Effect of vascular endothelial growth factor receptor 2 antagonism on adiposity in obese mice

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Abstract

Development and maintenance of fat depots require angiogenesis, in which vascular endothelial growth factor (VEGF) and its receptors play a crucial role. We have evaluated the effect of blocking VEGF receptor 2 (VEGF-R2) with a MAB (DC101) on adipose tissue of mice with established obesity. Therefore, obese male wild-type C57B1/6 mice were treated with i.p. injection of DC101 (40 mg/kg body weight, twice weekly during 13 weeks) or of the control antibody 1C8. Treatment with DC101 resulted in a slightly lower body weight but had no effect on subcutaneous (SC) or gonadal (GON) white adipose tissue mass, as monitored by MRI. Histochemical analysis of isolated SC and GON fat pads did not reveal significant effects of DC101 treatment on adipocyte or blood vessel size or density. Plasma levels of the liver enzymes aspartate aminotransferase and alanine aminotransferase as well as liver triglyceride levels were significantly decreased following DC101 treatment. Plasma glucose levels were markedly lower upon DC101 treatment, whereas insulin and adiponectin levels were not affected. Furthermore, Akt phosphorylation in adipose tissues was not affected. Thus, *in vivo* VEGF-R2 blockade in mice with established nutritionally induced obesity did not significantly affect insulin signaling in adipose tissue or adiposity.

Key Words

- ▶ adipose tissue
- ▶ obesity
- ▶ angiogenesis
- ▶ VEGF
- ▶ VEGF receptor
- ▶ insulin

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Introduction

Expansion of adipose tissue is linked to the development of its vasculature. Indeed, adipogenesis is tightly associated with angiogenesis, as shown by the findings that adipose tissue explants trigger blood vessel formation (Castellot *et al.* 1982), whereas in turn adipose tissue endothelial cells promote preadipocyte differentiation (Varzaneh *et al.* 1994). Furthermore, adipose tissue growth in mice can be impaired with angiogenesis inhibitors (Rupnick *et al.* 2002).

Many pro- and anti-angiogenic components have been identified in adipose tissue. Vascular endothelial growth factor (VEGF) family members are major pro-angiogenic factors that stimulate proliferation and migration of

endothelial cells (Carmeliet *et al.* 1996). They bind to transmembrane tyrosine kinase receptors (VEGF-R). A previous study has revealed expression of different isoforms of VEGF-A, VEGF-B, and VEGF-C and the receptors VEGF-R1, VEGF-R2, and VEGF-R3 in subcutaneous (SC) and gonadal (GON) adipose tissues of mice (Voros *et al.* 2005). Furthermore, blockade of VEGF-R2 but not VEGF-R1 in mice was shown to limit diet-induced fat tissue expansion and suggested a role in early phase fat tissue development (Tam *et al.* 2009). In addition, several studies have suggested a link between the VEGF/VEGF-R2 pathway and insulin sensitivity and glucose tolerance (Elias *et al.* 2012, Wada *et al.* 2010).

Figure 1

Effect of VEGF-R2 antagonism with DC101 on the evolution of body weight of obese mice. Data are means $\pm$ s.e.m. of nine animals treated with DC101 (filled square) or of ten animals treated with 1C8 (filled circle).

In this study, we have investigated whether DC101, a VEGF-R2 blocking MAB (Bocci *et al.* 2004), has the potential to affect glucose metabolism or adipose tissue-related angiogenesis and adiposity in mice with established obesity (which would be more clinically relevant).

Materials and methods

Male wild-type C57Bl/6 mice were generated in the KU Leuven animal facility. Five-week-old mice were kept in individual microisolation cages on a 12 h light:12 h darkness cycle and fed a high-fat diet (HFD, Harlan Teklad, TD88137, Zeist, Netherlands; 42% kcal as fat, caloric value 20.1 kJ/g) for 28 weeks. Water was always available *ad libitum*. Age- and weight-matched mice were then treated with i.p. injection of the VEGF-R2 blocking MAB DC101 or of the control MAB 1C8 (40 mg/kg body weight, twice weekly during 13 weeks). DC101, a rat MAB against mouse VEGF-R2, and 1C8, a mouse MAB against human tissue type plasminogen activator (t-PA) not cross-reacting with mouse t-PA, were kind gifts of ThromboGenics (Leuven, Belgium).

