Table 1 Baseline characteristics of 50 patients with type 2 diabetes mellitus

	Pioglitazone (n = 25)	*Control (n = 25)*	*P-value*
Age (years)	63.8±12.6	61.4±12.3	NS
Male/female	15/10	14/11	–
Height (m)	159.9±6.6	161.2±7.9	NS
Weight (kg)	61.4±7.7	61.6±12.8	NS
BMI (kg/m^2)	24.0±2.5	23.6±3.6	NS
Systolic blood pressure (mm Hg)	131.9±12.7	133.4±12.2	NS
Diastolic blood pressure (mm Hg)	75.0±9.2	78.1±7.2	NS
Asparate aminotransferase (IU/l)	31.2±19.9	26.9±13.0	NS
Alanine aminotransferase (IU/l)	29.0±23.2	27.8±16.6	NS
γ-Glutamyl transpeptidase (IU/1)	48.9±39.0	43.2±32.9	NS
Serum creatinine (μmol/l)	72.1±31.8	67.1±20.2	NS
Fasting blood glucose (mmol/l)	9.73±4.63	9.76±3.69	NS
HbA_{1c} (%)	8.17±0.87	7.95±0.87	NS
Total cholesterol (mmol/l)	5.12±1.02	5.30±0.98	NS
Triglyceride (mmol/l)	1.42±0.84	1.45±0.92	NS
High-density lipoprotein cholesterol (mmol/l)	1.41±0.45	1.42±0.38	NS
Low-density lipoprotein cholesterol (mmol/l)	3.01±0.80	3.10±0.85	NS
HGF (ng/ml)	0.256±0.044	0.259±0.079	NS
VEGF (pg/ml)	262.0±188.6	261.7±254.5	NS

Values are expressed as mean±s.d. Abbreviations: HGF, hepatocyte growth factor; NS, not significant; VEGF, vascular endothelial growth factor.

Results

In vitro *tube formation induced by supernatant of 3T3-L1 cells*
To evaluate whether the adipocyte-derived growth factors promote tube formation of vascular endothelial cells *in vitro*, the supernatant of the 3T3-L1 cells was overlaid on type 1 collagen mixture containing HUVECs (Figure 1). The supernatant of the 3T3-L1 cells treated with dexamethasone, 3-isobutyl-1-methyxanthine and insulin dramatically induced tube formation compared with control (9.03-fold increase). The supernatant conditioned with pioglitazone after induction of adipocyte differentiation further promoted the tube formation compared with control (12.36-fold increase). Growth medium with 10 μM pioglitazone did not induce tube formation. These results suggested that 3T3-L1 cells secreted some tube-forming factors, and these factors might be increased with differentiation of the adipocytes.

Hepatocyte growth factor and/or vascular endothelial growth factor mRNA expression in 3T3-L1 cells
To identify the tube-forming factor(s) secreted by 3T3-L1 cells, HGF and VEGF mRNA in the cells were studied as candidates (Figure 2). Expression of HGF and VEGF mRNA in 3T3-L1 cells was demonstrated by RT-PCR. Strong HGF mRNA expression and VEGF mRNA expression in 3T3-L1 cells were detected by RT-PCR. Both HGF and VEGF mRNA expression in 3T3-L1 cells were suppressed by treatment with siRNAs for HGF and VEGF, respectively. These results indicated that 3T3-L1 cells expressed growth factors, notably HGF and VEGF. Furthermore, the data confirmed that siRNA for HGF and VEGF effectively interfered with the mRNA expression.

Hepatocyte growth factor and/or vascular endothelial growth factor protein expression in 3T3-L1 cells
Expression of HGF and VEGF protein in 3T3-L1 cells was demonstrated by Western blot analysis (Figure 3). Both HGF and VEGF protein expressions in 3T3-L1 cells were detected by RT-PCR and they were suppressed by treatment with siRNAs for HGF and VEGF, respectively, as well as those mRNA expressions detected by RT-PCR.

