Adipocyte Produces Matrix Metalloproteinases 2 and 9
Involvement in Adipose Differentiation

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Adipocyte hypertrophy and hyperplasia together with angiogenesis contribute to the growth of the fat mass. Because changes in the extracellular matrix (ECM) components are often associated with such cellular remodeling, we studied the adipocyte expression of the matrix metalloproteinases (MMPs) 2 and 9, two key enzymes involved in the modulation of ECM. The present study provides the first evidence that human adipose tissue produces and secretes MMP-2 and -9 as shown by gelatin zymography analysis performed on media conditioned by human subcutaneous adipose tissue and human preadipocytes in primary cultures and by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis on transcripts from mature human adipocytes. The further characterization performed on the murine 3T3F442A preadipocyte cell line demonstrates that MMP expression, assessed by RT-PCR and Western blot analysis, as well as activity, assessed by gelatin zymography analysis, increased during the adipocyte differentiation, whereas the expression of tissue inhibitor metalloproteinases 1 and 2 were abolished or not affected, respectively. Finally, preadipocyte treatment with MMP inhibitors such as batimastat and captopril, as well as neutralizing antibodies, markedly decreased adipocyte differentiation as demonstrated by the inhibition in the appearance of lipogenic (triglycerides) and lipolytic (glycerol release and hormone-sensitive lipase expression) markers. These data suggest that MMP-2 and -9 could be important key regulators of adipocyte differentiation. Thus, the adipocyte-derived MMPs might represent a new target for the inhibition of adipose tissue growth. *Diabetes* 50:2080–2086, 2001

Obesity is associated with an excessive growth of adipose tissue, the development of which is dependent on cellular events concerning both adipose tissue cell populations, i.e., adipocytes and their precursor cells, preadipocytes, as well as microvascular endothelial cells. Indeed, during the settlement of obesity, enhanced lipogenesis together with decreased lipolysis will result in a net increase in lipid storage within the adipocyte, leading to adipocyte hypertrophy. Adipocyte hyperplasia due to preadipocyte proliferation and differentiation will contribute to adipose mass expansion (1). Concomitant with both hypertrophic and hyperplasic events, stimulation of angiogenic process will provide new blood vessels in the growing tissue, thus permitting the supply of oxygen and nutrients necessary for adipose tissue metabolism (2).

It is quite obvious that dramatic tissue remodeling occurs within the adipose tissue during the fat mass development. However, whereas mechanisms leading to adipocyte hypertrophy and hyperplasia have been extensively studied, few data are available concerning the regulation of the angiogenic processes as well as the modification of the extracellular matrix (ECM) during fat mass increment and the settlement of obesity. Adipocytes, besides their metabolic activities, are able to produce several factors, such as growth factors and cytokines, which may play a role in the paracrine regulation of the adipose tissue remodeling (3). Indeed, adipocytes also secrete proangiogenic factors, such as vascular endothelial growth factor (VEGF) (4), tumor necrosis factor–α (5), monobutyrin (6), and leptin (7). These factors, originating from adipocytes, may contribute to the formation of new blood vessels inside the fat pad. Moreover, because these secretions are increased during adipocyte differentiation, it further strengthens the hypothesis of the existence of a paracrine loop between adipocyte differentiation and the stimulation of angiogenic process.

Besides these secreted factors, proteases and ECM components might also play an important role in regulating adipose tissue remodeling. Indeed, it is now well established that degradation of ECM represents the first step in the angiogenic process. Matrix metalloproteinases (MMPs), especially MMP-2 and -9, have been shown to be necessary for this event (8). No data report the presence of MMPs in adipose tissue and adipocytes. However, ECM components are synthesized and degraded during the process of adipocyte differentiation (9–11), and some studies have shown that different ECM context modulates adipocyte differentiation (12).

We performed the present study to determine whether adipocytes and preadipocytes produce MMPs. We provide the first evidence that human adipose tissue releases MMP-2 and -9 and that this secretion is modulated during adipocyte differentiation. Moreover, MMP activities are directly involved in the regulation of adipocyte differentiation, since their inhibition resulted in a blockade of adipocyte differentiation. It is then tempting to speculate that the adipocyte-derived MMPs might represent a new

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BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; F12, nutrient mix F12; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSL, hormone-sensitive lipase; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase–polymerase chain reaction; TIMP, tissue inhibitor metalloproteinase; VEGF, vascular endothelial growth factor.

pharmacological target for the inhibition of adipose tissue growth by inhibiting adipose differentiation as well as angiogenic process.

