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Effect of matrix metalloproteinase inhibition on adipose tissue development

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Abstract

The effect of Ro 28-2653, a synthetic matrix metalloproteinase (MMP) inhibitor, on adipose tissue development was studied in mice kept on a high fat diet (HFD). Five-week-old male wild-type (C57Bl/6J) mice were fed the HFD (42% kcal as fat, 20.1 kJ/g) and received daily p.o. instillations of inhibitor (30 mg/kg) or vehicle. After 15 weeks of the HFD, the body weight gain was lower in the inhibitor-treated group (7.4 ± 0.88 g versus 10 ± 1.4 g) whereas the weights of the isolated subcutaneous (SC) or gonadal (GON) fat deposits were 10–15% lower. The number of adipocytes in adipose tissues of the inhibitor-treated mice was somewhat higher (10–17%) but their diameter was smaller (about 10%). In situ zymography showed reduced gelatinolytic activity in SC (about 2.7-fold) and GON (1.4-fold) adipose tissue of inhibitor-treated mice, whereas their fibrillar collagen content was higher (1.5- and 4.7-fold, respectively). In both SC and GON adipose tissues of inhibitor-treated mice, MMP-2 (gelatinase A) and MMP-14 (membrane type-1 MMP) were 2- to 3-fold upregulated, whereas MMP-9 (gelatinase B) mRNA levels were not affected. Thus, in this in vivo model partial inhibition of gelatinolytic activity is associated with moderate effects on adipose tissue development and cellularity. Possibly, enhanced MMP expression to some extent counteracts the in vivo effect of the inhibitor in adipose tissue.

Keywords: Adipose tissue; Obesity; Matrix metalloproteinases; Angiogenesis

Development of obesity is associated with extensive modifications in adipose tissue involving adipogenesis, angiogenesis, and extracellular matrix (ECM) remodeling [1]. Matrix metalloproteinases (MMPs) may contribute to tissue remodeling by degradation of ECM and basement membrane components or activation of latent growth factors [2,3]. Several lines of evidence suggest a potential role of MMPs in development of adipose tissue. Conditioned medium of rat adipocytes contains a MMP-2 (gelatinase A) like gelatinolytic activity, that may play a role in their organization into large multicellular clusters [4]. High expression of MMP-2 was reported in adipose tissue of mice with nutritionally induced obesity [5,6] as well as in genetically obese mice [5], and in human adipose tissue [7]. A detailed analysis of the expression of MMP system components revealed upregulation of mRNA levels of MMP-3, -11, -12, -13, -14, and TIMP-1, and downregulation of mRNA levels of MMP-7, -9, -16, -24, and TIMP-4 in adipose tissue of obese mice [8]. Recent studies in mice with nutritionally induced obesity revealed that MMP-3 (stromelysin-1) deficient mice developed more adipose tissue [9], whereas TIMP-1 deficient mice had lower body and adipose tissue weight [10]. Also during in vitro adipogenesis, i.e., differentiation of murine 3T3-F422A preadipocytes, MMP and TIMP expression is modulated [8]. The hypothesis that MMP activity is important in adipose tissue development is further supported by the finding that administration of galardin, a broad-spectrum MMP inhibitor, to mice on a high fat diet resulted in

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significantly lower SC and GON fat pad weights. It is however unclear which specific MMPs were targeted by this inhibitor [11].

In the present study, we have investigated the effect of Ro 28-2653, a synthetic MMP inhibitor with enhanced selectivity for MMP-2, MMP-9, and MMP-14 (membrane type 1- MMP) [12], on adipose tissue development in a murine model of nutritionally induced obesity.

