Cytotechnology (2004) 46:25–36
DOI 10.1007/s10616-005-1477-4

Differential effect of green tea catechins on three endothelial cell clones isolated from rat adipose tissue and on human umbilical vein endothelial cells

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Received 31 January 2005; accepted 24 January 2005

Key words: Adipose tissue, Angiogenesis, Catechin, Endothelial cells, Obesity, Tea

Abstract

By single colony isolation from the cells in stromal vascular fraction (SVF) dispersed from rat adipose tissues, we isolated three independent clones with different proliferation potential. All clones showed cobblestone-like morphology at the confluence and incorporated fluorescent Dil acetylated low density lipoprotein. When plated on Matrigel, they formed a capillary network-like structure. These rat adipose tissue endothelial cell (RATEC) clones showed higher expression of wnt2, wnt4, wnt5a, wnt5b, fzd1 and fzd5 whereas lower expression of cell cycle controlling genes such as CIP1, KIP1, KIP2, CDKN2A, CDKN2B, CDKN2C and CDKN2D compared to human umbilical vein endothelial cell (HUVEC). As reported for HUVEC, the growth of RATEC was inhibited by green tea catechins such as epigallocatechin, epicatechin gallate, epicatechin and epigallocatechin gallate but with higher sensitivity than HUVEC. The sensitivity of RATEC to catechins was higher for the cultures with low plating density and for the clone with higher proliferation potential.

Abbreviations: DMEM – Dulbecco's modified Eagle's medium; FBS – Fetal bovine serum; HUVEC – human umbilical vein endothelial cell; PCR – Polymerase chain reaction; RATEC – Rat adipose tissue endothelial cell; RT-PCR – Reverse transcription PCR; SVF – Stromal vascular fraction; TIMP – Tissue inhibitor of metalloproteinase; VEGF – Vascular endothelial growth factor

Introduction

Obesity has become a problem of epidemic proportions in Western societies and is commonly associated with a broad range of health risk factors including accelerated atherosclerosis, thrombosis, hypertension, hyperinsulinemia, insulin resistance and type 2 diabetes (Mokdad et al. 2003). Means of controlling obesity by daily food and drinking have attracted wide attention also in the recent Japanese society. Excessive expansion of adipose tissue can result from adipocyte hypertrophy, a consequence of increased triglyceride storage, and/or from adipocyte hyperplasia, a process that requires the proliferation and differentiation of preadipocytes.

Adipose tissue is highly vascularized, and the increase in fat mass during the development of obesity in the adult appears to be accompanied by an increase in the size of the microcirculation (reviewed in Crandall et al. 1997; Bouloumie et al. 2002). Expanding adipose tissue thus represents one of the few sites of active angiogenesis in the adult body. A tight link has been demonstrated between adipogenic precursor cells and the capillary network. The formation of capillary convolutions is a decisive phase in the development of fat lobules during embryonic development (Wassermann 1965). Adipose lineage cells have been shown to release potent angiogenic factors such as monobutyril, vascular endothelial growth factor and leptin (Castellot et al. 1982; Dobson et al. 1990; Claffey et al. 1992; Bouloumie et al. 1998; Sierra-Honigmann et al. 1998). During the process of *de novo* adipogenesis in mice at the site of injection of Matrigel and basic fibroblast growth factor (Kawaguchi et al. 1998; Toriyama et al. 2002), endothelial cells first migrated into the space of Matrigel to form a capillary network and then adipocyte precursors migrated, proliferated and differentiated to form a fat pad. Interaction between adipogenesis and blood vessel formation has been shown by loss of adipose tissue by administration of antiangiogenic agents (Rupnick et al. 2002).

The stromal vascular fraction (SVF) is the sediment fraction of cell suspension dispersed from adipose tissues by collagenase-digestion and represents a heterogeneous cell population. Its culture was classically used to investigate preadipocyte differentiation into mature adipocytes, but recently found to be a source of multipotent cells able to differentiate into osteogenic, chondrogenic, and myogenic lineages (Erickson et al. 2002; Rupnick et al. 2002; Zuk et al. 2002). In these studies, limited dilution culture or single colony isolation culture of the cells in SVF resulted in the isolation of an actively proliferating cell population having the potential of multiple differentiation along with mesenchymal cell linage. Transplantation of such cells could reconstitute the function of tissues in lethally irradiated mice (Cousin et al. 2003). Recently, their differentiation potential was even extended to the neurogenic lineage (Ashjian et al. 2003). Although isolation and culture of microvascular endothelial cells have been also reported from SVF (Hutley et al. 2001; Frye and Patrick 2002), we hypothesized that endothelial cells in SVF may have specific characters to support the adipose tissue formation and have independently isolated three clones of rat adipose tissue endothelial cells by the single colony isolation technique.

Using human umbilical vein endothelial cells (HUVEC) as a model system, several independent researches (Abou-Agag et al. 2001; Kondo et al. 2002; Sartippour et al. 2002; Singh et al. 2002) have shown the inhibition of angiogenesis by green tea catechins and substantiated the *in vivo* data that drinking tea suppresses angiogenesis (Cao and Cao 1999; Kao et al. 2000). In this study, we also confirmed the sensitivity of endothelial cells to catechins. To our surprise, we found that endothelial cells isolated from adipose tissue are much more sensitive to catechins than HUVEC. Their growth was markedly inhibited by catechins at a concentration of less than 10 μg/ml. Thus, specific effects of catechins on adipose tissue endothelial cells may explain recently the appreciated anti-obesity effect of drinking green tea.