RESEARCH DESIGN AND METHODS

Materials. Chemicals were obtained from Sigma (Saint Quentin Fallavier, France) and cell culture reagents either from Life Technologies (Cergy Pontoise, France) or Roche Diagnostics (Meylan, France). The monoclonal mouse antibody against MMP-2 was obtained from NeoMarkers (Microm, Francheville, France) and the polyclonal rabbit antibodies against MMP-9 and tissue inhibitor metalloproteinases (TIMPs) 1 and 2 were from Chemicon (Euromedex, Souffelweyersheim, France). The neutralizing antibodies against MMP-2 and MMP-9 were obtained from NeoMarkers. The polyclonal chicken antibody against the rat hormone-sensitive lipase (HSL) was provided by Dr. Cecilia Holm, Department of Cell and Molecular Biology, Lund University, Lund, Sweden. Batimastat was provided by British Biotech (Oxford, England). The peroxidase-conjugated antibodies were obtained from Calbiochem (France Biochem, Meudon, France) and the enhanced chemiluminescence kit was obtained from Amersham (Les Ulis, France). The prestained protein marker and the protein determination kit were from Bio-Rad (Ivry/Seine, France). Triglycerides, glycerol, and lactate dehydrogenase were determined using kits from Sigma.

Human adipose tissue. Human subcutaneous adipose tissue was obtained from moderately overweight women undergoing plastic surgery (mean BMI 25.5 ± 1.4 kg/m^2). This study was approved by the Ethics Comittee of Toulouse University Hospital.

For the ex vivo experiments, sterile adipose tissue was cut into small pieces and rinsed four times, and 1 g of 50- to 100-mg fragments were incubated in 3 ml of Dulbecco's modified Eagle's medium (DMEM), supplemented with 0.1% bovine serum albumin (BSA) and an antibiotic mixture (500 units/ml penicillin and 50 μg/ml streptomycin) in an atmosphere of 95% air/5% CO_2 at 37°C. Media were collected for zymography analysis after 2–24 h.

Isolated mature adipocytes were obtained according to the method of Rodbell (13) by collagenase digestion of freshly collected adipose tissue.

The isolation of human adipose tissue–derived stromal cells and the culture of stromal preadipocytes differentiated into adipocytes were performed as previously described (14) with minor modifications (15). Briefly, after isolation and overnight attachment, stromal cells were cultured in an atmosphere of 95% air/5% CO_2 at 37°C in the defined serum-free adipogenic medium consisting of DMEM/nutrient mix F12 (DMEM/F12), containing 50 μg/ml gentamicin, 10 μg/ml transferrin, 100 nmol/l cortisol, 66 nmol/l insulin, and 200 pmol/l triidothyronine supplemented the first 2 days with 20 μmol/l 3-isobutyl-1-methylxanthine and 1 μg/ml troglitazone. After a 2-day priming period, cells were then cultured in the adipogenic medium alone for 0, 5, 10, or 15 days. The cells were then washed with phosphate-buffered saline (PBS) and maintained in DMEM/F12 supplemented with 50 μg/ml gentamicin and 0.1% BSA for 24 h. Medium was then collected and used in zymography analysis.

3T3F442A cell culture. Cells from 3T3F442A preadipocyte cell line were grown as previously described (16) in an atmosphere of 95% air/5% CO_2 at 37°C. Cells were cultured until confluence in DMEM supplemented with 10% donor calf serum and an antibiotic mixture (500 units/ml penicillin and 50 μg/ml streptomycin) and then cultured in adipogenic medium consisting of DMEM supplemented with 10% fetal calf serum, 50 nmol/l insulin, and the antibiotic mixture for 0, 3, 6, 8, or 10 days. At the indicated time period, cells were washed with PBS and maintained in DMEM supplemented with 0.1% BSA for 24 h. Medium was then collected and aliquots were used directly in zymography analysis or after alcoholic precipitation in Western blot analysis. Cells were rinsed twice in PBS and stored at −80°C until analysis. Treatments with MMP inhibitors (batimastat, captopril, MMP-2, and MMP-9 neutralizing antibodies) were performed on confluent cells cultured in the adipogenic medium for 5–10 days. After treatments, media were collected for glycerol determination and cells were rinsed twice and stored at −80°C until analysis.