Methods

Obesity model. Five-week-old male C57Bl/6J mice were kept in microisolation cages on a 12 h day/night cycle and fed ad libitum with a high fat diet (HFD, Harlan TD 88137, Zeiss, The Netherlands; 42% kcal as fat, 20.1 kJ/g). One group ($n = 10$) received daily p.o. instillations of the MMP inhibitor Ro 28-2653 (5-biphenyl-4-yl-5-[4-(4-nitrophenyl)-piperazin-1-yl]pyrimidine-2,4,6-trione) (a kind gift from Roche Diagnostics GmbH, Penzberg, Germany) in a suspension of 45% polyethylene glycol 400, 10% *N*,*N*-dimethylformamide in PBS, at a dose of 30 mg/kg per day [12]. A control group ($n = 9$) was treated with vehicle only. The IC_{50} values of Ro 28-2653 for human MMP-2, MMP-9, and MMP-14 are 10, 12, and 10 nM, respectively, as compared to 4310 or 245 nM for MMP-1 (collagenase-1) or MMP-13 (collagenase-3) [12].

For comparison, a group of mice ($n = 10$) was also kept on normal chow (KM-04-k12, Muracon, Carfil; 13% kcal as fat, 10.9 kJ/g). Mice were weighed weekly and after 15 weeks, following overnight fasting, they were anesthetized by intra-peritoneal injection of 60 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL). Blood was drawn from the retro-orbital sinus with addition of trisodium citrate (final concentration 0.01 mol/L) and plasma was stored at −20 °C. Intra-abdominal (gonadal, GON) and inguinal subcutaneous (SC) fat pads were removed and weighed; portions were either snap-frozen in liquid nitrogen or processed for histological analysis. Both unfixed cryosections (12 μm) and paraffin sections (8 μm) were prepared. Several organs including kidneys, lungs, liver, and heart were also removed, weighed, and snap-frozen.

Food uptake was measured for several 24 h periods and expressed as g per 24 h per mouse. Spontaneous physical activity was evaluated by placing mice in a separate cage equipped with a turning wheel linked to a computer to register full turning cycles. Data from a continuous 48 h period were collected per mouse and expressed as number of cycles per night (12 h), as daytime activity was very low. Data were first averaged per mouse for the different periods and are given as means ± SEM for the number of animals studied.

All animal experiments were approved by the Local Ethical Committee and were performed in accordance with the guiding principles of the American Physiological Society and the International Society on Thrombosis and Haemostasis [13].

Histological analysis. Computer-assisted image analysis was used to determine mean adipocyte size on hematoxylin–eosin-stained paraffin sections. Results on 10–12 sections per animal were averaged, and data were expressed as means ± SEM of the number of mice studied. Sirius red staining specific for fibrillar collagens and blood vessel staining using the biotinylated *Bandeiraea simplicifolia* BSI lectin (Sigma) [10,14] were performed and analyzed by computer-assisted image analysis. The Cy3 TSA fluorescence kit (Perkin-Elmer Life Sciences) was used according to manufacturer's instructions. Images were taken using a LSM510 scanning fluorescence microscope (Zeiss) using red fluorescence for the stained area and green (auto)fluorescence to visualize adipocytes.

Gelatinase activity assays. Extracts of adipose tissues were prepared and analyzed by gelatin zymography as described [15]; lysis zones of the substrate were quantified using computer-assisted image analysis and data were expressed in arbitrary units (AU) of lysis per g of tissue. In situ zymography on cryosections of adipose tissue using pig skin gelatin Oregon Green (Molecular Probes) was performed as described [16]. Lysis area was expressed as a percentage of the total section area and given as mean ± SEM. In addition, homogenates of adipose tissues were prepared in 50 mmol/L Tris–HCl buffer, pH 7.3, containing 10 mmol/L $CaCl_2$, and 1 μmol/L $ZnCl_2$. Gelatinolytic or total MMP activity in samples containing equal amounts of total protein (60 μg, determined by BCA assay) was determined using excess of dye-quenched (DQ) gelatin (Molecular Probes) [17] or of a quenched fluorescently labeled substrate (Mca-PLGLDpaAR; Biomol) which is cleaved by most MMPs [18]. Data were expressed as relative fluorescence units (FU) generated per time unit, corrected for controls without sample.

