Matrix Metalloproteinase Inhibition Impairs Adipose Tissue Development in Mice

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Abstract—The effect of galardin, a broad-spectrum matrix metalloproteinase (MMP) inhibitor, was studied in mice kept on a high fat diet (HFD). Five-week-old male wild-type mice were fed the HFD (42% fat) for up to 12 weeks and were daily injected intraperitoneally with the inhibitor (100 mg/kg) or with vehicle. After 12 weeks of the HFD, the body weights of both groups were comparable, but the weight of the isolated subcutaneous (SC) or gonadal (GON) fat deposits was significantly lower in the inhibitor-treated group than in the control group (88±11 versus 251±66 mg, respectively, for SC fat [$P<0.05$]; 90±24 versus 217±30 mg, respectively, for GON fat [$P<0.02$]). The number of adipocytes was somewhat higher and the diameter was somewhat smaller (but not significantly) in adipose tissues of the inhibitor-treated group. Adipose tissue of the inhibitor-treated mice contained more collagen than did that of the vehicle-treated mice (Sirius red–stained area of 42±2.6% versus 22±4.4%, respectively, for SC fat [$P<0.05$]; 21±5.1% versus 4.7±0.92%, respectively, for GON fat [$P<0.01$]); a distinct collagen-rich cap was formed around the inhibitor-treated tissue. In situ zymography with casein- or gelatin-containing gels confirmed a reduced MMP activity in SC and GON adipose tissues of inhibitor-treated mice. Thus, in this model, growth and development of adipose tissue appears to be limited by the formation of a collagen-rich matrix cap around the inhibitor-treated tissue. These data suggest a functional role for MMPs in the development of adipose tissue. **(*Arterioscler Thromb Vasc Biol*. 2002;22: 374-379.)**

Key Words: matrix metalloproteinases ■ obesity ■ galardin ■ adipose tissue ■ adipocytes

Obesity is a common disorder, and its related diseases, such as non–insulin-dependent diabetes mellitus, atherosclerosis, and hypertension, are major causes of death and disability in Western societies. The development of obesity is associated with extensive modifications in adipose tissue involving adipogenesis, angiogenesis, and extracellular matrix (ECM) proteolysis.[1] Proteolytic systems, eg, the plasminogen/plasmin (fibrinolytic) and matrix metalloproteinase (MMP) system, contribute to tissue remodeling by degradation of the ECM and basement membrane components or by activation of latent growth factors.[2,3] Specific molecular interactions between both proteolytic systems suggest that they may cooperate in achieving ECM degradation.[4] Plasminogen activator inhibitor-1 (PAI-1), the main physiological plasminogen activator inhibitor, is highly expressed in adipose tissue and may play a role in the development of obesity.[5–9] An MMP-2 (gelatinase A)–like gelatinolytic activity was observed in conditioned medium of rat adipocytes and may play a role in their organization into large multicellular clusters.[10] We have recently observed high expression of MMP-2 in the adipose tissue of mice with nutritionally induced obesity as well as in genetically obese mice. Its localization in adipocytes has been suggested by immunoprecipitation of the culture medium of isolated adipocytes, by mRNA determination and by immunogold electron microscopy.[11] Recently, it has been reported that the addition of MMP inhibitors accelerated the accumulation of lipid during differentiation of murine fibroblastic 3T3-L1 progenitor cells and that differentiated adipocytes express and activate MMP-2.[12]

To establish a potential role of the MMP system in adipose tissue development, we have studied the effect of *N*-[2*R*-2-(hydroxamido-carbonylmethyl)-4-methylpentanoyl]-L-tryptophan methyl amide (galardin), a hydroxamate-based broad-spectrum MMP inhibitor,[13,14] in a mouse model of nutritionally induced obesity.[9]