Food intake was measured daily and body weight and temperature at weekly intervals. Physical activity at night was monitored in cages equipped with a turning wheel linked to a computer to register full turns/12 h (1900–0700 h). MRI to determine body and fat volumes was performed as described elsewhere (Hemmerlyck *et al.* 2010).

At the end of the experiments, after overnight fasting, mice were killed by i.p. injection of 60 mg/kg

Nembutal (Abbott Laboratories). Intra-abdominal (GON) and inguinal SC fat pads were removed and weighed; portions were snap-frozen in liquid nitrogen and fixed in 1% formaldehyde to prepare paraffin sections (10 μ m) for histology. Other organs including kidneys, lungs, spleen, pancreas, liver, heart, and brain were also removed and weighed. Liver enzymes were determined with routine clinical assays. Triglyceride levels in liver tissue extracts were measured using the Triglycerides FSkit (DiaSys Diagnostic Systems, Holzheim, Germany). Plasma glucose levels were measured after overnight fasting using Glucocard strips (Menarini Diagnostics, Firenze, Italy); insulin (Mercodia, Uppsala, Sweden) and adiponectin (R&D Europe, Lille, France) levels were determined using commercial ELISAs.

TaqMan gene expression assays (Life Technologies) were used to analyze the mRNA levels in SC and GON adipose tissues of adiponectin (Mm 00456425_m1) and *Glut4* (*Slc2a4*; Mm 00436615_m1), with β -actin (Mm 01205647_g1) as housekeeping gene, as described elsewhere (Scroyen *et al.* 2012). For peroxisome proliferator-activated receptor γ (PPAR γ), the following primer and probe set was used: fw, ctgtcgggttcagaagtgct; rev, atctccgccaacagcttctc; probe: cccaaacctgatggcattgtagaca.

To monitor insulin signaling, analysis of Akt and Phospho (P) Akt in extracts of SC or GON adipose tissues was performed using the primary antibodies Akt or P-Akt (9272 or 9271, Cell Signaling Technology, Danvers, MA, USA), as described (Scroyen *et al.* 2012). Data are normalized to β -actin as internal control and expressed as the ratio P-Akt/Akt.

Table 1 Effect of VEGF-R2 antagonism with DC101 on body and organ weight of obese mice. Data are means $\pm$ s.e.m. of *n* experiments

	DC101 (n=9)	1C8 (n=10)
Body weight start (g)	52 $\pm$ 0.90	51 $\pm$ 0.80
Body weight end (g)	50 $\pm$ 1.2*	54 $\pm$ 1.2
SC fat (mg)	2456 $\pm$ 139	2579 $\pm$ 101
GON fat (mg)	2311 $\pm$ 91	2408 $\pm$ 157
Liver (mg)	2751 $\pm$ 267 [†]	4155 $\pm$ 142
Spleen (mg)	307 $\pm$ 19 [†]	164 $\pm$ 8.3
Kidney (mg)	293 $\pm$ 8.1	263 $\pm$ 6.2
Pancreas (mg)	374 $\pm$ 11	398 $\pm$ 11
Lungs (mg)	251 $\pm$ 19	209 $\pm$ 6.2
Heart (mg)	216 $\pm$ 9.0	185 $\pm$ 3.4
Brain (mg)	67 $\pm$ 2.8	67 $\pm$ 2.5

* $P < 0.05$ and [†] $P \leq 0.001$ vs control.

Table 2 Effect of VEGF-R2 antagonism with DC101 on body and fat composition of obese mice, as monitored by MRI. Data are means $\pm$ S.E.M. of four determinations in each group

	DC101	1C8
Total body volume (ml)	83 $\pm$ 1.9	81 $\pm$ 1.7
Abdomen volume (ml)	27 $\pm$ 0.96	25 $\pm$ 2.7
Total fat volume (ml)	41 $\pm$ 2.2	36 $\pm$ 1.6
Total body fat (%)	49 $\pm$ 1.7	44 $\pm$ 1.2
Visceral fat volume (ml)	12 $\pm$ 0.87	11 $\pm$ 0.05
SC fat volume (ml)	28 $\pm$ 2.9	27 $\pm$ 0.76

The size and density of adipocytes or blood vessels in the adipose tissues were determined by staining (not shown) with hematoxylin/eosin or with the *Bandeiraea simplicifolia* lectin (Sigma–Aldrich), a pan marker for rodent endothelial cells (Laitinen 1987), followed by signal amplification with the Tyramide Signal Amplification Cyanine System (Perkin Elmer, Boston, MA, USA). Images were quantitatively analyzed by computer-assisted image analysis, as described (Van Hul *et al.* 2012a).