Determination of MMP activity by zymography. Proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS in 10% polyacrylamide gels containing 1 mg/ml gelatin, due to the proteins' capacity to digest the gelatin substrate. Briefly, culture media (20 μl) were loaded directly on gels; after electrophoresis, proteins were renatured by exchanging SDS with 2.5% Triton X-100 (15-min incubation repeated twice). The gels were then incubated for 16 h at 37°C in 50 mmol/l Tris-HCl, pH 8, 5 mmol/l $CaCl_2$, and 0.02% NaN_3 and stained with Coomassie blue. The gelatinolytic activity was visualized as areas of lytic activity on an otherwise blue gel. Migration of proteins was compared with that of a prestained molecular weight marker. The gels were scanned by an imaging densitometer and quantified using the NIH Image program (developed at the U.S. National Institutes of Health).

Western blot analysis. For Western blot analysis of MMPs and TIMPs, proteins in the cell media from preadipocyte cultures were precipitated with cold ethanol at −70°C for 2 h. Pelleted proteins (12,000*g* for 20 min) were washed with ethanol and subsequently lyophilized. For Western blot analysis of HSL expression, cells were washed twice with PBS and scraped. After brief centrifugation (1,000*g*, 2 min, 4°C), pellets were resuspended in 200 μl of lysis buffer containing 10 mmol/l Tris-HCl (pH 7.5), 0.15 mol/l NaCl, 2 mmol/l sodium vanadate, 0.1% SDS, 1% Nodinet P-40, 1% sodium deoxycholate, 2 mmol/l phenylmethylsulfonyl fluoride, and a mix of protease inhibitors. The lysate was then centrifuged at 13,000*g* for 30 min at 4°C and the supernatant protein concentration was determined using a protein determination kit. Fifty micrograms were separated by SDS-PAGE under denaturing conditions. After they were transferred to nitrocellulose membranes and Ponceau staining was performed to verify equal loading of the lanes, membranes were blocked overnight and then incubated with the primary antibody for 90 min, followed by incubation with the secondary antibody for 60 min. The immunocomplexes were detected using a chemiluminescence reagent kit. The autoradiography was analyzed and quantified using the public domain NIH Image program.

Extraction of RNA and reverse transcriptase–polymerase chain reaction analysis. Total RNAs were extracted by the standard acid guanidium phenol-chloroform method. For the reverse transcription (RT), 2 μg total RNA was incubated with 200 units reverse transcriptase (GIBCO), dNTP (175 μmol/l), oligo (dT) (200 ng), dithiothreitol (1 mmol/l), and reaction buffer in a final volume of 20 μl at 37°C for 60 min. In some reaction mixtures, reverse transcriptase of total RNA was omitted to determine the amplification of contaminating genomic DNA or cDNA. After a final denaturation at 94°C for 7 min, 6 μl of cDNA was subjected to polymerase chain reaction (PCR) consisting of denaturation at 94°C for 1 min, followed by 90 s of annealing at 58°C and 90 s of elongation at 72°C for 28 cycles. The last cycle was ended by 7 min of elongation at 72°C. The primers used for the amplification of MMP-2 and MMP-9 cDNAs were derived from the sequence of the cloned human and mouse MMP-2 and -9 (accession numbers hMMP-2 180670, mMMP-2 198465, hMMP-9 177204, and mMMP-9 286079). The primers used for amplification of VEGF were derived from the sequence of cloned human cDNA and have previously been shown to amplify all VEGF splice variants (17). The primers used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) derived from the cloned human and rat GAPDH cDNA as previously reported (17).