Differentiation of preadipocytes. 3T3-F442A preadipocytes were passaged subconfluently in basal medium (DMEM/Ham's F12 1:1) supplemented with 100 mmol/L pantothenate, 1 mmol/L biotin, 2.5 mmol/L glutamine, 15 mmol/L Hepes, and 10% (v/v) fetal calf serum. To induce differentiation, cells were seeded at 3.6×10^4 cells/cm^2 in 24-well plates and grown to confluency in basal medium (day 0). At day +2, differentiation was induced with basal medium supplemented with ITS (10 mg/L insulin, 5.5 mg/L transferrin, and 5 μg/L selenium), 10 mmol/L dexamethasone, 0.25 mmol/L methylisobutylxanthine, and 1 nmol/L triiodothyronine (T_3). At day +7, the medium was changed for differentiation medium (basal medium supplemented with ITS and T_3) and maintained for 2 weeks, with daily changes [8]. To evaluate the effect of MMP inhibition, pure Ro 28-2653 was dissolved in DMSO and added to a final concentration of 1 μmol/L, whereas control wells were treated with DMSO alone. This was the highest non-toxic concentration, as determined by measuring cell viability by trypan blue exclusion assay and by WST-1 assay (Roche Molecular Biochemicals). At the end of the experiment, the extent of preadipocyte differentiation was evaluated using Oil red O staining and extraction essentially as described [19]. Briefly, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS. Lipids were stained with Oil red O in isopropanol/water for 2 h at 37 °C, washed several times with water, and the cells were finally dried at 100 °C. The retained dye was extracted with 100% isopropanol and the absorbance at 510 nm was determined. To account for differences in cell numbers, total DNA was extracted from the cells and the absorbance at 260 nm was determined; data from the dye extraction method were normalized against this value.

Analysis of mRNA expression. Quantification of the relative expression levels of different genes was performed essentially as described [8]. DNA-free total RNA extractions of SC and GON adipose tissues or cell cultures were performed using the RNEasy kit (Qiagen) according to manufacturer's instructions. RNA concentrations were determined with the RiboGreen RNA quantification kit (Molecular Probes). Aliquoted samples were stored at –80 °C.

The expression levels of different mRNAs were determined using semi-quantitative RT-PCR. The RT step was performed on 10 ng total RNA for 15 min at 70 °C using the GeneAmps Thermostable RNA PCR kit (Applied Biosystems) and the respective reverse primers (Table 1). The subsequent PCR cycles were performed with the respective target specific primers in PTC-200 thermal cycler. The final number of cycles per target was chosen as to not reach 'plateau' levels, with water as negative controls. The PCR products were separated on 10% acrylamide gels and stained with SYBR Green (Molecular Probes). The gels were scanned using the Gel Doc 2000 system and band intensities were determined using Quantity One software (Bio-Rad). Data were normalized to 28S rRNA levels used as internal standard.

Statistical analysis. Data are reported as means ± SEM. Statistical analysis was performed by repeated measures ANOVA (for body weight evolution) or Student's t test with or without Welch correction.

Table 1
Sequence of primers used for RT-PCR analysis (5′–3′)

Target	Sense primer	Antisense primer	Product size	Annealing temperature (°C)
MMP-2	AGATCTTCTTCTTCAAGGACCGGTT	GGCTCCTCAGTGGCTTGGGGTA	225	68
MMP-9	AGGGTCCACCTTGTTCACC	GGCGTGTCTGGAGAT	190	55
MMP-14	GGATACCCAATGCCCATTGGCCA	CCATTGGGCATCCAGAAGAGAGC	221	68
TIMP-1	GGCATCCTCTTGTTGCTATCACTG	GTCATCTTGATCTCATAACGCTGG	174	68
28S rRNA	GTTCACCCACTAATAGGGAACGTGA	GGATTCTGACTTAGAGGCGTTCAGT	212	68