Materials and methods

Isolation of three clones of rat adipose tissue endothelial cells (RATEC)

Epididymal adipose tissue from a F344 NSIc rat was washed three times with FD20 (Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Nissui Pharmaceutical) containing 20% fetal bovine serum (FBS) (ICN Biomedicals, Lot: 8022E), 100 units/ml streptomycin (Meijiseika Ltd.), 100 μg/ml penicillin (Meijiseika) cut finely and digested with 1 ml of 1 mg/ml type I collagenase (Collagenase Type I, 274 unit/mg, Worthington) for 1 h at 37 °C in a water bath with reciprocal shaking. Cells obtained after filtrating the digested tissue through 250 μm meshes were washed three times with FD20 and 500 ng/ml fungizone (GIBCO 15290-018) by centrifugation at 1200 rpm for 5 min at room temperature, and the cells in sediment were used as SVF. SVF cells were cultured and passaged once with above DMEM/F12 medium and once with DMEM (Nissui Pharmaceutical) containing 10% FBS, 50 units/ml penicillin and 50 mg/ml streptomycin (the DMEM) and single colony isolation was initiated from these

SVF cells at second passage using the DMEM throughout. Trypsinized SVF cell suspension was diluted to the density to have one cell, in average, per well when 0.1 ml aliquots were distributed into three 96-well plates (NUNC). After 10 days of culture with medium change, altogether 5 wells (three in the 1st, two in the 2nd and zero in the 3rd plate) were found to have enough cells for the transfer into a 24-well plate (NUNC). The colonies transferred into the well numbered B2, B3 and C2 grew to fill the wells but the colonies transferred into B4 and B5 did not grow further. Clone C2 showed best growth during further transfer into one, two wells of 6-well plates and T-flask (with 25 cm^2 of the culture surface; NUNC), which corresponded to 5th, 6th and 7th passage. Clone B2 needed longer period to reach the size of T-flask culture but showed good growth afterward. Clone B3 did not grow well on the first 6-well plate, but its growth became faster after one additional transfer into 6-well plate. After obtaining T-flask size culture of these clones, they were maintained by passage into three new T-flasks. Since they attached to the culture surface strongly after reaching the confluence, only half of the cells could be detached by trypsinization for the transfer. The cells at 8th to 12th passage were used for the experiments.

The reference cell of HUVEC were kindly provided by Prof. S. Iijima (Department of Biotechnology, Graduate School of Engineering, Nagoya University) and maintained under humidified 5% CO_2/95% air at 37 °C in the DMEM.

Characterization of endothelial cells

To identify the isolated clones to be endothelial cells, their activity of endocytosis of acetylated low density lipoprotein (Stein and Stein 1980; Tokida et al. 1990) was tested. For this, fluorescent Dil acetylated low density lipoprotein (Molecular Probes, l-03484) was added to the culture media, incubated overnight and micrograph of the cultures was taken with a fluorescent microscope (Olympus IX70) with Rhodamin filter.

For further identification of endothelial cells, their ability of forming capillary-like network structure (Ouchi et al. 2004) was tested. For this, HUVEC or RATEC C2 clone was serum starved by culturing in endothelial cell basal medium-2 (EBM-2; Cambrex Bio Science, CC3156) with 0.5% FBS for 16 h. The serum starved cells were plated at the density of 4×10^5 cell/well on Matrigel, which was coating the wells of 24-well plate and had been equilibrated with EBM-2 medium (containing catechins when indicated). When cultured on Matrigel, endothelial cells aligned themselves into network structure within 12 h.

Effect of catechins on cell growth

The cells were plated into 24-well plates at a density of either 3×10^3 (low density) or 2×10^4 (high density) cells/well in 1.0 ml of the DMEM. After 24 h of pre-culture, (−)-epigallocatechin (Wako, 056-06761), (−)-epicatechin gallate (Wako, 052-06741), (−)-epicatechin (Wako, 059-06751), epigallocatechin gallate (Wako, 059-05411) were added to the DMEM at various concentrations. After three days, the cell number was counted with a Nucleocounter (New Brunswick Scientific, USA). Each dose of catechins was tested with duplicated cultures and all experiments were performed at least twice.

Semi-quantification of expressed genes by reverse transcription-polymerase chain reaction (RTPCR)