	Reverse	Forward
MMP-2	TGTGTCTTCCCCTTCACTTT	GATCTGAGCGATGCCATCAA
MMP-9	TGTACCGCTATGGTTACAC	CCGCGACACCAAACTGGAT
VEGF	GGAGAGATGAGCTTCCTACAG	TCACCGCCTTGGCTTGTCACA
GAPDH	TATGACAACTCCCTCAAGAT	AGATCCACAACGGATACATT

The PCR contained 0.4 μmol/l of each primer, dNTP (200 μmol/l), reaction buffer, and 2.5 units *Taq* polymerase in a final volume of 50 μl. The amplified cDNAs were size-fractionated by agarose gel electrophoresis and stained with ethidium bromide. Analysis of the scanned gels was performed using the public domain NIH Image program.

Statistical analysis. Values are expressed as means ± SEM. Statistical analysis was performed by one-way analysis of variance followed by Dunnett's multiple-comparison post hoc test or Student's *t* test when appropriate. Statistical significance was set at $P < 0.05$.

RESULTS

Human adipocytes produce MMP-2 and -9. Detection of gelatinase activity was performed on media from human subcutaneous adipose tissue explants maintained ex vivo for 24 h. As shown in Fig. 1*A*, after a 2-h incubation period, one area of lytic activity was detected in the media migrating at a molecular weight of 72 kDa. After 24 h, the intensity of the 72-kDa band markedly increased (fivefold increase, $P < 0.01$, $n = 3$), whereas another gelatinase activity appeared at a higher molecular weight of 92 kDa. Both gelatinase activities identified in the 24-h conditioned human adipose tissue media migrated at the same molecular weight as the ones detected in the media from primary cultures of human umbilical vein endothelial cells. More-