Results

Obesity model

Body weight and adipose tissue composition

After 15 weeks on the HFD, body weight gain, and SC and GON fat pad weights were somewhat, but not significantly, lower in the inhibitor-treated mice as compared to the vehicle-treated mice (Fig. 1 and Table 2). The weights of other organs, including kidney, lung, heart, and liver, were also comparable for inhibitor- and vehicle-treated mice (not shown), indicating that no aspecific toxic effect occurred. In vehicle-treated mice kept on normal chow for 15 weeks, total weight gain (11 ± 0.86 g) was comparable to vehicle-treated mice on HFD, but the SC and GON fat mass was significantly lower (0.20 ± 0.03 and 0.23 ± 0.03 g, respectively). Progressive analysis of the evolution of total body weights over 15 weeks revealed a significant effect of the diet ($p < 0.001$) but not of the inhibitor ($p = 0.83$).

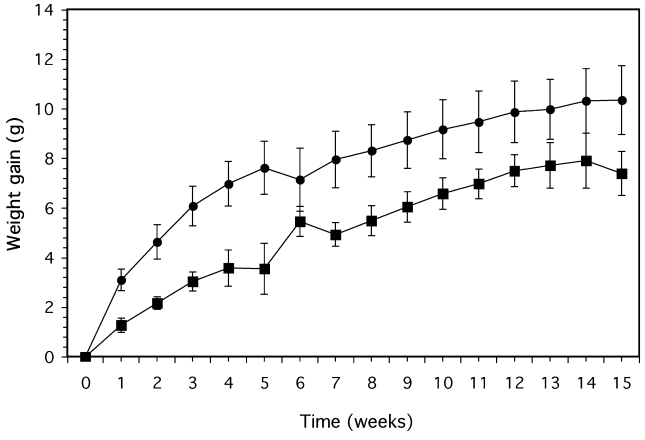

Fig. 1. Body weight gain as a function of time for C57Bl/6J mice kept on a high fat diet for 15 weeks and treated with inhibitor (■) or vehicle (●). Data are means ± SEM of results obtained with 9 or 10 animals.

Table 2
Body weight gain, adipose tissue weight, and cellularity of inhibitor-treated and vehicle-treated mice after 15 weeks of HFD

	Vehicle	Inhibitor
Initial weight (g)	17 ± 1.3	20 ± 0.66
Final weight (g)[a]	28 ± 0.57	27 ± 0.89
Weight gain (g)	10 ± 1.4	7.4 ± 0.88
SC fat (g)	0.71 ± 0.10	0.63 ± 0.07
GON fat (g)	0.60 ± 0.11	0.51 ± 0.08
Adipocyte number ($\times 10^{-6}/\mu m^2$)		
SC	700 ± 88	780 ± 84
GON	400 ± 39	470 ± 41
Adipocyte diameter (μm)		
SC	46 ± 3.2	43 ± 1.7
GON	58 ± 2.6	54 ± 2.2

Data are means ± SEM of results obtained with 9 or 10 animals.
[a] Weight before overnight fasting.

Over the entire HFD period, physical activity was higher in inhibitor-treated mice ($11{,}600 \pm 1180$ versus 8600 ± 620 cycles per 12 h; $p = 0.038$), whereas the average food intake was lower (2.9 ± 0.18 versus 3.7 ± 0.01 g per 24 h; $p = 0.057$).

Analysis of the cellularity of the fat deposits after 15 weeks of the HFD revealed that the diameter of the adipocytes in tissue treated with inhibitor was somewhat smaller than in the tissue without inhibitor, and that the number of adipocytes per surface unit was somewhat higher. As observed previously, the HFD induced adipocyte hypertrophy as compared to normal chow (adipocyte diameter of 36 ± 2.8 or 38 ± 2.2 μm in SC or GON adipose tissue of vehicle-treated mice on normal chow).

Staining of adipose tissue sections with Sirius red revealed more collagen throughout the inhibitor-treated tissues compared with the vehicle-treated tissues (stained area of $13 \pm 1.5\%$ versus $8.7 \pm 1.5\%$ of the total area ($p = 0.04$) for SC tissue, with corresponding values of $6.6 \pm 0.99\%$ versus $1.4 \pm 0.15\%$ ($p = 0.0008$) for GON tissue).