Tube formation by supernatant of 3T3-L1 cells treated with small interfering RNA
While the data confirmed that 3T3-L1 cells express HGF and VEGF, it remains unclear whether HGF and VEGF are actually potent tube-forming factor secreted from adipocytes. To identify the tube formation-inducing factor, we studied whether the supernatant of 3T3-L1 cells treated with siRNAs for HGF or VEGF suppresses the tube formation *in vitro* (Figure 4). Addition of control siRNA (100 nM) did not suppress the increase in tube formation induced by the supernatant of 3T3-L1 cells. The supernatant of adipocytes treated with siRNA for VEGF also did not suppress the tube formation compared with the addition of control siRNA. The supernatant of adipocytes treated with 50 or 100 nM of siRNA for HGF suppressed the tube formation compared with the addition of control siRNA. The siRNA for HGF (100 nM) suppressed the *in vitro* tube formation by 61% compared with the control siRNA.

Hepatocyte growth factor and/or vascular growth factor mRNA expression in 3T3-L1 cells conditioned with pioglitazone
Figure 1 shows that the supernatant of differentiated 3T3-L1 cells conditioned with pioglitazone further promoted the

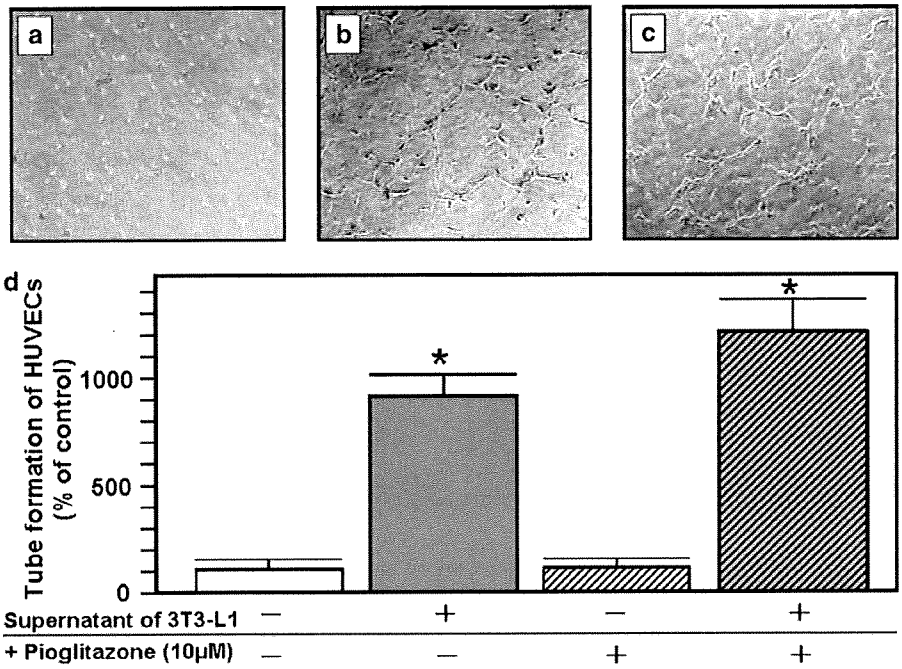

Figure 1 *In vitro* tube formation stimulated by supernatant of 3T3-L1 cells. After 1.0 ml of the supernatant of 3T3-L1 cells was treated by dexamethasone, 3-isobutyl-1-methyxanthine and insulin or medium was added into the type 1 collagen mixture containing human umbilical vascular endothelial cells (HUVECs), and phase-contrast photomicrographs (× 100 magnification) were recorded on a digital camera. (**a**) Control: growth medium. (**b**) Supernatant of 3T3-L1 cells. (**c**) Supernatant of 3T3-L1 cells treated with 10 μM of pioglitazone. (**d**) Quantitative analysis of network structures. Total length of the network structures was measured and total length per field was calculated and expressed as a ratio to control. Values are expressed in mean ± s.d. *$P<0.001$ compared with control.

