treatment with various concentrations of curcumin (Figs. B, C and D), and with doxorubicin (Fig. E) are shown.

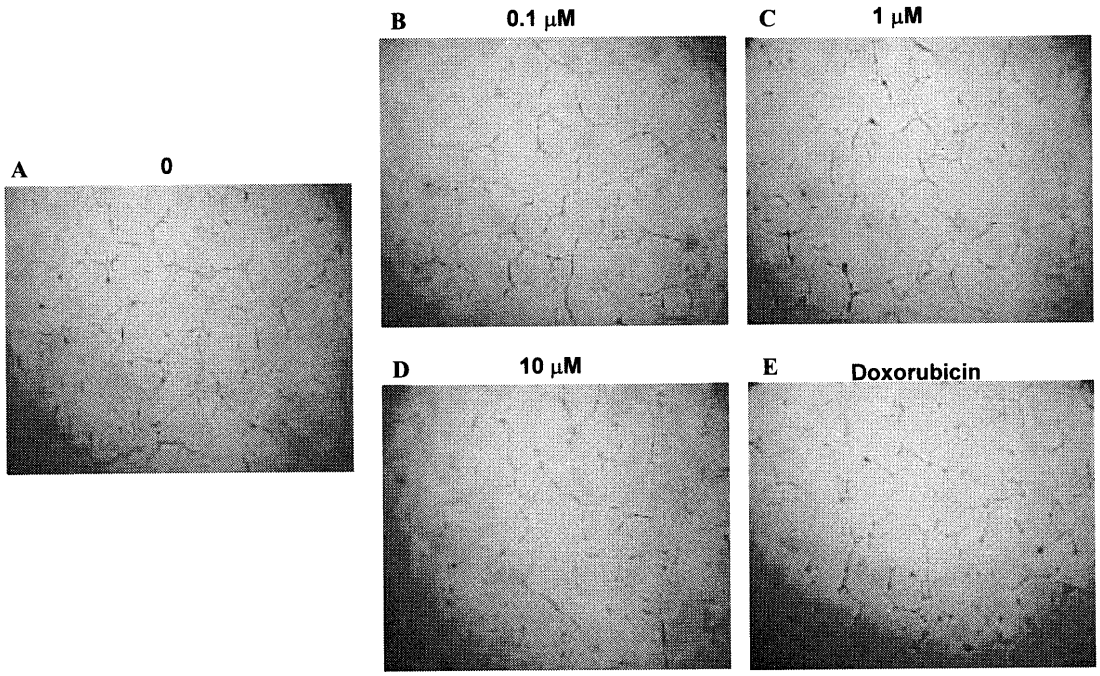

Fig. 4 Effect of curcumin ED on the formation of tube-like structures by HUVEC. Cells seeded in 6-well plates were pretreated with the indicated concentrations of compounds for 24 h. The cells were washed once with PBS, starved with Trypsin-EDTA and centrifuged at 2,000 rpm for 5 min. Cell suspensions ($3x10^4$ cells/well/500 μl) were then prepared in 0.2% FBS-supplemented HuMediaEG-2 medium (250 μl) and seeded on a 48-well plate, previously coated with 10 mg/ml Matrigel$_{\text{Becton-Dickinson}}$ (120 μl). The formation of tube-like structures was monitored under a microscope every 1 h for 6 h. Photos of the tube formation (6 h) of control (vehicle, Fig. A), those after treatment with various concentrations of curcumin ED (Figs. B, C and D), and with doxorubicin (Fig. E) are shown.