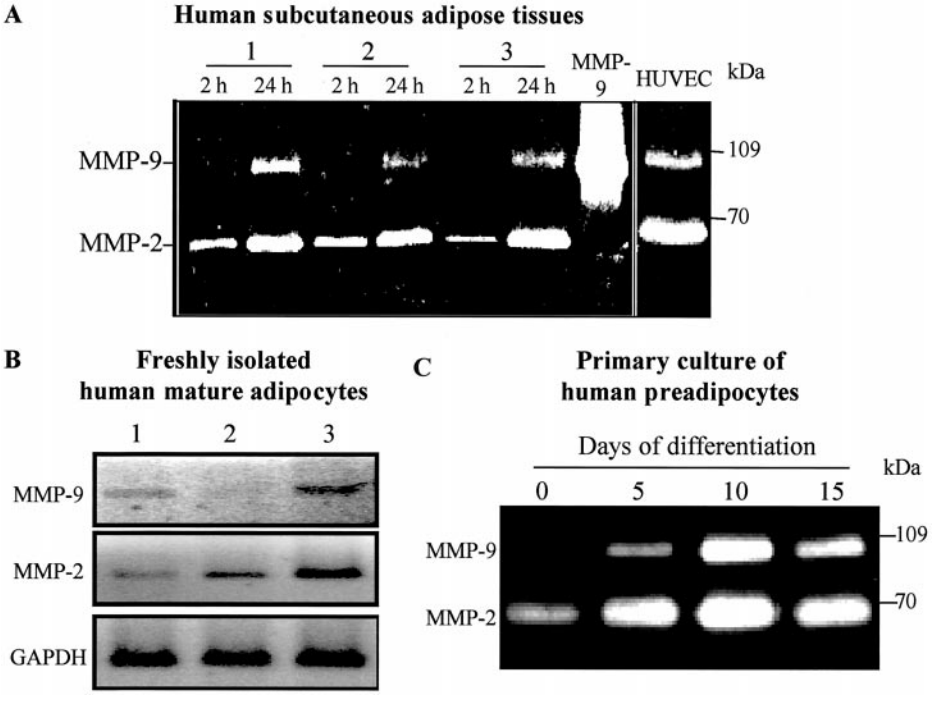

FIG. 1. MMPs in human adipose tissue. *A*: Gelatinase activity released by human adipose tissue. Twenty microliters of media conditioned by 1 g of explants of human subcutaneous adipose tissue originated from three patients for 2–24 h were analyzed in gelatin zymography together with positive controls (human recombinant MMP-9 and culture medium from human umbilical vein endothelial cells). A representative Coomassie blue staining of electrophoresis is shown. Three independent experiments gave similar results. *B*: Expression of MMP-2 and -9 in human adipocytes. RT-PCR experiments using specific primers for the amplification of MMP-9, MMP-2, and GAPDH cDNAs were performed on total RNAs extracted from freshly isolated mature human subcutaneous adipocytes. Representative ethidium bromide stainings of electrophoresis are shown. Two other experiments gave the same results. *C*: Gelatinase activity released during human preadipocyte differentiation. Stroma vascular cells from human subcutaneous adipose tissue were cultured in adipogenic medium for 0, 5, 10, or 15 days. At the indicated time, cells were washed and maintained in DMEM/F12 supplemented with 50 μg/ml gentamicin and 0.1% BSA for 24 h. Media were then collected and 20 μl analyzed in gelatin zymography. A representative Coomassie blue staining of electrophoresis is shown. Three independent experiments gave similar results.

over, when 0.01 mol/l EDTA, an inhibitor of gelatinase activity, was added to the zymography, no lytic band could be detected (data not shown), further demonstrating the identity of the gelatinase activities as MMP-2 or 72-kDa collagenase and MMP-9 or 92-kDa collagenase.

To further confirm the adipocyte origin of the secreted MMPs, experiments were performed on freshly isolated mature adipocytes. Due to the presence of collagenase used to digest the adipose tissue, it was impossible to perform gelatin zymography experiments on media conditioned by mature adipocytes, even after extensive washings. RT-PCR analysis using specific primers for MMP-9 and -2 cDNAs revealed the presence of both transcripts in human adipocytes, although the expression of MMP-9 was lower than that of MMP-2 and exhibited high individual variations (Fig. 1*B*). Finally, experiments were performed on primary cultures of human preadipocytes. Gelatin zymography analysis was performed on 24-h conditioned media by nondifferentiated and 5-, 10-, and 15-day differentiated preadipocytes. As shown in Fig. 1*C*, MMP-2 activity was present in the media of nondifferentiated preadipocytes and increased significantly with the differentiation process to reach a maximum after 10-day differentiation (2.5-fold increase compared with nondifferentiated preadipocytes, $P < 0.05$, $n = 3$). MMP-9 activity was not detected, or was at a very low level, in the media conditioned by nondifferentiated preadipocytes. A strong significant induction of MMP-9 activity was observed with the adipocyte differentiation (Fig. 1*C*).

MMP-2 and -9 are expressed and released during differentiation by 3T3F442A adipocytes. To further characterize the adipocyte-derived MMP-2 and -9, we performed experiments on a murine preadipocyte cell line, the 3T3F442A cell line. We collected 24-h conditioned media from nondifferentiated and 3-, 6-, 8-, and 10-day differentiated preadipocytes to perform gelatin zymography analysis and Western blot analysis.

Gelatin zymography analysis clearly showed the presence of two lytic areas in preadipocyte-conditioned media, the molecular weights of which corresponded to MMP-2