The cells plated at high density and pre-cultured for 24 h as above but in T-flasks were subjected to RNA extraction after 72 h of exposure to catechins. The total cellular RNA was isolated using TRIzol™ (Invitrogen Life Technologies). One milligram of RNA was used for first strand cDNA synthesis with random primer sets by M-MLV reverse transcriptase without RNase H activity due to point mutant (Promega). PCR was carried out for 35 cycles with a AmpliTaq (ABI) with cycling parameters after pre-heating at 94 °C for 10 min of 94 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s followed by a final extension at 72 °C for 10 min. PCR products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. Used PCR primers were as follows: Rat TIMP-1 forward primer: 5′-ATG GAG AGC CTC TGT GGA TAT G-3′; rat TIMP-1 reverse primer: 5′-GAT CTG ATC TGT CCA CAA GCA A-3′; rat MMP-7 forward primer: 5′-CTA CAG ACT TGC CTC GGT TCT T-3′;

rat MMP-7 reverse primer: 5′-CGT CCA GTA CTC ATC CTT GTC A-3′; rat MMP-12 forward primer: 5′-GAA ATT GGA GGC AGA AAT CAA C-3′; rat MMP-12 reverse primer: 5′-TAG AAA TCA GCT TGG GGT AAG C-3′; human/rat Wnt2 forward primer: 5′-TGG ACA GCT GCG AAG TTA TG-3′; human/rat Wnt2 reverse primer: 5′-AGT TGT CCA GTC GGC ACT CT-3′; human/rat Wnt4 forward primer: 5′-GCC ACG CAC TAA AGG AGA AG-3′; human/rat Wnt4-reverse primer: 5′-GGC CTT AGA CGT CTT GTT GC-3′; rat Wnt5A forward primer: 5′-TGC CAC TTG TAT CAG GAC CA-3′; rat Wnt5A reverse primer: 5′-TGT CTC TCG GCT GCC TAT TT-3′; human/rat Wnt5B forward primer: 5′-CGG TGC AGA GAC CCG AGA TG-3′; human/rat Wnt5B reverse primer: 5′-GTG AAG GCA GTC TCT CGG CT-3′; rat Fzd1 forward primer: 5′-CCA AGG AGG TGG AGG CGT C-3′; rat Fzd1 reverse primer: 5′-GTG TGT GTC TGG CGA GGA AG-3′; rat Fzd2 forward primer: 5′-AAG CCA GCA CT GCA AGA GCC TA-3′; rat Fzd2 reverse primer: 5′-CAC GGT TGC AGT CCG GAC CTG-3′; rat Fzd5 forward primer: 5′-TCT TGT CTG CGT GCT ACC TG-3′; rat Fzd5 reverse primer: 5′-CCC GGA CAC GCG CAG GTG AG-3′; rat Fzd6 forward primer: 5′-CTT ACC AGG CAA ACC CAA AA-3′; rat Fzd6 reverse primer: 5′-TTG CCA TGC TTC TTC TTG TG-3′; rat CIP1 forward primer: 5′-AAT ACC GTG GGT GTC AAA GC-3′; rat CIP1 reverse primer: 5′-GTG TGA GGA CTC GGG ACA AT-3′; rat KIP1 forward primer: 5′-GAT TTT TAC CTG GCC TGT TCT G-3′; rat KIP1 reverse primer: 5′-AAA TTA TTC CTC CCC ACC AAG T-3′; rat KIP2 forward primer: 5′-CTC TAC TCG CTC TCA GTG CAA A-3′; rat KIP2 reverse primer: 5′-GCT GGT GAT TTC TAG TGC CTT T-3′; rat CDKN2A forward primer: 5′-CTT GGT CAC TGT GAG GAT TCA G-3′; rat CDKN2B reverse primer: 5′-AGT TCG AAT CTG CAC CGT AGT T-3′; rat CDKN2B forward primer: 5′-AGT ACC TTC CCC TGT GAA CTG A-3′; rat CDKN2B reverse primer: 5′-CTG TGG GTC ACA GTT TAG TGG A-3′; rat CDNK2C forward primer: 5′-TCA GAG GTG CTA ATC CCA ATT T-3′; rat CDNK2C reverse primer: 5′-GCT TCA TAA GGA ACT CCA CCA C-3′; rat CDKN2D forward primer: 5′-TTA AAG CCC TAG CCT CAG AGT G-3′; rat CDKN2D reverse primer: 5′-TGG AAA CCT TCC AAG AAA GAA A-3′; mouse GAPDH forward primer: 5′-TCA CGG CAA ATT CAA CGG CAC AGT-3′; mouse GAPDH reverse primer: 5′-TCG GCA GAA GGG GCG GAG ATG AT-3′.

Results

Isolation of three RATEC clones

The cells in SVF dispersed from rat epididymal adipose tissues showed the potential for almost unlimited proliferation in DMEM and could be sub-cultured over 9 passages which corresponded to an average population doubling level of 25. Among the heterogenous population of the cells expanded from SVF, many endothelial cells appeared to be included together with fibroblastic cells, some of them may have the potential for multiple differentiation along mesenchymal linage (Zuk et al. 2002). When such a cell suspension of SVF was highly diluted to transfer a single cell (in average) into each well of 96-well plate, however, most cells stopped growth and only a limited number of cells formed colonies. After maintaining such cultures for 10 days, 5 wells out of 280 wells had a colony of more than 100 cells enough for transferring into 24-well plate. Our interesting observation was that all cells in these 5 wells exhibited the morphology of endothelial cells.