All animal experiments were approved by the KU Leuven ethics committee and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (1996).

Statistical significance between groups was evaluated by nonparametric Mann–Whitney *U* test. Values of $P < 0.05$ are considered statistically significant.

Results

After 13 weeks of administration of the anti-VEGF-R2 MAB DC101 ($n=9$) or of the control MAB 1C8 ($n=10$) to obese age- and weight-adjusted mice, body weight was slightly lower in the DC101 group (50 ± 1.2 vs 54 ± 1.2 g; $P < 0.05$). Evolution of the body weight over time is shown in Fig. 1. The slight drops in body weight at weeks 2 and 6 are due to overnight fasting before blood sampling for glucose measurements. Food intake (3.6 ± 0.1 vs 3.7 ± 0.1 g/day), body temperature (35.0 ± 0.22 vs 35.3 ± 0.26 °C), and physical activity (1170 ± 210 vs 1580 ± 275 turns/12 h) were comparable for DC101 and 1C8 treatment respectively. Analysis of whole blood cell counts for mice treated with DC101 or 1C8 revealed a similar contribution of lymphocytes (54 ± 4.3 vs $50 \pm 2.4\%$), neutrophils (29 ± 3.2 vs $32 \pm 2.6\%$), and monocytes ($10 \pm 1.2\%$ both). Also platelet counts were not significantly different for DC101 and 1C8 treatment (1010 ± 143 vs $1290 \pm 78 \times 10^3/\mu\text{l}$).

The weight of isolated SC and GON adipose tissues was also comparable for both groups, as was the weight of

other main organs, with the exception of lower liver and higher spleen weight in the mice treated with DC101 (Table 1). Noninvasive MRI at the end of the experiment did not reveal significant differences in body or fat (total, visceral, or SC) volumes (Table 2). Histochemical analysis of SC and GON adipose tissues did not reveal significant effects of DC101 treatment on adipocyte or blood vessel size or density (Fig. 2 and Table 3).

Plasma total cholesterol and HDL-cholesterol levels at the end of the experiment were significantly lower for DC101 versus 1C8 treatment, whereas triglyceride levels were higher (Table 4). During treatment with 1C8, glucose levels significantly increased whereas they decreased during DC101 treatment, resulting in lower levels at the end of DC101 treatment. Insulin levels were not affected by either treatment, whereas adiponectin levels increased to the same extent during both treatments.

Figure 2

Histological staining with hematoxylin/eosin (left panels) or *Bandeiraea simplicifolia* lectin (right panels) of subcutaneous (A, B, C and D) and gonadal (E, F, G and H) adipose tissues of mice treated with DC101 (A, B, E and F) or 1C8 (C, D, G and H). The scale bars correspond to 100 μm .

Table 3 Effect of VEGF-R2 antagonism with DC101 on adipocyte and blood vessel size and density of obese mice. Data are means $\pm$ S.E.M. of eight to ten determinations

	DC101	1C8
Adipocyte size (μm^2)		
SC fat	4064 $\pm$ 92	3648 $\pm$ 131
GON fat	5621 $\pm$ 377	5842 $\pm$ 291
Adipocyte density ($\times 10^{-6}/\mu\text{m}^2$)		
SC fat	250 $\pm$ 5.2	283 $\pm$ 10
GON fat	185 $\pm$ 12	172 $\pm$ 12
Blood vessel size (μm^2)		
SC fat	43 $\pm$ 2.3	40 $\pm$ 1.8
GON fat	42 $\pm$ 1.8	38 $\pm$ 2.7
Blood vessel density ($\times 10^{-6}/\mu\text{m}^2$)		
SC fat	310 $\pm$ 12	342 $\pm$ 13
GON fat	195 $\pm$ 15	210 $\pm$ 19

Determination of liver enzymes revealed a significant decrease during DC101 treatment of aspartate aminotransferase (AST) and alanine aminotransferase (ALT); during 1C8 treatment, such differences were not observed, resulting in lower AST and ALT levels at the end of DC101 treatment. Levels of alkaline phosphatases were not affected by DC101 treatment but decreased during 1C8 treatment. Plasma triglyceride levels decreased upon administration of DC101 and 1C8 but were higher at the end of DC101 treatment (Table 4). In liver tissue extracts, triglyceride levels were significantly lower for DC101-treated mice (29 ± 5.7 vs 70 ± 3.3 mg/g tissue for 1C8 treatment; $P < 0.0001$). Total protein content in the liver extracts was comparable for DC101 and 1C8 treatment (13 ± 0.42 vs 13 ± 0.55 mg/g tissue).