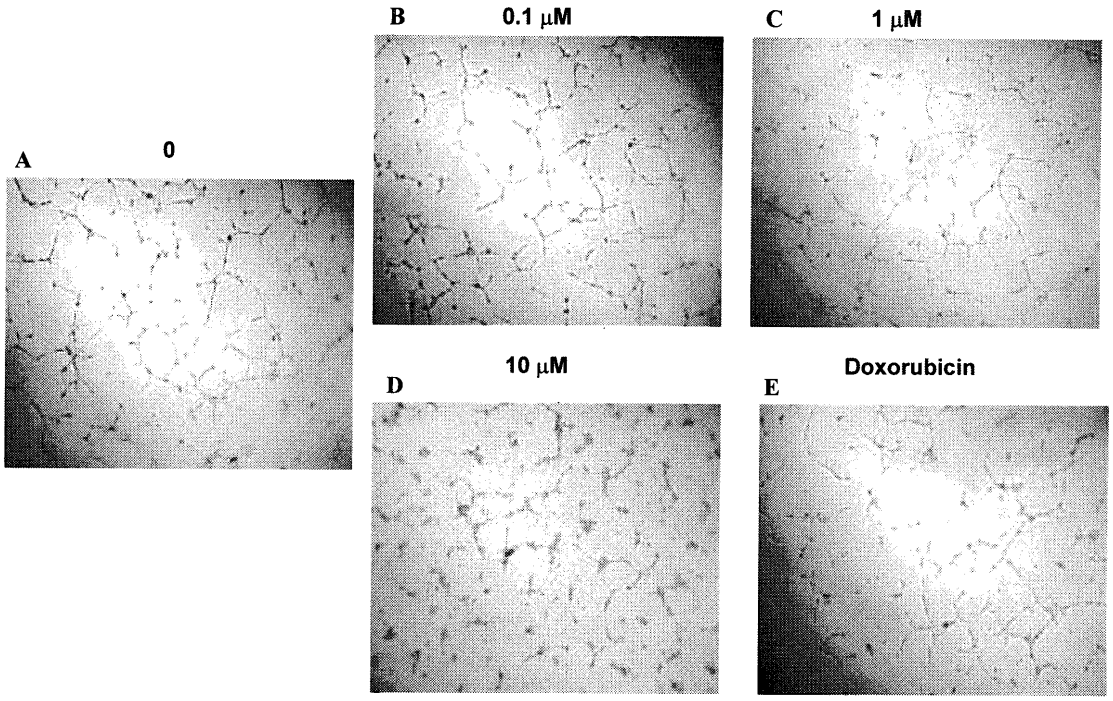

Fig. 5 Effect of curcumin EDMn on the formation of tube-like structures by HUVEC. Cells seeded in 6-well plates were pretreated with the indicated concentrations of compounds for 24 h. The cells were washed once with PBS, starved with Trypsin-EDTA and centrifuged at 2,000 rpm for 5 min. Cell suspensions ($3x10^4$ cells/well/500 μl) were then prepared in 0.2% FBS-supplemented HuMediaEG-2 medium (250 μl) and seeded on a 48-well plate, previously coated with 10 mg/ml Matrigel$_{\text{Becton-Dickinson}}$ (120 μl). The formation of tube-like structures was monitored under a microscope every 1 h for 6 h. Photos of the tube formation (6 h) of control (vehicle, Fig. A), those after treatment with various concentrations of curcumin EDMn (Figs. B, C and D), and with doxorubicin (Fig. E) are shown.

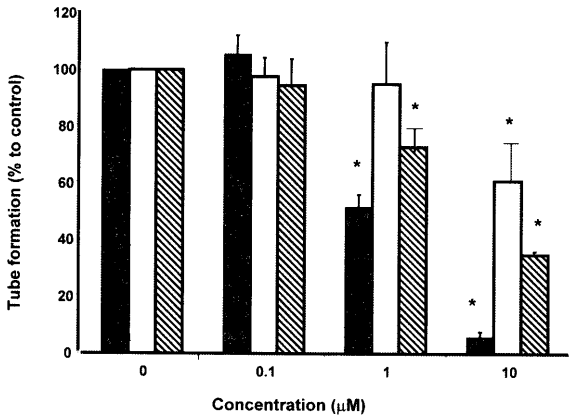

Fig. 6 Comparison of the inhibitory effects of curcumin, curcumin ED and curcumin EDMn on tube formation. The extent of inhibition of tube formation by HUVEC in presence of curcumin (filled bar), curcumin ED (empty bar) and curcumin EDMn (stripped bar) was analyzed by measuring the length of tube-like structures. The data are expressed as the means (% of control) ± S.D. of groups of triplicate cultures. *: $p<0.05$ compared with the untreated control by Student's two-tailed t-test.

tion of curcumin and curcumin EDMn at 1 μM (Figs. 3C and 5C, respectively) and 10 μM (Figs. 3D and 5D, respectively) led to the formation of poorly branching structures of shorter length. Interestingly, at the concentration of 10 μM the inhibitory effect of both compounds was stronger than that of 1.84 μM doxorubicin (Figs. 3E and 5E).

On the other hand, no inhibitory effect of curcumin ED was observed at concentrations lower than 10 μM (Fig. 4D). In summary, the inhibitory effect of all compounds at the concentrations of 1 and 10 μM was in the following order: curcumin > curcumin EDMn > curcumin ED (Fig. 6).