A colony we referred to as C2 showed high potential for proliferation over the repeated expansion cultures with a doubling time of less than 16 h, which was shorter than that of HUVEC. Two other colonies we referred to as B2 and B3 grew slowly with doubling times of more than 1.5 days even after acceleration of growth during the expansion culture. The remaining two colonies obtained in the first 96-well culture could not be maintained in the expansion culture. As shown in Figures 1A–C, these colonies exhibited cobblestone morphology with centrally located nuclei, which is typical for endothelial cells. An apparent minor difference in micrographs in Figure 1 was only due to different duration reaching the confluence, and they resembled to each other during a round of culturing cycle between passages. Specific incorporation of fluorescent Dil acetylated low density lipoprotein by these colonies is shown in Figures 1D and E. This activity is

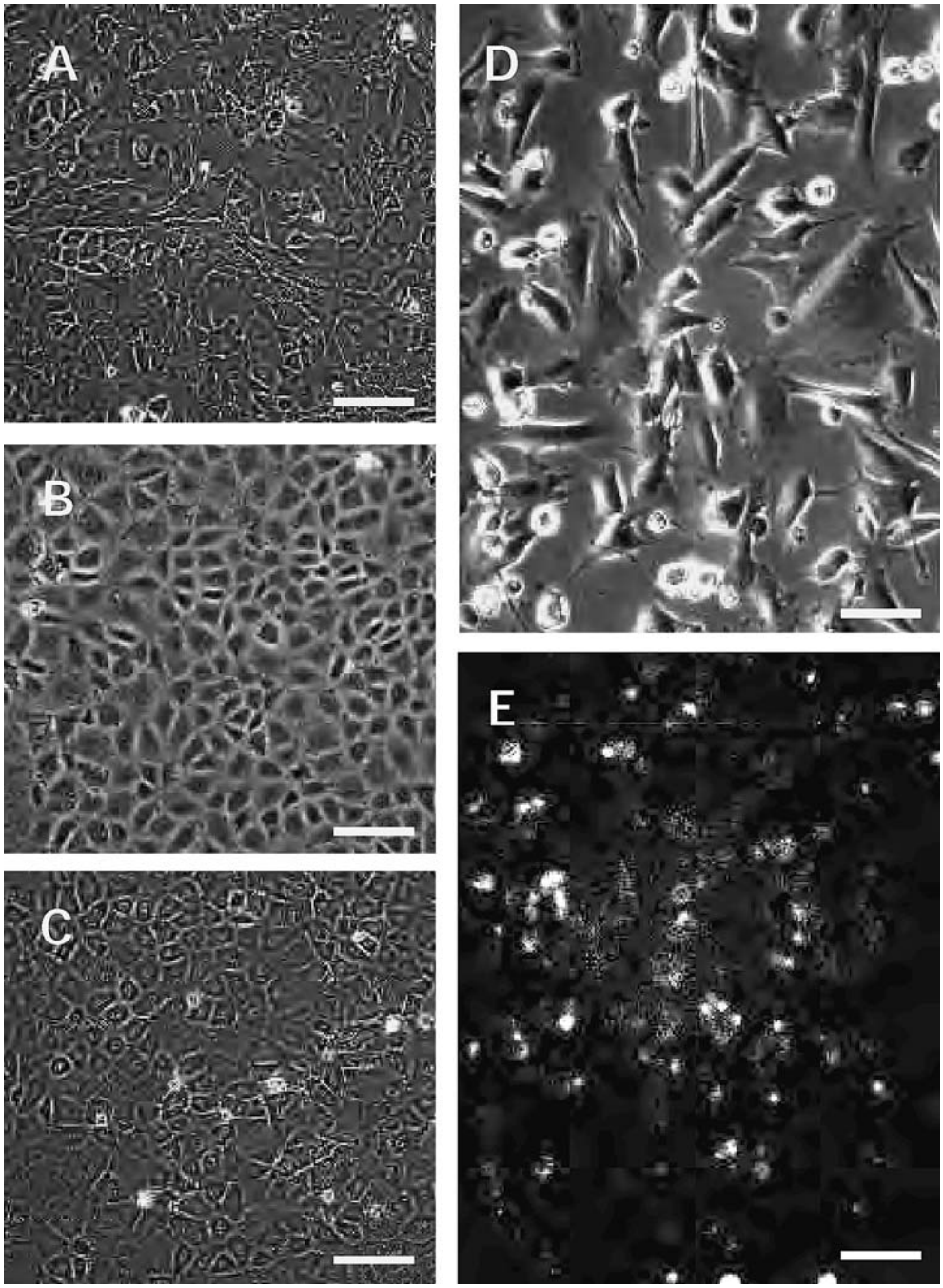

Figure 1. Isolation of three rat adipose tissue endothelial cell (RATEC) clones. Micrographs of RATEC C2 (A), B2 (B) and B3 (C) clones at different time after reaching the confluence are shown. Specific incorporation of fluorescent Dil acetylated low density lipoprotein by C2 clone is shown by fluorescent micrograph (E) and phase contrast micrograph (D) of a same field of view. Scale bar: 100 μm.

only known for macrophages and endothelial cells (Stein and Stein 1980). For further identification of the obtained colonies to be endothelial cells, we confirmed their ability of forming a capillary-like network structure on Matrigel as later shown in Figure 3.