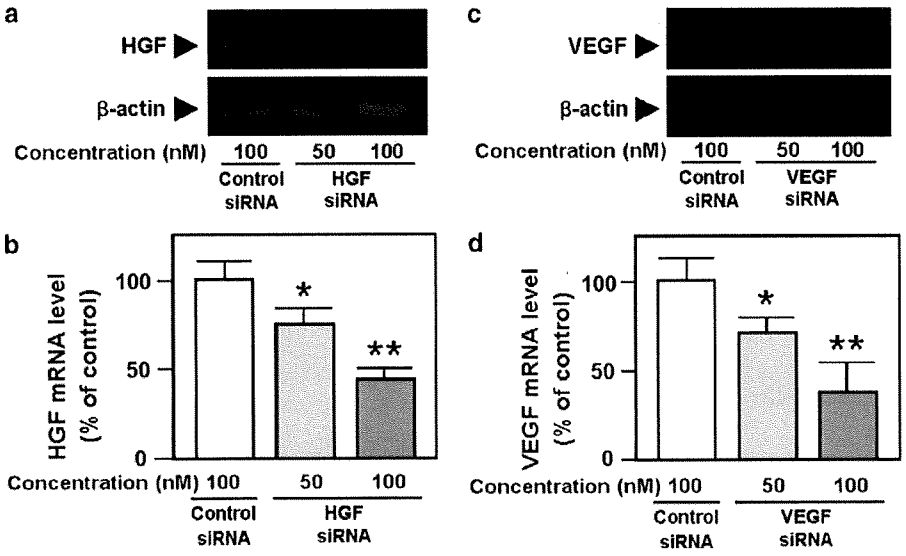

Figure 2 Hepatocytic growth factor (HGF) and vascular endothelial growth factor (VEGF) mRNA expression in 3T3-L1 cells. Hepatocytic growth factor and VEGF mRNA in 3T3-L1 cells treated with dexamethasone, 3-isobutyl-1-methyxanthine and insulin was detected by reverse transcription-polymerase chain reaction (RT-PCR). (**a**) Hepatocytic growth factor mRNA expression in 3T3-L1 cells detected by RT-PCR using a pair of gene-specific PCR primers designed for HGF. Far left lane: HGF mRNA expression in the cells treated with 100 nM of control small interfering RNA (siRNA); lanes 2 and 3 from the left: HGF mRNA expression in the cells treated with 50 and 100 nM of HGF siRNA, respectively. (**b**) Quantitative analysis of HGF mRNA expression in the cells treated with control siRNA or HGF siRNA. (**c**) Vascular endothelial growth factor mRNA expression in the cells detected by RT-PCR using a pair of gene-specific PCR primers designed for VEGF. Far left lane: VEGF mRNA expression in the cells treated with 100 nM of control siRNA; lanes 2 and 3 from the left: VEGF mRNA expression in the cells treated with 50 and 100 nM of VEGF siRNA, respectively. (**d**) Quantitative analysis of VEGF mRNA expression in the cells treated with control siRNA or VEGF siRNA. The products were quantified by digital scanning and Scion Image software. Values are expressed in mean ± s.d. *$P<0.05$, and **$P<0.005$ compared with control.