Staining of SC or GON adipose tissue with an endothelial cell specific lectin revealed somewhat higher blood vessel density in inhibitor-treated as compared to vehicle-treated mice on HFD (Table 3). In SC adipose tissue, the stained area and vessel size were comparable for both groups, whereas in GON adipose tissue these were higher in inhibitor-treated mice. These differences were, however, not statistically different.

Gelatinolytic activity

Zymography on gelatin-containing gels revealed the presence of proMMP-2 and active MMP-2 species in extracts of SC or GON adipose tissue, whereas MMP-9 levels were not detectable. Addition of Ro28-2653 (final concentration 10 μmol/L) to the incubation buffer re-

Table 3
Characterization of blood vessels in SC and GON adipose tissue of inhibitor- or vehicle-treated mice after 15 weeks of HFD

	SC		GON	
	Vehicle	Inhibitor	Vehicle	Inhibitor
Stained area[a]	4.9 ± 1.5	5.1 ± 1.7	1.7 ± 0.36	3.9 ± 0.96
Vessel density[b]	520 ± 100	610 ± 95	280 ± 41	430 ± 74
Vessel size[c]	85 ± 17	82 ± 16	58 ± 4.0	86 ± 8.6

Data are means ± SEM of results obtained with three to five animals.
[a] Lectin stained area in percent of the total area.
[b] Number of vessels per mm^2.
[c] Vessel size in μm^2.

sulted in inhibition of the lysis zones on the gel, confirming the activity of the inhibitor (data not shown).

Gelatinolytic activity determined in adipose tissue homogenates with the use of a quenched fluorescent substrate hydrolyzed by most MMPs was somewhat reduced in GON but not SC tissue of inhibitor-treated mice compared to vehicle-treated controls. Activity against the gelatin substrate was lower in both inhibitor-treated tissues (Fig. 2A). In situ gelatin zymography with cryosections confirmed lower gelatinolytic activity in SC ($9.5 \pm 0.76\%$ versus $26 \pm 4.9\%$) and GON ($17 \pm 1.4\%$ versus $24 \pm 6.5\%$) adipose tissue of inhibitor-treated mice on HFD. These differences were, however, not statistically significant.

mRNA expression

The mRNA expression levels of MMP-2, -9, and -14 (the main targets of Ro 28-2653) and of TIMP-1 in SC and GON adipose tissues were determined using semi-quantitative RT-PCR. In both SC and GON tissues, a statistically significant two- to threefold upregulation was observed in inhibitor-treated animals for MMP-2 ($p = 0.0004$ for SC and $p = 0.02$ for GON) and for MMP-14 ($p = 0.001$ for SC and $p = 0.0002$ for GON), as compared to vehicle-treated animals. The expression levels of MMP-9 and TIMP-1 were not significantly affected by inhibitor treatment (Fig. 2B).

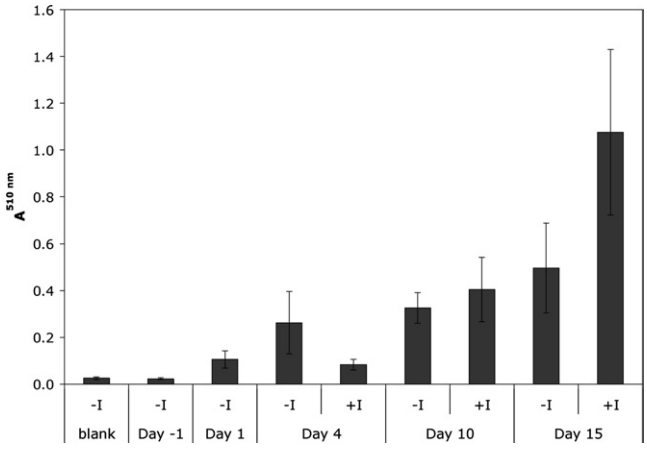

Fig. 3. Differentiation of 3T3-F442A preadipocytes as an in vitro model of adipogenesis. Differentiation was monitored in the absence (−I) or in the presence (+I) of the inhibitor. Intracytoplasmatic lipids were stained with Oil red O at different time points, extracted, and quantified from the absorbance at 510 nm ($A^{510\ nm}$). Blank, corresponds to wells without cells; day 1–15, are days after the induction of differentiation in confluent cultures. Data are mean values of two experiments, performed with a variability of less than 10%.