Differential effect of catechins on three RATEC clones and HUVEC

Using HUVEC as a model system, several independent researches have shown the inhibition of angiogenesis by green tea catechins (Abou-Agag et al. 2001; Kondo et al. 2002; Sartippour et al. 2002; Singh et al. 2002). Reflecting multiple mechanisms of the effect possible for these polyphenols (see Discussion), their effects on the growth of HUVEC have been controversial and were variable depending on the experimental conditions. In our experiment shown in Figure 2B for example the addition of 1 μg/ml of green tea cate-

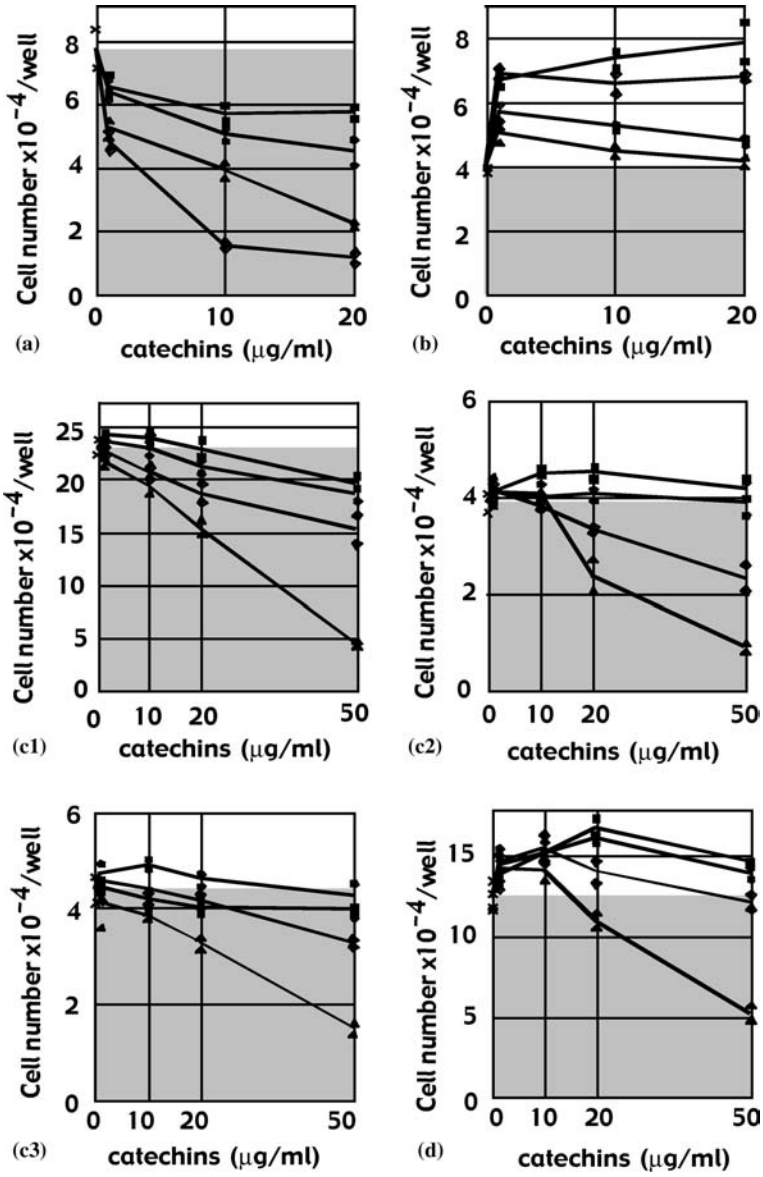

Figure 2. Differential effect of catechins on the growth of three RATEC clones and HUVEC plated at different plating cell density. RATEC C2 (A and C1), B2 (C2) , B3 (C3) clones and HUVEC (B and D) were plated into 24-well plates at a cell density of either 3×10^3 (A and B) or 2×10^4 (C1, C2, C3 and D) cells per well in 1.0 ml of the DMEM. After 24 h of preculture, epigallocatechin gallate (rectangle), epicatechin gallate (triangle), epigallocatechin (circle) or epicatechin (square) was added at indicated concentration. After three days, the cell number was counted. Each dose of catechins was tested with duplicated cultures.

chins was growth stimulatory on HUVEC when the cells were initially plated at a low density of 3000 cells per well of 24-well plates (1600 cells/cm^2).

Epicatechin gallate and epigallocatechin gallate showed an inhibitory effect on HUVEC at the 20–50 μg/ml range only when the cells were plated at a high density of 2×10^4 cells per well of 24-well plate (10,500 cells/cm^2) (Figure 2D). In a clear contrast, the growth of RATEC C2 was markedly inhibited by green tea catechins even at 1 μg/ml when the cells were plated at a low density Figure 2A). The inhibitory effects of chatechins were in the order of epigallocatechin gallate > epicatechin gallate > epigallocatechin > epicatechin, and the growth of RATEC C2 was almost completely inhibited at 10–20 μg/ml of epigallocatechin gallate.

We also found that the effect of catechins on RATEC was strongly dependent on the cell density. When RATEC C2 was plated at initial density of 2×10^4 cells per well of 24-well plate, they became resistant to 20 μg/ml of catechins and inhibition was observed by 50 μg/ml of epigallocatechin (Figure C1). A series of experiments summarized in Figures C1–C3 is to compare the effect of catechins on three RATEC clones having different growth rate. The effect was basically similar among the clones but tended to be stronger on C2, which had the highest proliferation potential. For slow growing clones B2 and B3, the stimulatory effect of epigallocatechin and epicatechin was observed for HUVEC at low concentration range .