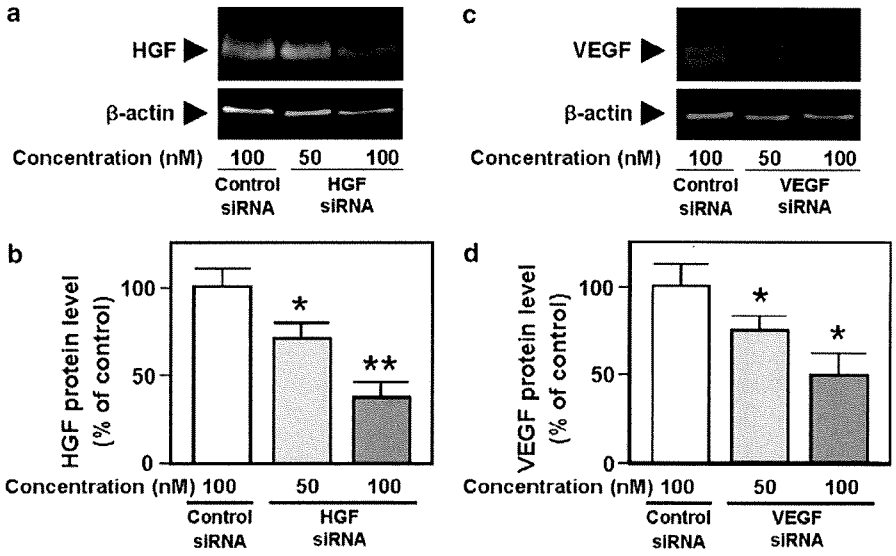

Figure 3 Hepatocytic growth factor (HGF) and vascular endothelial growth factor (VEGF) protein expression in 3T3-L1 cells. Hepatocytic growth factor or VEGF protein in 3T3-L1 cells treated by dexamethasone, 3-isobutyl-1-methyxanthine and insulin was detected by Western blot analysis. (**a**) Hepatocytic growth factor protein expression in the cells was detected by anti-murine HGF antibody. Far left lane: HGF protein expression in the cells treated with 100 nM of control small interfering RNA (siRNA); lanes 2 and 3 from the left: HGF protein expression in the cells treated with 50 and 100 nM of HGF siRNA, respectively. (**b**) Quantitative analysis of HGF protein expression in the cells treated with control siRNA or HGF siRNA. (**c**) Vascular endothelial growth factor protein expression in the cells was detected by Western blot analysis using anti-murine VEGF antibody. Far left lane: VEGF protein expression in the cells treated with 100 nM of control siRNA; lanes 2 and 3 from the left: VEGF protein expression in the cells treated with 50 and 100 nM of VEGF siRNA, respectively. (**d**) Quantitative analysis of VEGF protein expression in the cells treated with control siRNA or VEGF siRNA. The products were quantified by digital scanning and Scion Image software. Values are expressed in mean ± s.d. $^{*}P<0.05$ and $^{**}P<0.005$ compared with control.