Methods

Experimental Protocol

Five-week-old male wild-type mice (mixed 75% C57/Bl6 and 25% 129SVj genetic background) were kept in microisolation cages on a 12-hour day-night cycle and fed a high fat diet (HFD) containing 42% fat (Harlan TD 88137). One group (n=14) received daily intraperitoneal injections of galardin at a dose of 100 mg/kg body wt (sterile suspension of 20 mg/mL in 4% carboxymethylcellulose in PBS, pH 7.3), and a control group (n=14) received the vehicle only.[15] After 6 or 12 weeks, after they had fasted overnight, the mice

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were anesthetized by intraperitoneal injection of 60 mg/kg Nembutal (Abbott Laboratories). Blood was collected with or without the addition of trisodium citrate (final concentration 0.01 mol/L). Gonadal (GON) and subcutaneous (SC) fat pads were removed, and the wet weight was determined. Portions were immediately frozen at −20°C for extraction or were prepared for histology. The SC fat pad represented the adipose tissue overlying the posterior iliac crest and was dissected from its attachment sites to the skin.

All animal experiments were approved by the local ethics committee and were performed in accordance with the guiding principles of the American Physiological Society and the International Society on Thrombosis and Hemostasis.[16] Galardin was synthesized as described.[13] The final product was precipitated from a mixture of H_2O and ethyl acetate and was >98% pure, as evaluated by high-performance liquid chromatography. The inhibitor was suspended in 4% carboxymethylcellulose and sterilized before use.

Assays

Numbers of adipocytes (expressed as $\times 10^{-6}$ per μm^2) in 15-μm frozen-cut adipose tissue sections, stained with hematoxylin-eosin under standard conditions, and their mean diameters were determined by using a computer-assisted image analysis system (Axioplan 2, Zeiss).

For each animal, 3 to 5 areas in 4 different sections each were analyzed. The data were first averaged per section and then per animal. Staining of fibrillar collagen with Sirius red and of blood vessels with a rabbit polyclonal antibody raised against rat thrombomodulin[17] (a kind gift of Dr R. Jackman, Harvard University, Boston, Mass) was performed on paraffin sections and quantified by computer-assisted image analysis. Four to 9 sections were analyzed per animal and then averaged.

Extraction of adipose tissue (≈250 mg/mL) was performed by overnight incubation at 4°C on a tilting table in 10 mmol/L sodium phosphate buffer, pH 7.2, containing 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.2% NaN_3. After centrifugation, the protein concentration of the supernatant was determined (BCA assay, Pierce). Zymographic analysis of gelatinase activity was performed on 10% Tris-glycine gels with 0.1% gelatin, and zymographic analysis of plasminogen activator (tissue-type plasminogen activator [tPA] or urokinase-type plasminogen activator [uPA]) activity was performed on 12.5% acrylamide gels with 1% nonfat dry milk and 5 μg/mL human plasminogen.[18,19] The lysis of the substrate gel (area×intensity) was quantified by image analysis and expressed in arbitrary units (AU) of lysis per milligram total protein in the extract.[19]

In situ zymography on cryosections of adipose tissue using casein- or gelatin-containing gels was performed essentially as described.[20] The substrate gel (0.5% agarose) contained 1.0 mg/mL resorufin-labeled casein (Boehringer-Mannheim) or pig skin gelatin (Oregon Green 488 conjugate, Molecular Probes Europe BV). Overlays were analyzed by computer-assisted image analysis after incubation for 48 hours in a moist chamber at 37°C. Lysis is expressed as percentage of the total section area.

Blood glucose concentrations were measured with the use of Glucocard strips (Menarini Diagnostics). White blood cell, red blood cell, platelet, hemoglobin, and hematocrit levels were determined by using standard laboratory assays. PAI-1, tPA, uPA, plasminogen, and α_2-antiplasmin antigen levels were measured with specific ELISAs.[21,22]

Data are reported as mean±SEM, and statistical analysis was performed by the nonparametric Student *t* test.