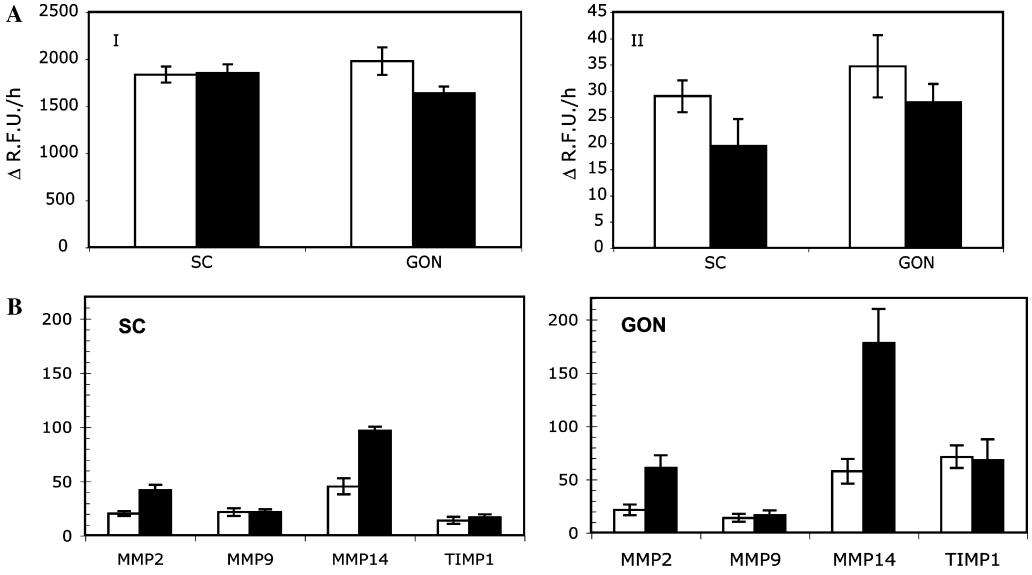

Fig. 2. (A) Activity of subcutaneous (SC) or gonadal (GON) adipose tissue homogenates from mice treated with Ro 28-2653 ($n = 8$, filled bars) or vehicle ($n = 10$, open bars) against an aspecific MMP-substrate (I) and against a gelatinase-specific substrate (II). Data are mean changes in fluorescence intensity/h ± SEM. (B) mRNA expression levels of MMP-2, -9, -14, and TIMP-1 in adipose tissues from mice treated with Ro 28-2653 ($n = 5$, filled bars) or vehicle ($n = 5$, open bars). Data are normalized to 28S rRNA levels and shown as means ± SEM.

Differentiation of preadipocytes

Differentiation of 3T3-F442A preadipocytes was characterized by their conversion into spherical lipid-filled adipocytes. Staining with Oil red O and extraction of cellular lipids revealed somewhat reduced differentiation after 4 days in the presence of 1 μmol/L Ro 28-2653, whereas subsequently (up to day 15) differentiation appeared somewhat but not significantly enhanced (Fig. 3). At the end of the experiment, FACS analysis using Nile red indicated over 85% of differentiated cells (not shown). Inhibitor treatment induced enhanced mRNA expression for MMP-2 (by 60%) and MMP-9 (by 100%), but not for MMP-14 (11% increase), and a decreased expression of TIMP-1 (by 40%).