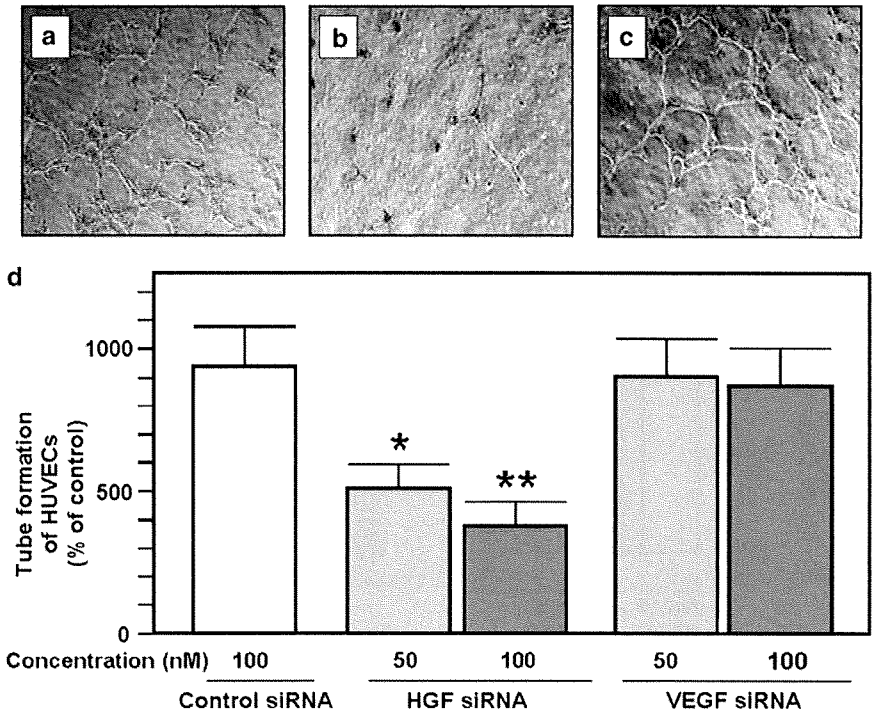

Figure 4 Effect of the supernatants of differentiated 3T3-L1 cells treated with small interfering RNAs (siRNAs) for hepatocytic growth factor (HGF) and vascular endothelial growth factor (VEGF) on tube formation *in vitro*. Phase-contrast photomicrographs (× 100 magnification) were recorded on a digital camera. (**a**) Supernatant of 3T3-L1 cells treated with 100 nM of control siRNA was added onto type 1 collagen mixture containing human umbilical vascular endothelial cells (HUVECs). (**b**) Supernatant of the cells treated with 100 nM of HGF siRNA was added. (**c**) Supernatant of the cells treated with 100 nM of VEGF siRNA was added. (**d**) Quantitative analysis of tube formation of HUVECs overlaid with supernatant of 3T3-L1 cells treated with control siRNA, HGF siRNA or VEGF siRNA. Values are expressed as mean ± s.d. $^{*}P<0.05$ and $^{**}P<0.005$ compared with control.

tube formation compared with the supernatant of the cells conditioned without pioglitazone. To clarify the tube-forming factor(s) in 3T3-L1 cells enhanced by addition of pioglitazone, expressions of HGF and VEGF mRNA in the cells that were conditioned with pioglitazone were demonstrated by RT-PCR (Figure 5). Reverse transcription-polymerase chain reaction showed that HGF mRNA expression in 3T3-L1 cells conditioned with pioglitazone was enhanced by 33% compared with that in the cells conditioned without pioglitazone. On the other hand, VEGF mRNA expression in the cells was not enhanced when the cells were conditioned with pioglitazone.

Effects of pioglitazone on plasma hepatocyte growth factor and vascular endothelial growth factor levels in patients with type 2 diabetes mellitus

As shown in Figure 6, the changes of BMI and HbA_{1C} in the pioglitazone group were significantly higher than those in the control group. The change of plasma HGF levels in the pioglitazone group was also significantly higher than those in the control group ($P=0.0479$), whereas no significant differences in the change of plasma VEGF levels were observed between the pioglitazone group and the control group. In the pioglitazone group, the change of plasma HGF levels showed a positive correlation with the change of BMI

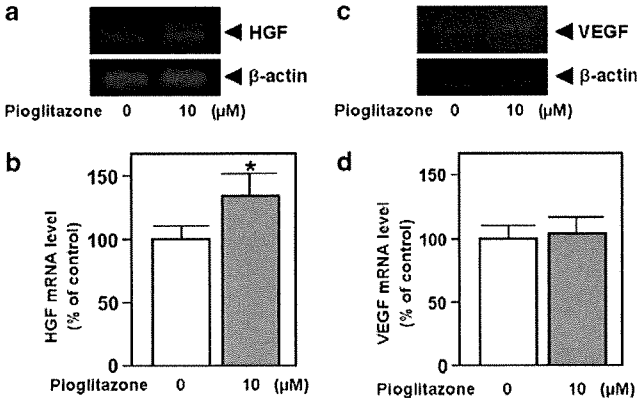

Figure 5 Hepatocytic growth factor (HGF) and vascular endothelial growth factor (VEGF) mRNA expression in 3T3-L1 cells treated with pioglitazone. The cells treated with 10 μM of pioglitazone after induction of differentiation. Hepatocytic growth factor and VEGF mRNA in the cells was detected by reverse transcription-polymerase chain reaction (RT-PCR). (**a**) HGF mRNA expression in the cells detected by RT-PCR using a pair of gene-specific PCR primers designed for HGF. Far left lane: HGF mRNA expression in the cells (control); lane 2 from the left: HGF mRNA expression of cells treated with 10 μM of pioglitazone. (**b**) Quantitative analysis of HGF mRNA expression in the cells treated with 10 μM of pioglitazone. (**c**) Vascular endothelial growth factor mRNA expression in the cells detected by RT-PCR using a pair of gene-specific PCR primers designed for VEGF. Far left lane: VEGF mRNA expression in the cells (control); lane 2 from the left: VEGF mRNA expression of cells treated with 10 μM of pioglitazone. (**d**) Quantitative analysis of VEGF mRNA expression in the cells treated with 10 μM of pioglitazone. The products were quantified by digital scanning and Scion Image software. Values are expressed in mean $\pm$ s.d. *$P<0.05$ compared with control.