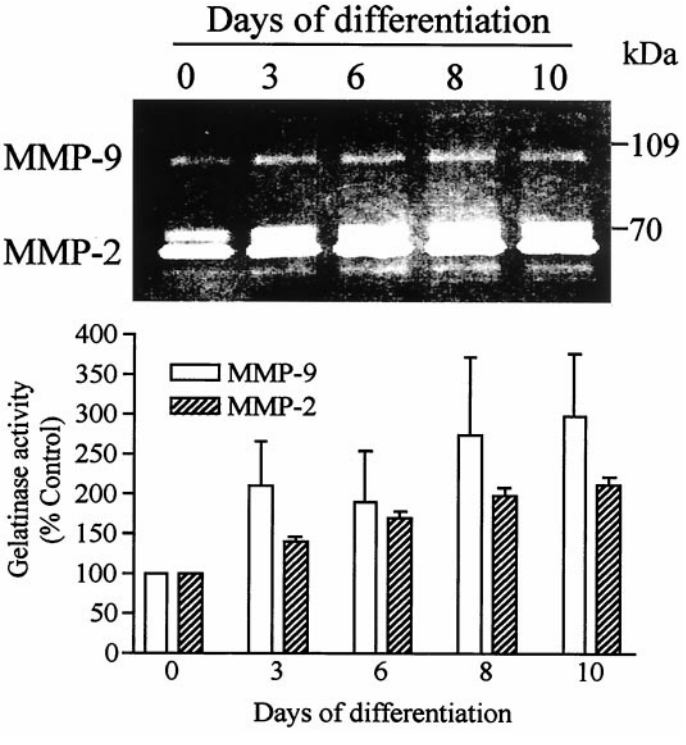

FIG. 2. MMP activity secreted by 3T3F442A preadipocytes during differentiation. Once confluent (day 0), 3T3F442A preadipocytes were cultured in differentiating medium for 0, 3, 6, 8, and 10 days. At the indicated time, cells were washed and maintained in serum-deprived medium supplemented with 0.1% BSA for 24 h. Media were then collected and analyzed in gelatin zymography. A representative Coomassie blue staining of electrophoresis is shown. Densitometric analysis of the lytic area was performed and results are expressed as percentage of the values obtained for day 0. Values are means ± SEM of eight independent experiments. Lower panel: ▨, MMP-2; □, MMP-9.

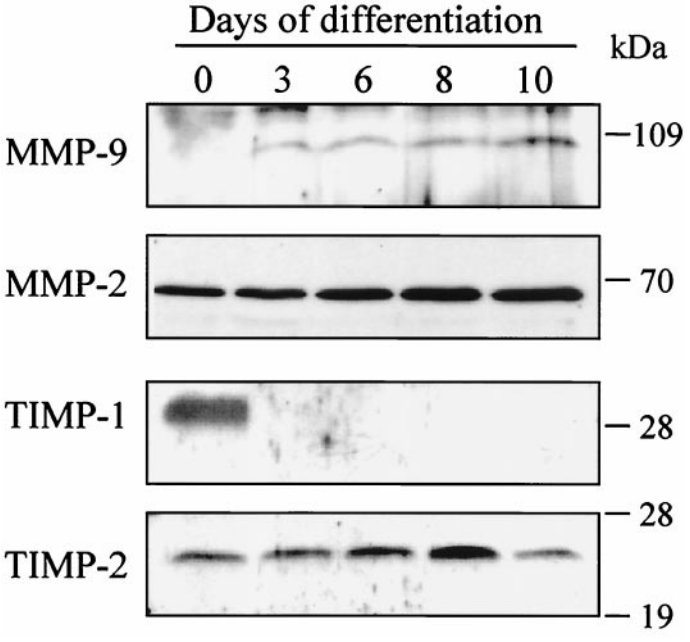

FIG. 3. Secretion of MMPs and TIMPs during 3T3F442A preadipocyte differentiation. Once confluent (day 0), 3T3F442A preadipocytes were cultured in differentiating medium for 0, 3, 6, 8, and 10 days. At the indicated time, cells were washed and maintained in serum-deprived medium supplemented with 0.1% BSA for 24 h. Media were then collected, concentrated, and analyzed by Western blot using specific antibodies directed against MMP-9, MMP-2, TIMP-1, and TIMP-2. Representative autoradiographies from eight independent experiments are shown.

and -9 (Fig. 2). As observed in human adipose tissue, the activity of MMP-2 was markedly higher than that of MMP-9. Moreover, both pro–MMP-2 and mature MMP-2 were detected. Densitometric analysis of the lytic area showed that MMP activities were increased during the differentiation, with a maximal value reached after 10 days of differentiation (three- and twofold increases for MMP-2 and -9, respectively, $P < 0.05$, $n = 8$). Addition of 0.01 mol/l EDTA to the zymography totally inhibited the appearance of both gelatinolytic activities (data not shown).

The Western blot analysis performed on the same conditioned media but after a concentration step confirmed the differentiation-dependent expression of MMPs. Indeed, as shown in Fig. 3, the secretion of MMP-2 and -9 increased during the differentiation process, with a maximum reached after 8 days of differentiation (two- and fourfold increases, respectively, $P < 0.05$, $n = 8$). In parallel, Western blot analysis performed with antibodies directed against TIMP-1 and -2 showed a total disappearance of TIMP-1 protein when preadipocytes enter in differentiation, whereas the secretion of TIMP-2 was not significantly modified during the differentiation process ($n = 8$).