Discussion

To test the hypothesis that MMP activity plays a role in development of adipose tissue, we have administered a synthetic MMP inhibitor to mice kept on HFD for 15 weeks. Ro 28-2653 has a relative specificity/selectivity for gelatinases (MMP-2 and -9), but also efficiently inhibits MMP-14, and less efficiently MMP-1 and MMP-13. The published IC_{50} values [12] relate to the MMPs of human origin and are not known for their murine counterparts. The minimal effective dose for daily oral administration to mice and rats was determined to be 30 mg/kg (H.-W. Krell, personal communication). In a standard prostate cancer model in rats, this dosing was shown to reduce tumor growth and to prolong survival [20]. Ro 28-2653 was also effectively used at a dose of 50 mg/kg in mice with T-cell lymphoma [12]. We have administered the inhibitor to mice by daily p.o. instillations at a dose of 30 mg/kg. Higher doses were not investigated because we wanted to limit the effect on food intake of the mice. At the time of sacrifice, reduced gelatinolytic activity was observed by in situ zymography on adipose tissue sections of inhibitor-treated mice, whereas the fibrillar collagen content was higher, compatible with in situ inhibitory activity of Ro 28-2653. The metabolism of the inhibitor within adipose tissue is, however, unknown. The residual gelatinolytic activity may be caused by MMPs, that are not inhibited; there are indeed 17 other MMPs besides MMP-2 and MMP-9 with reported activity against gelatin [2]. In addition, some of the ADAM (a disintegrin and metalloprotease) family members, e.g., ADAM-15, also degrade gelatin [21]. We observed an enhanced expression of MMP-2 and MMP-14 in adipose tissues of inhibitor-treated animals; in contrast, MMP-9 and TIMP-1 expression was not affected. During in vitro differentiation of 3T3-F442A preadipocytes, we observed enhanced expression of MMP-9 in the presence of the inhibitor. It is known from other studies that the gene expression profile in these cells differs from the in vivo situation [22,23]; their in vitro differentiation is associated with abundant deposition of extracellular matrix and secretion of gelatinases [8]. It is not clear if modulation of MMP expression has resulted in enhanced MMP activity in adipose tissue, which may counteract the in vivo effect of the inhibitor. In previous studies in a T-cell lymphoma model, no effect of Ro 28-2653 was observed on MMP expression, in contrast to unspecific inhibitors such as Batimastat [12]. Also in HT1080 cells, upregulation of proMMP-9 was reported with aspecific MMP inhibitors, but not with Ro 28-2653 [24,25]. Another broad-spectrum inhibitor, galardin (Ilomastat), was shown to enhance the expression of MMP-3, -9, and -13 in murine keratinocytes [26].

In any case, only minor effects of partial MMP inhibition were observed on adipose tissue development and cellularity. In a previous study, we have shown that—in the same murine model—administration of galardin resulted in impaired adipose tissue development [11]. Galardin was also found to accelerate lipid accumulation during in vitro differentiation of fibroblastic 3T3-L1 progenitor cells [27]. Ro 28-2653 also resulted in somewhat, but not significantly, enhanced differentiation of 3T3-F442A cells. These data thus suggest an inhibitory effect of some MMPs on adipocyte differentiation, possibly by preventing the assembly of basement membrane.

Involvement of MMPs in angiogenesis is supported by the finding that galardin reduces the number and area of new blood vessels in rat corneas implanted with a malignant carcinoma [28]. In our murine model of nutritionally induced obesity, galardin impaired adipose tissue mass, resulting in apparently higher blood vessel density [11]. Also in the present study, in vivo administration of Ro 28-2653 resulted in somewhat enhanced blood vessel density. In contrast, in the ex vivo rat aortic ring assay, Ro 28-2653 completely blocked neovessel formation, indicating an anti-angiogenic potential [25]. If we normalize the blood vessel density in our study to the adipocyte number, the effect of the inhibitor largely disappears. The number and/or size of adipocytes in adipose tissue may indeed affect blood vessel density [9,10].

Taken together, it appears that the inhibitory spectrum of Ro 28-2653 is not adequate to affect adipose tissue development significantly. Alternatively, one could argue that the MMPs targeted by the inhibitor do not play key roles in this process. It remains to be shown whether more extensive or more specific MMP inhibition will allow one to modulate adipose tissue development in vivo.

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