Results

Adipose Tissue Weight and Cellularity

During the experimental period, 5 of 14 animals died in the control group compared with 2 of 14 animals in the inhibitor-treated group. After 6 weeks on the HFD, the mice in the inhibitor-treated group were not different from the mice in the control group with respect to body weight (29±0.5 versus 29±0.9 g, respectively), weight gain (5.9±1.9 versus 7.4±1.3 g, respectively), SC fat mass (83±15 versus 89±27 mg, respectively), or GON fat mass (180±45 versus 220±41 mg, respectively). After 12 weeks on the HFD, body weight and weight gain were also comparable in the inhibitor-treated and vehicle-treated (control) mice (Table 1). However, untreated wild-type mice of the same genetic background kept on the HFD for 12 weeks had a significantly higher body weight (40.6±1.7 [mean±SEM] g, n=7; $P \leq 0.01$ versus treated mice). After 12 weeks of the HFD, the SC and GON fat pad weights were significantly lower in the inhibitor-treated group (Table 1). However, the total fat mass was lower than that in the untreated wild-type mice kept on the HFD for 15 weeks (≥1.0 g in SC and GON territories). Hematoxylin-eosin staining of adipose tissue sections did not reveal differences in appearance (not shown). Analysis of the cellularity of the fat deposits after 12 weeks of the HFD revealed that the diameter of the adipocytes in tissue with inhibitor was somewhat smaller than that in the tissue without inhibitor but that the number of adipocytes per surface unit was somewhat higher (both $P=0.06$ for GON tissue, and both $P=0.6$ for SC tissue; Table 1).

TABLE 1. Effect of MMP Inhibition on Body Weight Gain and on Adipose Tissue Weight and Cellularity in Mice Kept on HFD for 12 Weeks

	No Inhibitor	Inhibitor
Body weight, g	32±2.7	33±2.8
Weight gain, g	11±1.8	12±1.4
SC fat, mg	251±66	88±11*
GON fat, mg	217±30	90±24†
Adipocyte number, $\times 10^{-6}/\mu m^2$		
SC	1070±120	1460±420
GON	670±41	950±93
Adipocyte diameter, μm		
SC	36±2	31±5
GON	45±2	38±2

Values are mean±SEM of 5 or 6 animals in control or inhibitor groups. *$P<0.05$ and †$P<0.02$ vs corresponding group without inhibitor.

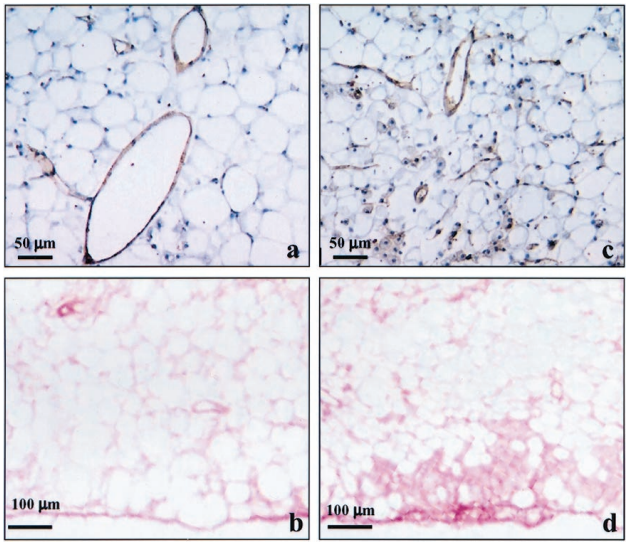

Figure 1. Light-microscopic analysis of adipose tissue. GON adipose tissue derived from mice treated with vehicle (a and b) or with inhibitor (c and d) for 12 weeks was stained with an antibody against thrombomodulin (a and c) or with Sirius red (b and d).