Discussion

We examined the *in vitro* effects of curcumin, curcumin ED and curcumin EDMn on the growth and tube formation by HUVEC, which are considered to be important steps in the angiogenic process.[18] None of these three compounds showed cytotoxic effect against HUVEC at concentrations of 10 μM and less (Fig. 2). All compounds were cytotoxic to HUVEC at the concentration of 100 μM. A comparable effect of curcumin was investigated in immortalized mouse fibroblast (NIH3T3) cells, Ehrlich ascites tumor (EAT) cells, and HUVEC cells.[12] Gururaj and coworker reported that curcumin was not cytotoxic to any of the cell types tested (e.g. NIH3T3, EAT, HUVEC) in the concentration range of 0.001-1 mM, but curcumin reduced the HUVEC cell number with a maximum reduction at 0.1 mM.[12]

Additionally, we observed that curcumin, curcumin ED and curcumin EDMn were effective in inhibiting the tube formation of HUVEC cells (Figs. 3, 4 and 5). Curcumin and curcumin EDMn were active at 1 and 10 μM, whereas

curcumin ED was effective at 10 μM. Among the investigated compounds, curcumin showed the most potent inhibitory effect, followed by curcumin EDMn and curcumin ED. Our results are in good agreement with those reported by Gururaj and co-workers, who showed that curcumin inhibited angiogenesis in two *in vivo* angiogenesis assay systems, peritoneal angiogenesis and choriollantoic membrane assay.[12] Some studies described similar effects of other inhibitors on the formation of tube-like structures by endothelial cells. To mention, our group previously showed that a matrix metalloproteinase (MMP) inhibitor (MMI270) led to 40% of inhibition of tube formation by hepatic sinusoidal endothelial (HSE) cells at the concentration of 12.5 μg/ml (31 μM).[19] Moreover a specific proteasome inhibitor, lactacystin, caused approximately 60 and 90% of inhibition of the endothelial tube formation of human dermal microvascular endothelial (HDME) cells at the concentrations of 3 and 10 μM, respectively.[15]

Although curcumin ED and curcumin EDMn showed the inhibitory effect on the tube formation of HUVEC cells in a lesser extent than that of curcumin, both compounds might be clinically useful for anti-lipid peroxidation and SOD activity[17] in addition to their anti-angiogenic effect.

The inhibitory effect of curcumin and its derivatives on the tube formation by HUVEC is possibly due to the radical scavenging ability of the complexes via the combined modes as anti-lipid peroxidation and SOD mimics. Most antioxidants commonly scavenge radicals (NO, $\cdot O_2^-$, HO$^\cdot$, RO$^\cdot$, *etc.*) non selectively while SOD selectively mimics dismute superoxide anions to hydrogen peroxide. The decrease of NO and $\cdot O_2^-$ that are the signaling messengers in the angiogenesis may lead to the inhibition of tube formation.[17] Curcumin EDMn showed a stronger inhibitory effect on the tube formation of HUVEC as compared with curcumin ED, possibly due to its combined modes of action, as antioxidation and SOD mimic, owing to the incorporation of manganese atom into the structure.

The active sites of the curcumin are the phenolic hydroxy groups and the keto-enol group in the linkage between two phenolic groups. The conjugated double bond system in the molecule also contributes to the radical scavenging ability, the more conjugation system in the structures results in the more reactivity of the scavenging action. Curcumin is the most active compound among the three compounds, since it contains three active hydroxy groups and more conjugation in the structure. Concerning curcumin ED, it showed a less strong effect than curcumin EDMn possibly because the cyclic ring in the linkage reduced the number of active hydroxy groups, from three to two groups, besides decreasing the number of conjugation in the system.

In conclusion, the present study demonstrated *in vitro* the effects of curcumin ED and curcumin EDMn on the formation of tube-like structures by HUVEC, and compared them with those effects of curcumin. Both compounds may be further modified to develop anti-angiogenic drugs.

Acknowledgement

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Japanese abstract

クルクミン（curcumin），クルクミン・エチレンジアミン誘導体（curcumin ED）及びそのマンガン複合体（curcumin EDMn）を用いて，ヒト臍帯静脈内皮細胞の管腔様構造の形成能を指標にして血管新生阻害効果を検討した。10 μM の curcumin, curcumin ED 及び curcumin EDMn は，管腔様構造の形成をそれぞれ94%，40%及び65%程度阻害した。これらの結果は，curcumin とともに curcumin ED 及び curcumin EDMn が以前に報告した抗脂質過酸化作用やスーパーオキシドジスムターゼ活性を有することに加えて，血管新生の阻害薬として有効であることが示唆された。

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