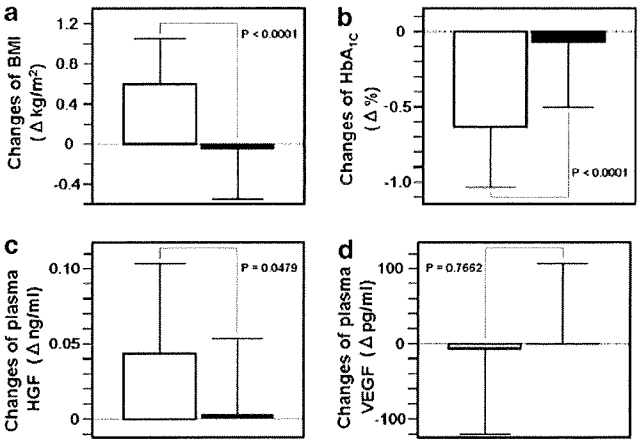

Figure 6 The changes of body mass index (BMI) (**a**), HbA_{1C} (**b**), plasma HGF (**c**) and plasma VEGF (**d**) in 50 subjects randomized to receive either 30 mg pioglitazone orally once daily ($n=25$, white bars) or control ($n=25$, black bars) for 12 weeks. The data are shown as mean $\pm$ s.d.

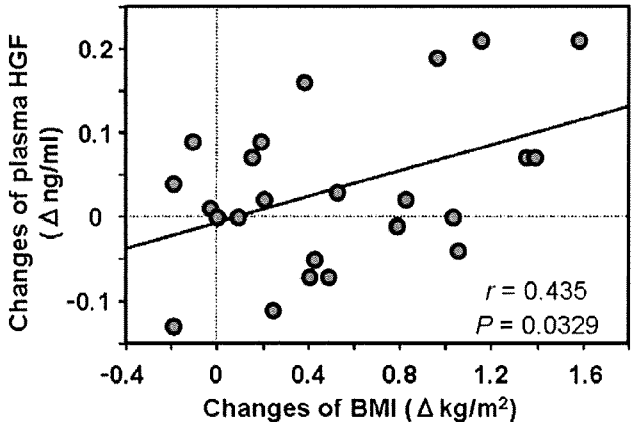

Figure 7 Correlation between change of body mass index (BMI) and change of plasma hepatocytic growth factor (plasma HGF). Increase in BMI and increase in plasma HGF show a significant correlation ($r=0.435$, $P=0.0329$).

($r=0.435$, $P=0.0329$) (Figure 7). However, the change of plasma HGF levels did not show correlation with asparate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, fasting blood glucose, HbA_{1C} and plasma lipid levels in the group (data not shown).

Discussion

In this study, the supernatant of 3T3-L1 cells, which were induced of differentiation by dexamethasone, 3-isobutyl-1-

methyxanthine and insulin, strongly promoted tube formation of HUVECs cultured in type 1 collagen gel. Reverse transcription-polymerase chain reaction showed that 3T3-L1 cells expressed both HGF and VEGF mRNA. Western blot analysis also demonstrated both HGF and VEGF protein. The supernatant of 3T3-L1 cells treated with siRNA for HGF suppressed the tube formation of HUVECs compared with control siRNA, whereas the supernatant of the cells treated with siRNA for VEGF did not suppress. These results indicate that VEGF may also contribute in part to promote tube formation by adipocytes. However, our results suggest that HGF is the major angiogenetic factor secreted by adipocytes, because the supernatant of differentiated 3T3-L1 cells treated with siRNA for HGF failed to promote tube formation. Although HGF has been reported to be secreted by mature adipocytes,[18] no report has shown that the HGF secreted by mature adipocytes is associated with angiogenesis. Our report provides the first evidence that HGF secreted by adipocytes promotes tube formation of HUVECs.