TABLE 2. Effect of MMP Inhibition on Metabolic and Hematologic Parameters in Mice Kept on HFD for 12 Weeks

	No Inhibitor	Inhibitor
Glucose, mg/dL	124±12	98±10
PAI-1, ng/mL	9.7±0.60	26±9.9
Plasminogen, μg/mL	99±3.9	85±5.8
α_2-Antiplasmin, μg/mL	120±12	93±9.3
WBCs, $\times 10^9$/L	3.1±0.3	3.2±0.2
RBCs, $\times 10^{12}$/L	6.9±0.2	6.0±0.6
Hemoglobin, g/dL	12±0.6	11±0.9
Hematocrit, %	31±1.1	27±3.0
Platelets, $\times 10^9$/L	700±66	660±53

WBC indicates white blood cell; RBC, red blood cell. Values are mean±SEM of 5 or 6 animals in control or inhibitor groups.

Staining of GON adipose tissue sections with an antibody against thrombomodulin (Figure 1a and 1c) revealed a higher blood vessel density in the inhibitor-treated animals compared with the control animals (stained area was 4.9±0.43% versus 2.8±0.26% of the total area, respectively; $P=0.038$).

Staining of adipose tissue sections for collagen with Sirius red (Figure 1b and 1d) revealed the presence of more collagen throughout the inhibitor-treated samples compared with the control samples (stained area was 42±2.6% versus 22±4.4%, respectively, of the total area [$P<0.05$] for SC tissue, with corresponding values of 21±5.1% versus 4.7±0.92% [$P<0.01$] for GON tissue). In the inhibitor-treated samples, collagen was much more abundant at the borders of the adipose tissue, forming a cap around the tissue. Similar observations were made for SC and GON adipose tissue. Red to orange birefringent collagen was more pronounced in inhibitor samples than in control samples, indicating more collagen disorganization in the control samples (not shown).

Blood and Adipose Tissue Parameters

Glucose levels were comparable in the control and inhibitor-treated groups at 12 weeks, and plasma plasminogen and α_2-antiplasmin levels were normal in both groups. As observed previously with this HFD,[9] plasma PAI-1 levels were strongly elevated in both groups (normal levels on regular chow are 1 to 3 ng/mL); however, differences between control and inhibitor-treated groups were statistically not significant. Other hemostatic parameters, including white blood cell, red blood cell, and platelet counts and hemoglobin and hematocrit levels, were not different (Table 2). Similar data were obtained in the inhibitor- and vehicle-treated groups after 6 weeks of HFD (not shown).

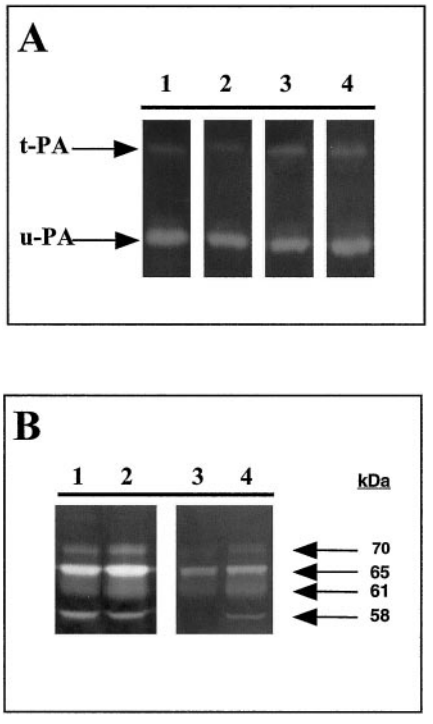

Figure 2. Zymographic analysis of adipose tissue. Extracts of SC (lanes 1 and 2) or GON (lanes 3 and 4) adipose tissue obtained from mice kept on HFD for 12 weeks without (lanes 1 and 3) or with (lanes 2 and 4) inhibitor administration were applied to casein-containing (A) or gelatin-containing (B) gels.