Finally, RT-PCR analysis performed on RNAs extracted from nondifferentiated and 2-, 4-, 7-, and 9-day differentiated cells showed a marked differentiation-dependent increase of MMP-2 mRNA level (13-fold increase in mRNA levels after 9 days of differentiation compared with nondifferentiated cells, $n = 3$, $P < 0.01$) (Fig. 4). A similar profile was obtained for the expression of the VEGF mRNAs, already described to be increased during adipocyte differentiation (4). MMP-9 transcript amount followed a moderate increase during differentiation, reached maximal level after 7 days, and decreased afterward (Fig. 4).

Inhibition of MMP-2 and -9 inhibits adipocyte differentiation. To determine whether MMP activity released by adipocytes might play a role in the differentiation process, confluent 3T3F442A preadipocytes were treated with increasing concentrations of MMP inhibitors, batimastat (from 0.5 to 10 μmol/l), and captopril (from 10 to 1,000 μmol/l) in the presence of adipogenic medium. After 5–10 days of treatment, total cellular triglyceride content was quantified as well as the expression of the HSL in cells maintained for 24 h in serum-deprived medium and basal extracellular glycerol released in the medium was determined.

As shown in Fig. 5, 10 μmol/l batimastat as well as 1 mmol/l captopril together with adipogenic medium for 5 days strongly inhibited the lipid storage within the preadipocytes as well as the change in cell morphology occurring when cells were cultured in adipogenic medium alone. Other protease inhibitors such as aprotinin and leupeptin did not affect the adipocyte differentiation induced by the adipogenic medium (data not shown). The Western blot analysis using an antibody directed against HSL showed that both inhibitors markedly and signifi-

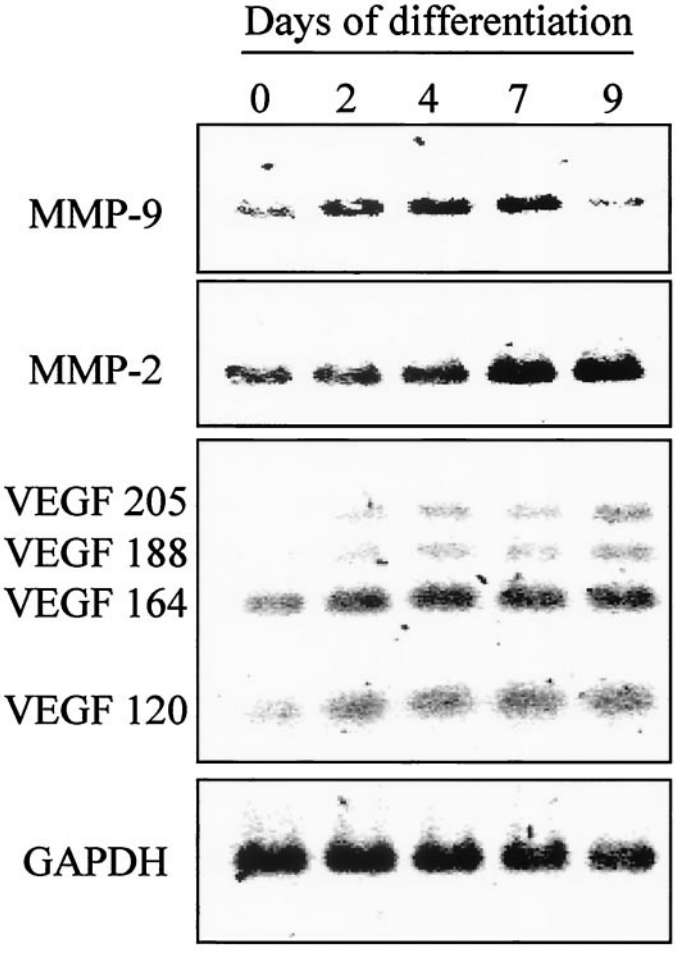

FIG. 4. Expression of MMPs during 3T3F442A preadipocyte differentiation. Once confluent (day 0), 3T3F442A preadipocytes were cultured in differentiating medium for 0, 2, 4, 7, and 9 days. At the indicated time, cells were washed and maintained in serum-deprived medium supplemented with 0.1% BSA for 24 h. Total RNAs were extracted and RT-PCR analysis using specific primers for MMP-2, MMP-9, VEGF, and GAPDH cDNAs was performed. Representative ethidium bromide stainings of electrophoresis gels are shown from three independent experiments.