As described above, our RATEC colonies showed the potential of forming capillary-like structures when cultured on Matrigel (Figure 3). Compared with the structures formed by HUVEC, the tube-like structures were thinner and irregular in the length. In a recent study by Singh et al. the capillary-like structure formation by HUVEC on Matrigel was inhibited by the treatment with epigallocatechin gallate at 10–80 μg/ml (Singh et al. 2002). In a similar experiment on HUVEC, we also observed a strong effect of epigallocatechin gallate at 50 μg/ml (Figure 3C), but the consequence was somewhat different. Compared to the experiment without drug (Figure 3A), HUVEC formed a denser and irregular network with shorter tube-like structures connecting the crossing points. Treatment with epicatechin gallate at 50 μg/ml also gave similar results (Figure 3B). This capillary-like network formation is a complicated process including apoptosis of the cells excluded from the process of network formation (Pollman et al. 1999). Actually, many dead cells were observed in the presence of these catechins (Figures 3B, C, E and F). Thus, it is difficult to estimate the effect of catechins on microvessel formation only by this Matrigel assay. The capillary-like structure formation of RATEC C2 on Matrigel was also affected by both epicatechin gallate (Figure 3E) and epigallocatechin gallate Figure 3F) at 50 μg/ml. We could not find clear a difference between HUVEC and RATEC in their response to catechins during the capillary-like structure formation on Matrigel.

Different gene expression profiles of RATEC and HUVEC

Growing evidence indicates that Wnt signaling is involved in angiogenesis and endothelial cell function. Members of the Wnt and Frizzled families are expressed in different types of endothelial cells, but the expression profiles of Wnt signaling molecules on endothelial cells are still incomplete. The mRNAs encoding Wnt-7a, Wnt-10b, and Frizzled-1 have been shown by RT-PCR to be present in mouse primary brain microvascular endothelial cells (MBMEC), and Wnt-5a and Frizzled-3 are expressed by HUVEC (Wright et al. 1999). FrzA, the bovine homolog of sFRP1, is expressed in bovine aortic endothelial cells (BAEC) (Duplaa et al. 1999). FrzB-1 (also known as sFRP3) is expressed in rat aortic endothelial cells (RAEC) (Mao et al. 2000). Several functional studies further suggest that Wnt signaling is required for angiogenesis. Ishikawa et al. found that Frz5 mRNA is expressed in the yolk sac, eye, and lung bud during mouse embryogenesis by *in situ* hybridization (Ishikawa et al. 2001). Taking advantage of isolated RATEC clones, we serviced the expression of Wnt- Frizzled families in these endothelial cells. As shown in Figure 4 and Table 1, expression of Wnt4, Wnt5a, Fzd1 and Fzd5 was stronger in RATEC than HUVEC. Their expression was affected by neither epigallocatechin nor epicatechin gallate.

The expression profiles and the potential role of matrix metalloprotease and their tissue inhibitors (TIMPs) were shown to be related to the adipose