In SC or GON adipose tissues, no significant differences were observed in the expression of *Ppar γ* (*Pparg*),

adiponectin, or *Glut4* between treatment with DC101 or 1C8 (Fig. 3A, B and C). Furthermore, the ratio of P-Akt/Akt was similar in SC and GON adipose tissues for both treatments (Fig. 3D).

Discussion

Inhibition of angiogenesis may be a potential strategy to affect adipose tissue development (Rupnick *et al.* 2002). Furthermore, the VEGF/VEGF-R system may play a role in insulin resistance (Elias *et al.* 2012). In this study, we have evaluated whether VEGF-R2 blockade affects adipose tissue-related angiogenesis and fat mass or insulin signaling in mice with established nutritionally induced obesity. Using the same model, we have previously shown that matrix metalloproteinase inhibitors, such as Tolyllsam and ABT-518, have the potential to impair adipose tissue angiogenesis and development (Van Hul *et al.* 2012a,b). In this study, we have used the rat MAB DC101 that was previously shown to efficiently inhibit tumor angiogenesis in mouse models, using similar administration schemes for shorter duration (Prewett *et al.* 1999, Kadambi *et al.* 2001, Fischer *et al.* 2007). In addition, at a dose of 25 mg/kg i.p. given every 2 days, DC101 significantly impaired postnatal retinal or systemic vascular development (Van de Veire *et al.* 2010). In our study, DC101 at a dose of 40 mg/kg i.p. twice weekly did not, however, affect adipose tissue-related angiogenesis nor did fat mass in obese mice. A limitation of our study may be that we have not directly demonstrated *in vivo* blockade of VEGF-R2. The total body weight of the mice in our study was somewhat reduced compared with controls;

Table 4 Effect of VEGF-R2 antagonism with DC101 on metabolic parameters and liver enzymes in plasma of obese mice. Data are means $\pm$ S.E.M. of nine to ten determinations

	DC101		1C8	
	Start	End	Start	End
Glucose (mg/dl)	127 $\pm$ 12	112 $\pm$ 15 [†]	129 $\pm$ 7	169 $\pm$ 7.3
Insulin (ng/ml)	0.81 $\pm$ 0.19	1.08 $\pm$ 0.14	0.80 $\pm$ 0.23	0.95 $\pm$ 0.07
Adiponectin ($\mu\text{g}/\text{ml}$)	7.6 $\pm$ 1.1	12 $\pm$ 0.82	7.3 $\pm$ 0.48	12 $\pm$ 0.98
Total cholesterol (mg/dl)	440 $\pm$ 17	187 $\pm$ 19 ^{†,}	478 $\pm$ 19	320 $\pm$ 11
HDL-cholesterol (mg/dl)	181 $\pm$ 26	133 $\pm$ 13 [†]	252 $\pm$ 9	234 $\pm$ 5
LDL-cholesterol (mg/dl)	239 $\pm$ 19	40 $\pm$ 9	205 $\pm$ 14	76 $\pm$ 8
Triglycerides (mg/dl)	100 $\pm$ 9	76 $\pm$ 9 ^{*,§}	108 $\pm$ 7	51 $\pm$ 4
Alkaline phosphatases (U/l)	186 $\pm$ 10	133 $\pm$ 25	193 $\pm$ 12	116 $\pm$ 11
AST (U/l)	442 $\pm$ 27	279 $\pm$ 25 [§]	478 $\pm$ 36	438 $\pm$ 59
ALT (U/l)	513 $\pm$ 45	146 $\pm$ 24 ^{†,}	510 $\pm$ 39	360 $\pm$ 48

AST, aspartate aminotransferase; ALT, alanine aminotransferase. ^{*} $P < 0.05$, [†] $P < 0.005$, and [‡] $P < 0.0005$ vs end of 1C8 treatment. [§] $P < 0.05$ and ^{||} $P < 0.0005$ vs start of the same treatment.