In extracts of SC or GON adipose tissue, PAI-1, tPA, and uPA antigen levels were comparable in the control and inhibitor-treated groups (Table 3). Zymography of extracts on casein-containing gels confirmed the presence of tPA and uPA activity at comparable levels (Figure 2A). Zymography

TABLE 3. MMP and Fibrinolytic System Components in Adipose Tissue of Mice Kept on HFD for 12 Weeks

	No Inhibitor		Inhibitor	
	SC	GON	SC	GON
PAI-1 antigen,* ng/mg	1.1±0.10	1.1±0.41	1.7±0.87	0.52±0.14
tPA antigen,* ng/mg	0.72±0.20	1.5±0.54	0.57±0.06	1.0±0.07
tPA activity,† AU/mg	13±2.4	28±5.2	13±3.1	24±4.6
uPA antigen,* ng/mg	4.9±0.69	8.2±1.2	2.5±0.51	4.5±0.82
uPA activity,† AU/mg	92±13	150±12	93±10	140±86
70-kDa proMMP-2,† AU/mg	49±11	12±5.4	37±20	13±2.5
65-kDa proMMP-2,† AU/mg	160±23	52±11	300±87	67±8.5
61-kDa MMP-2,† AU/mg	93±5.0	13±3.8	120±11	29±7.1
58-kDa MMP-2,† AU/mg	69±13	1.2±0.8	99±26	13±6.9

Values are mean±SEM of 5 or 6 animals.

*Antigen levels are expressed as nanograms per milligram protein in the extract.

†Activity levels are expressed as AU of lysis per milligram protein in the extracts.

TABLE 4. Effect of MMP Inhibition on Gelatinolytic or Caseinolytic Activity in Adipose Tissue, as Measured by In Situ Zymography

	Lysis, % of Section Area	
	Gelatin	Casein
SC		
No inhibitor	26±2.6	24±2.3
Inhibitor	13±3.7*	13±3.7*
GON		
No inhibitor	19±3.3	20±1.0
Inhibitor	15±3.5	14±3.1

Values are mean±SEM of 5 or 6 animals.
*$P \leq 0.05$ vs control without inhibitor.

on gelatin-containing gels revealed the presence of 70- and 65-kDa proMMP-2 and of 61- and 58-kDa active MMP-2 species, whereas MMP-9 levels were not detectable (Figure 2B). Addition to the incubation buffer of EDTA (final concentration 20 mmol/L) or galardin (final concentration 0.5 mmol/L) totally abolished the lysis zones on the gel, confirming their MMP nature as well as the inhibitory activity of galardin. The addition of galardin (final concentration 0.05 mmol/L) to extracts of the control group did not abolish the detected gelatinolytic activity, suggesting that the inhibitor dissociates during electrophoresis (data not shown). The levels of the different molecular species of MMP-2 were somewhat lower in GON than in SC adipose tissue but were comparable with or without inhibitor treatment (Table 3). The total MMP-2 levels at 12 weeks (all 4 forms combined) were not significantly different in the absence or the presence of the inhibitor, either in the SC tissue (360±41 versus 550±120 AU/mg, respectively; $P=0.43$) or in the GON tissue (79±19 versus 120±15 AU/mg, respectively; $P=0.18$). Also, the ratio of active (61 plus 58 kDa) versus latent (70 plus 65 kDa) MMP-2 was comparable in the absence or presence of the inhibitor for the SC tissue (0.78 versus 0.67, respectively) but somewhat different for the GON tissue (0.22 versus 0.53, respectively). Very similar data were obtained for the fibrinolytic parameters and gelatinase levels in adipose tissue extracts of the inhibitor- and vehicle-treated groups after 6 weeks of HFD (not shown).

In situ zymography with casein- or gelatin-containing gels on cryosections of SC or GON adipose tissue confirmed a lower MMP activity in the tissue of inhibitor-treated animals (Table 4); these differences were statistically significant for the SC tissue (both $P \leq 0.05$) but not for the GON tissue ($P=0.12$ or $P=0.79$, respectively). The addition of a mixture of EDTA (final concentration 25 mmol/L) and 1,10-phenanthroline (final concentration 5 mmol/L) to the agarose gel resulted in inhibition of 60% to 85% of the lytic activity in the different experiments (not shown).