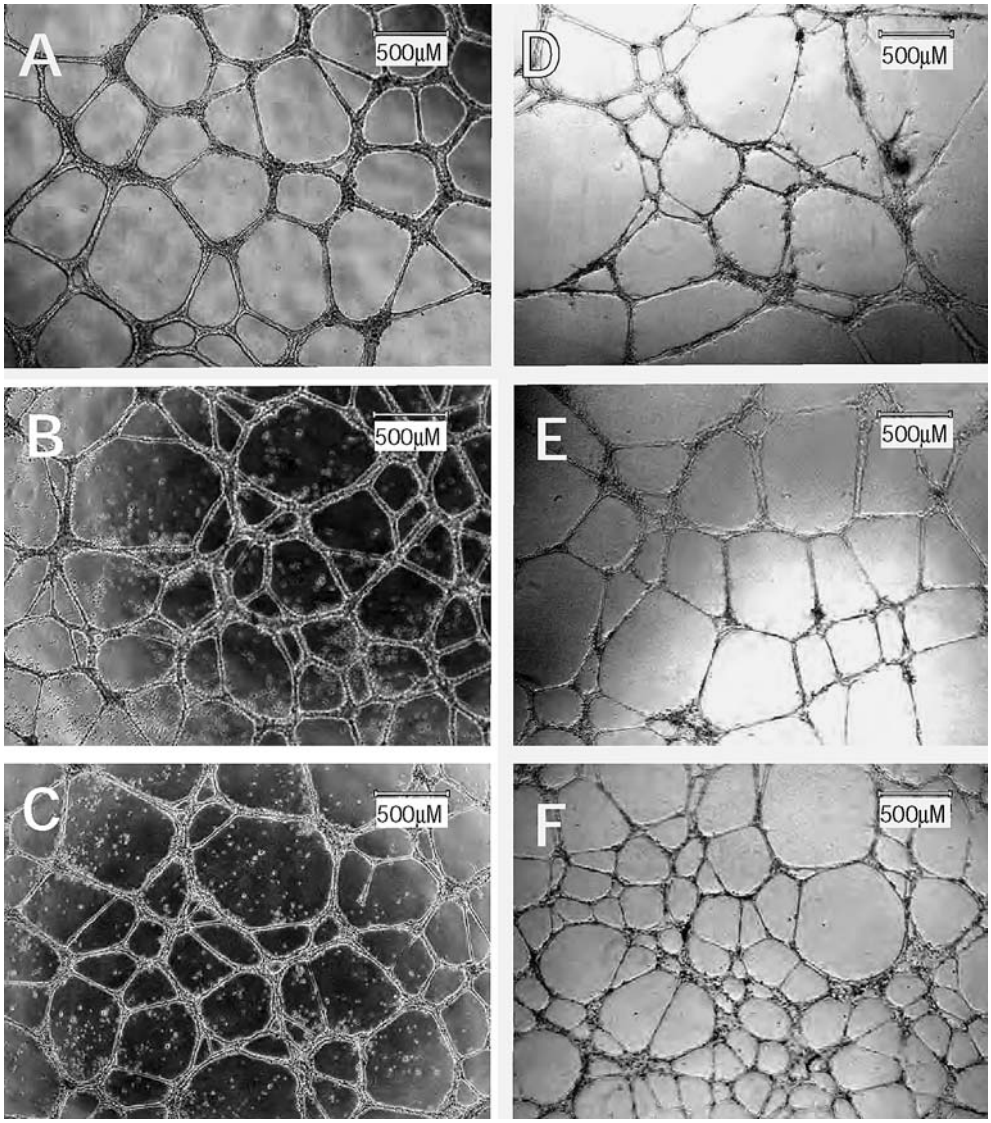

Figure 3. Capillary-like network structure formed by HUVEC and the RATEC C2 clone on Matrigel. The ability of forming capillary-like network structure by HUVEC (A, B and C) and RATEC (D, E and F) was tested by plating 4×10^4 cells on Matrigel coated 24-well plates after serum starvation for 16 h. The cultures contained none (A and D), 50 μg/ml epicatechin gallate (B and E) and 50 μg/ml epigallocatechin gallate (B and E) or 50 μg/ml epigallocatechin gallate (C and F). After 16 h of culture on Matrigel, the micrographs were taken. The bar indicates 500 μm

tissue remodeling during development of obesity in the mouse (Chavey et al. 2003). Despite some specific expression profiles are expected for RATEC in relation to dynamic changes of cell–matrix interactions and extracellular matrix remodeling during the development of obesity, RATEC expressed only TIMP-1 as HUVEC did and the expression was not affected by catechins (Table 1).

Expression of the cell cycle regulating genes was generally lower in RATEC than HUVEC and their levels did not respond to the addition of catechins (Table 1).

Discussion

By inhibiting tyrosine kinase and mitogen-activated protein kinase (MAPK), epigallocatechin gallate inhibited the growth of transformed but not of normal fibroblasts (Wang and Bachrach

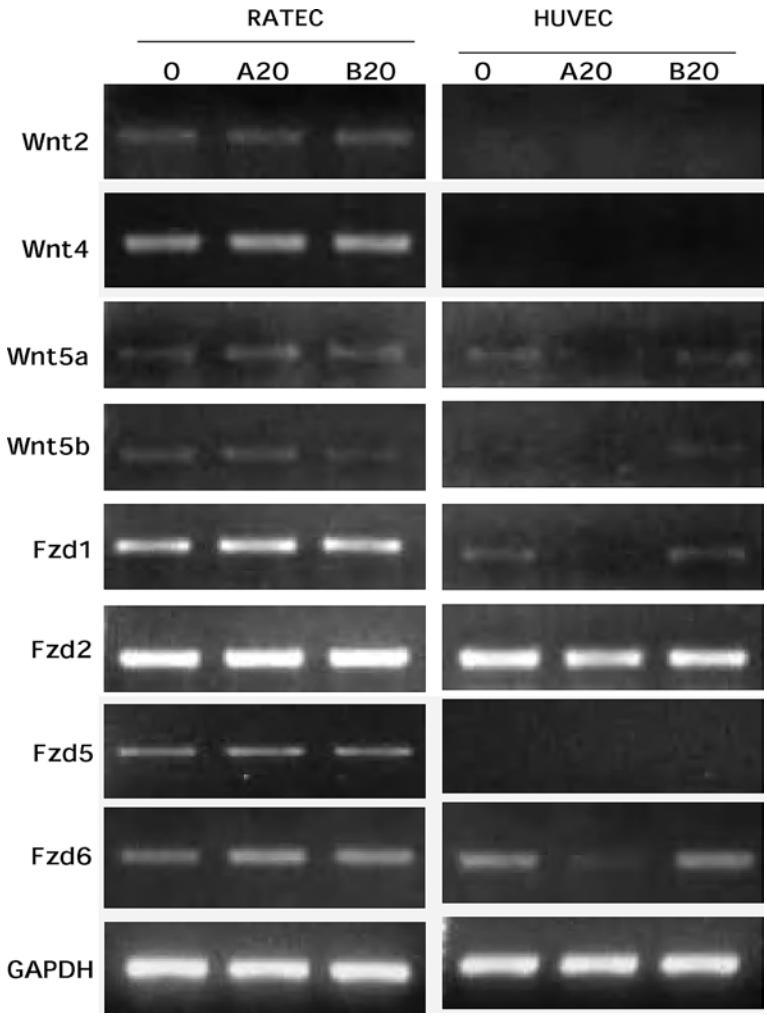

Figure 4. RT-PCR analysis of Wnt-related gene expression in RATEC and HUVEC. Total RNA was extracted from RATEC C2 clone and HUVEC cultured in medium containing 20 μg/ml of epigallocatechin (A20) or epicatechin gallate (B20) for 72 h. RT-PCR was carried out as described in Materials and methods. PCR products were separated by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. The mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) was an internal control for RT-PCR analysis.