Figure 3

Monitoring of insulin signaling pathways in SC or GON adipose tissues of mice treated with 1C8 (open bars) or DC101 (filled bars). Relative gene expression as determined by quantitative RT-PCR is shown for *Pparγ* (A), adiponectin (B), and *Glut4* (C). Data are means $\pm$ S.E.M. of seven or eight determinations. (D) Shows the ratio of P-Akt/Akt as determined by western blotting of protein extracts prepared from SC or GON adipose tissues. Data are means $\pm$ S.E.M. of four determinations.

Sun *et al.* (2012) also recently showed that VEGF-A/VEGF-R2 blockade in genetic obese *ob/ob* mice leads to reduced body weight gain. The similar distribution of white blood cells suggests that the rat antibody DC101 and the mouse antibody 1C8 did not elicit a markedly different immune response.

In a previous study, treatment of C57Bl/6 mice with DC101 (40 mg/kg every 3 days) had no effect on body weight during the first 5–6 weeks of HFD feeding, but afterwards (weeks 6–13), the rate of weight gain decreased significantly compared with the HFD controls (Tam *et al.* 2009). This was associated with a significantly decreased food intake in the DC101 group that was apparently not observed in the first 5–6 weeks. We did not observe a difference in food intake over the 13-week experimental period, and the evolution of body weight was similar to that in the control group. The main differences with our study are the start age (12 vs 33 weeks in our study) and the start weight (lean vs obese).

The data of Tam *et al.* (2009) thus suggest that blocking VEGF-R2-mediated angiogenesis may be effective during stages of active fat expansion. Furthermore, studies with low-molecular-weight tyrosine kinase inhibitors, such as SU5416, which selectively inhibit VEGF-R2, showed accumulation of active compound in adipose

tissue, and efficient inhibition of angiogenesis in a rat aortic ring model (Rasmussen *et al.* 2011) as well as in a mouse corneal neovascularization model (Keskin *et al.* 2012). The VEGF/VEGF-R2 pathway may also be important for angiogenesis during *de novo* adipose tissue formation from preadipocytes (Fukumura *et al.* 2003). By contrast, our study in obese mice indicates that anti-VEGF-R2 treatment did not affect established blood vessels and did not induce regression of fat mass.

DC101 treatment was associated with reduced liver weight in the obese mice, and with markedly lower levels of the liver enzymes AST and ALT compared with treatment with 1C8. Furthermore, liver triglyceride content was significantly reduced, suggesting reduced liver steatosis. Triglyceride levels indeed were previously reported to be indicative for liver steatosis (Donato & Gomez-Lechon 2012), although this was not histologically confirmed in this study. Furthermore, at the end of the experiment, plasma cholesterol and glucose levels were significantly lower for DC101 treatment compared with 1C8. It is conceivable that VEGF/VEGF-R2 blockade and associated higher circulating VEGF levels in this model result in an improvement in glucose metabolism and in insulin signaling, as previously observed with *ob/ob* mice (Sun *et al.* 2012). Furthermore, it was recently shown that overexpression of VEGF in the mouse was associated with improved whole body insulin sensitivity and glucose tolerance (Elias *et al.* 2012). It was also reported that soluble VEGF-R2 is increased in sera of subjects with metabolic syndrome and insulin resistance (Wada *et al.* 2012). These studies thus support a link between the VEGF/VEGF-R2 pathway and insulin sensitivity. In our study in obese mice, however, we did not observe effects of DC101 treatment on plasma levels of insulin or adiponectin, nor on adipose tissue gene expression of *Pparγ*, adiponectin, or of the insulin-responsive glucose transporter *Glut4*. Furthermore, a similar ratio of P-Akt/Akt in adipose tissues of mice treated with DC101 or 1C8 does not support an effect on insulin signaling in the obese mice.

Thus, whereas the VEGF/VEGF-R2 pathway has been shown to play an important role in angiogenesis during early phases of adipose tissue formation, we did not observe marked effects of VEGF-R2 antagonism on nutritionally induced established obesity. This anti-angiogenic approach thus does not have the potential to affect obese fat mass that developed during prolonged high-fat intake and does not result in improved insulin sensitivity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H R L contributed in the design and analysis of the study and writing of manuscript. I S was involved in analysis of samples and data acquisition.

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