Several studies have reported that adipocytes secrete angiogenetic factors. Claffey *et al.*[20] reported that VEGF expression was regulated by differentiation of 3T3-L1 cells. *In vivo*, Silha *et al.*[21] reported that serum concentrations of VEGF were significantly elevated in human overweight and obese subjects. Matrix metalloproteases are produced by human adipose tissue,[22] and are expressed in adipose tissue during obesity and modulate adipocyte differentiation.[23-] Dobson *et al.*[24] reported that 1-butyryl-glycerol was secreted by adipocytes, and that synthetic 1-butyryl-glycerol stimulated angiogenesis *in vivo* and *in vitro*. Leptin has also been reported to enhance tube formation *in vitro*.[25] A limitation of this study is that the findings are limited to demonstrating only about the HGF and the VEGF. In future studies, we should confirm whether those angiogenetic factor expressions in 3T3-L1 cells are shown and whether the supernatant of the cells treated with siRNAs for those factors suppress the tube formation of HUVECs. Also, the abnormalities of the VEGF receptor signaling pathway of the HUVECs might present under the conditions of this study. At least, the supernatant of 3T3-L1 cells did not suppress expressions of VEGF receptor 1 and 2 protein in HUVECs by Western blot analysis using phospho-specific antibodies to the VEGF receptor 1 and 2 compared with DMEM supplemented with 10% FCS (data not shown).

Pioglitazone is one of the thiazolidinediones that activate the peroxisome proliferator-activated receptor γ (PPARγ), which is expressed primarily in adipose tissue. Forced expression of PPARγ in the fibroblasts makes them differentiate into adipocytes.[26] The supernatant of the cells, differentiation in which was enhanced by addition of pioglitazone, potently promoted the tube formation of HUVECs. The enhancement of HGF mRNA expression in 3T3-L1 cells conditioned with pioglitazone was demonstrated by RT-PCR. Furthermore, plasma HGF levels increased in patients with type 2 diabetes mellitus after treatment with pioglitazone, and the change of plasma HGF levels showed a positive correlation with the change of BMI in the pioglitazone group. These results supported that the differentiation of adipocytes might enhance the HGF expression *in vitro* and *in vivo*. However, currently, the mechanism by which pioglitazone activates the HGF expression in adipocytes is not fully understood.

Hepatocyte growth factor was purified from rat platelets and characterized as a growth factor for mature parenchymal hepatocytes.[27] Several recent studies have reported that HGF exhibits angiogenetic actions. Morishita *et al.*[28] reported that treatment with human recombinant HGF improved hindlimb ischemia in rabbits. Human recombinant HGF has been applied in therapeutic angiogenesis, as a cytokine supplement for patients with arteriosclerosis obliterans.[29] Hepatocyte growth factor is also seen as a significant cardioprotective factor because of its potent angiogenetic properties[14,30] and because HGF can reduce restenosis after balloon injury by enhancing re-endothelialization.[31] On the other hand, plasma HGF levels are elevated in malignancies,[32] patients with atherosclerosis and proliferative diabetic retinopathy.[33,34] The findings indicate that obesity might affect processes such as tumor angiogenesis or proliferative diabetic retinopathy mediated by HGF. Future studies should investigate whether specific inhibitors or modulators of HGF production and signaling pathways might indeed serve as targets for vascular therapies.

In summary, the supernatant of differentiated 3T3-L1 cells strongly promotes tube formation of HUVECs cultured in type 1 collagen gel, and HGF secreted from 3T3-L1 cells may be the major factor regulating the tube formation. The supernatant supplemented with pioglitazone further enhances the tube formation. Pioglitazone enhanced HGF mRNA expression in 3T3-L1 cells and increased plasma HGF levels in patients with type 2 diabetes mellitus. These results suggest that agents that enhance the differentiation of adipocytes may promote tube formation of HUVECs mediated by HGF secreted by adipocytes.

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