2002). In this inhibition, epigallocatechin gallate preferentially decreased the level of the oncogenes Ras and Jun in transformed cells. By inhibiting the binding of vascular endothelial growth factor (VEGF) to HUVEC, green tea catechins inhibited VEGF-stimulated cell proliferation in a concentration dependent manner (Kondo et al. 2002). However, even the most effective epigallocatechin-3-gallate showed only 55% inhibition of growth at the concentration of 100 μM (30 μg/ml). Although epigallocatechin-3-gallate inhibited the capillary-like network formation of HUVEC on Matrigel, there was no significant inhibition in MTT reduction, a measure for cell growth and viability, after 6 h treatment at the range of 10–50 μg/ml. The inhibition was observed only after 48 h treatment (Singh et al. 2002). In our experiment on HUVEC with a comparable plating cell density (Figure 2D), we observed inhibition of the basal growth of HUVEC by epigallocatechin-3-gallate to a similar extent during 3 days of culture. However, other green tea catechins showed a stimulatory effect (Figure 2D). Such a stimulatory effect was more evident on HUVEC with lower plating cell density (Figure 2B).

Table 1. Expression of Wnt-related genes, TIMP genes and cell cycle regulating genes in RATEC and HUVEC estimated by RT-PCR.

GENES	RATEC (C2)		HUVEC			
	None	Epigallocatechin	Epicatechin gallate	None	Epigallocatechin	Epicatechin gallate
Wnt2	+	+	+	–	–	–
Wnt4	+ + +	+ + +	+ + +	–	–	–
Wnt5a	+	+	+	+	–	+
Wnt5b	+	+	+	–	–	+
Fzd1	+ + +	+ + +	+ + +	+	–	+
Fzd2	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Fzd5	+ +	+ +	+ +	–	–	–
Fzd6	+ +	+ +	+ +	+ +	+	+ +
TIMP-1	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
TIMP-7	–	–	–	–	–	–
TIMP-12	–	–	–	–	–	–
CIP1	–	–	–	+ +	+ +	+ +
KIP1	–	–	–	+ +	+ +	+ +
KIP2	+	+	+	+ + +	+ +	+ + +
CDKN2A	–	–	–	+ + +	+ + +	+ + +
CDKN2B	–	–	–	+ +	+ +	+ +
CDKN2C	+ +	+ +	+ +	+ + +	+ + +	+ + +
CDKN2D	–	–	–	+ +	+ +	+ +
GAPDH	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

Total cellular RNA was extracted from RATEC clone (C2) or HUVEC cultured in medium containing 20 μg/ml of epigallocatechin or epicatechin gallate for 72 h. RT-PCR was carried out as detailed in Materials and Method section. Estimated expression level of the corresponding gene base on the Intensity of amplified cDNA fragments as in Fig. 4 is listed. -: no expression, +: weak expression, + +: strong expression, + + +: very strong expression. *GAPDH*: glyceraldehydes-3-phospahe dehydrogenase gene.

Several studies have shown that epigallocatechin-3-gallate can act as an antioxidant by trapping peroxyl radicals and inhibiting lipid peroxydation. In a model experiment using membrane-bound ATPases in human erythrocyte membranes, *t*-butylhydroperoxide-induced lipid peroxidation was significantly blocked by epigallocatechin-3-gallate (Saffari and Sadrzandeh 2004). Amphiphilic properties of epicatechin made it capable of permeating cell membranes and murine aortic endothelial cells were demonstrated to accumulate epicatechin, which protected the cells against peroxynitrate-induced nitration of protein tyrosyl residues (Schroeder et al. 2003). Such activity of catechins may explain their stimulatory effect on the growth of HUVEC and our results have shown have that it is dependent on the plating cell density. An important finding was that RATEC was less competent to this stimulatory effect than HUVEC and only the inhibitory effect of catechins was evident on RATEC plated at low density (Figure 2A). Our results have demonstrated for the first time that apparent effects of catechins need to be understood on the balance of their stimulatory and inhibitory effects.

The cells in SVF dispersed from adipose tissues are recently attracting a broad attention by plastic surgeons working in the field of reconstructive therapy as a source of stem cells with high potential of endothelial differentiation for implantation into damaged tissues for neovascularization (Miranville et al. 2004; Planat-Benard et al. 2004). For this, a new stem cell population of mesoangioblast with potential for differentiation along both endothelial and mesenchymal linages (Minasi et al. 2002) is searched among a large population of $CD34^+$ cells in SVF. It is obvious that our RATEC clones are distinct from such stem cells, however, their high proliferation potential suggests that they may be worthwhile to be tested for the activity of neovascularization by transplanting into damaged sites.

Acknowledgements

We thank Prof. Shinji Iijima for providing us with HUVEC. This work was supported by a program of Promotion of Business Using Advised Technology provided by the Japan Science Technology

Agency and by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science awarded to YK (15208034) and ST (15390538). ATK was supported by Academic Frontiers Student Exchange Promotion Program from the Ministry of Education, Science, Culture and Sports of Japan.

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