Discussion

Fat pad formation, the concerted assembly of adipose, vascular, and nervous tissues, is a complex process. Formation of adipose tissue, the major cellular component, involves commitment of mesodermal stem cells to a preadipocyte lineage and differentiation of preadipocytes into adipocytes.[10] Differentiation is associated with an increased secretion of basement membrane components such as laminin, proteoglycans, and type IV collagen and decreased secretion of fibrillar type I collagen. The organization of adipocytes in spherical clusters requires accumulation in the extracellular space of sufficient basement membrane components to generate a surrounding basal lamina. ECM components are indeed synthesized and degraded during adipocyte differentiation[23–25] and may modulate it.[26] MMP-2 was suggested to play a role in adipocyte clustering, inasmuch as its inhibition prevented this process.[10] Furthermore, MMP-2 is highly expressed in the adipose tissue of obese mice.[11] It has also been reported that MMP-2 increases and that tissue inhibitor of MMPs type 1 (TIMP-1) decreases during adipocyte differentiation.[10,27] MMP-2 and MMP-9 expression and secretion have also recently been demonstrated in human adipose tissue.[28]

To test the hypothesis that MMP-mediated matrix degradation plays a role in the development of adipose tissue, we have studied the effect of galardin, a broad-spectrum MMP inhibitor, in a mouse model of nutritionally induced obesity. Galardin is a peptide-based zinc-chelating hydroxamate that inhibits several MMPs, including gelatinase A (MMP-2), gelatinase B (MMP-9), interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and stromelysin-1 (MMP-3).[13,14] In the present study, 5-week-old male wild-type mice were kept on the HFD for up to 12 weeks, whereas galardin was administered by daily intraperitoneal injections.

To monitor the effects of daily intraperitoneal injections of inhibitor on body weight gain, a control group was included that received daily injections of the vehicle (4% carboxymethylcellulose). Body weight gain during 12 weeks was comparable in the inhibitor- and vehicle-treated groups. Compared with wild-type mice of the same genetic background kept on the HFD for 12 weeks without injections, the body weight and the SC and GON fat mass in the present study were lower. This may be due to higher plasma PAI-1 levels observed in the present study for inhibitor-injected mice on the HFD compared with wild-type mice on the HFD without injections (10 to 25 ng/mL versus 5 to 10 ng/mL, respectively). This increase may be due to a combined effect of the HFD and of the carboxymethylcellulose injections and may contribute to the low body weight gain in all groups. It has indeed been previously shown that the HFD induces elevated PAI-1 levels and that these are associated with lower body weight gain during HFD feeding.[9] Alternatively, stress effects due to the daily injections and, possibly, loss of appetite may play a role. As discussed above, we have taken care to have an identical injection scheme in all experimental groups.

In the control group but not in the inhibitor group, the weight of the SC fat pad increased between 6 and 12 weeks of the HFD; in contrast, the weight of the GON fat pad was not further increased in the control group and even decreased in the inhibitor group. This suggests a differential effect of prolonged galardin administration on the development of SC and GON adipose tissue, which may partly be due to lower collagen content in the GON tissue but may also be due to a differential pattern of MMP expression. Comparison of mRNA expression between GON and SC fat from obese mice indeed revealed depot-specific expression patterns.[29] At present, we cannot exclude the possibility that the broad-spectrum

inhibitor galardin affects other MMPs in both types of adipose tissue. However, the overall effect of the inhibitor is a reduction of fat mass.

Overall, the inhibitor appeared to be well tolerated, which is in agreement with several previous studies in which no general toxicity of systemic galardin administration was observed.[15,30–34] In all these models, significant biological activity of galardin was reported, most likely as a result of MMP inhibition. We demonstrated by in situ zymography reduced gelatinolytic activity at the level of the inhibitor-treated adipose tissue, indicating that MMP-2 and/or MMP-9 is inhibited. Because galardin is a broad-spectrum inhibitor, it cannot be excluded that other MMPs are also inhibited, as suggested by the finding that in situ caseinolytic activity is also reduced (Table 4). In addition, SC and GON adipose tissues from inhibitor-treated animals contained more collagen than did those tissues from vehicle-treated mice. In an experimental balloon angioplasty injury model in the rabbit, it has previously been observed that galardin reduces collagen synthesis and degradation.[32] It is not clear whether in the present study in adipose tissue the inhibitor also had an effect on collagen synthesis. However, it was apparent that collagen was much more abundant at the borders of the adipose tissue of inhibitor-treated mice, forming a cap surrounding the tissue. It is also possible that in the vehicle-treated adipose tissue, other cell types are more abundantly present (eg, more stromal-vascular cells), which may contribute to larger fat pads. A similar observation with collagen has previously been reported in tumor tissues of mice treated with an MMP inhibitor.[35] By reducing the rate of collagen degradation and simultaneously stimulating collagen biosynthesis, the body may be able to encapsulate the invading tumor in collagen structures.[36] Studies with MMP inhibitors in tumor models have suggested that inhibition of growth could occur by the development of fibrotic tissue around the tumor, thereby preventing invasive growth, or by the inhibition of angiogenesis.[35] Hydroxamate-based MMP inhibitors have been shown to cause significant inhibition of SC rat prostate tumors in nude mice, but no obvious effect on tumor angiogenesis has been observed.[37]

MMPs may indeed be involved in direct matrix degradation, but they may also be involved in angiogenesis. Galardin has previously been shown to reduce the number and area of new blood vessels in rat corneas implanted with pellets containing extracts of a malignant carcinoma.[30] However, our in vivo data indicate that MMP inhibition with galardin results in a higher blood vessel density in adipose tissue. This may be explained by the fact that the total mass of the adipose tissue is lower in the inhibitor-treated animals, whereby a relatively comparable angiogenesis in inhibitor-treated and control groups would be perceived as a higher blood vessel density in the more compact inhibitor-treated tissue. Lund et al[15] have reported that daily administration of galardin to mice results in the enhanced expression of mRNAs for stromelysin-1, gelatinase B, and collagenease-3 in keratinocytes. In the present study, we did not see an enhanced expression of MMP-2 or MMP-9 in adipose tissue, at least not at the protein level, after the administration of galardin. However, gelatin zymography with extracts does not allow us to quantify active MMP, as it is presented in the tissue, because of dissociation from the inhibitor, but it did allow us to demonstrate that total amounts of gelatinases were comparable in the inhibitor-treated and control groups.

Recently, a regulatory role of MMP-3 (stromelysin-1) has been suggested in adipogenesis during mammary gland involution in mice; mice with MMP-3 deficiency or overexpression of TIMP-1 indeed showed accelerated differentiation and hypertrophy of adipocytes. In addition, galardin accelerated lipid accumulation during in vitro differentiation of fibroblastic 3T3-L1 progenitor cells. These data thus suggest an inhibitory effect of MMPs (MMP-3) on adipocyte metabolism and differentiation.[12] This was explained by preventing the assembly of basement membrane, possibly as a result of the MMP-3–mediated cleavage of entactin.[12] We observed a decrease in adipose tissue weight in the inhibitor-treated group. This may be due to an effect of MMP inhibition on other processes contributing to adipogenesis. It is also possible that the inhibitory effect of MMP-3 on adipogenesis is seen only in processes that occur relatively rapidly, such as mammary gland involution.

In view of the previous observations that PAI-1 is highly expressed in adipose tissue[5–9] and of the potential interactions between the fibrinolytic and MMP systems,[3,4] we have also determined fibrinolytic parameters in extracts of adipose tissue. We did not observe significant differences in PAI-1 antigen and in tPA or uPA antigen and activity levels.

Taken together, our findings of reduced collagen degradation on administration of the broad-spectrum MMP inhibitor indicate that the formation of a collagen-rich matrix cap impairs adipose tissue growth, suggesting a functional role for MMPs. It will be interesting to investigate whether other orally administered and eventually more specific MMP inhibitors will have a similar effect on adipose tissue development in vivo.

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