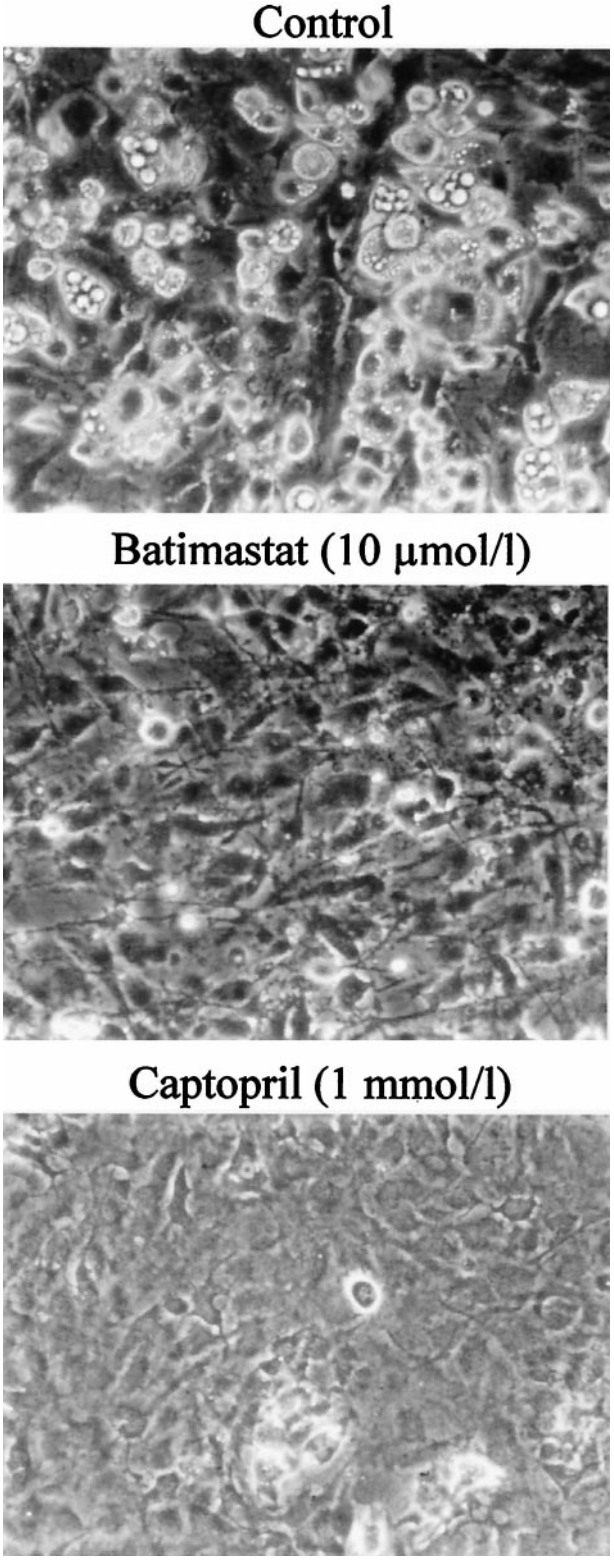

FIG. 5. Effect of MMP inhibitors on the differentiation of 3T3F442A preadipocytes. Once confluent, 3T3F442A preadipocytes were cultured in adipogenic medium in the presence of 10 μmol/l batimastat or 1 mmol/l captopril. Medium was changed every 2 days. Light microscopy analysis was performed after 5 days of treatment.

cantly inhibited HSL expression, a late adipocyte differentiation marker, in a concentration-dependent manner (Fig. 6). It has to be noticed that the inhibitory effect of captopril was significant only at high concentrations (0.5–

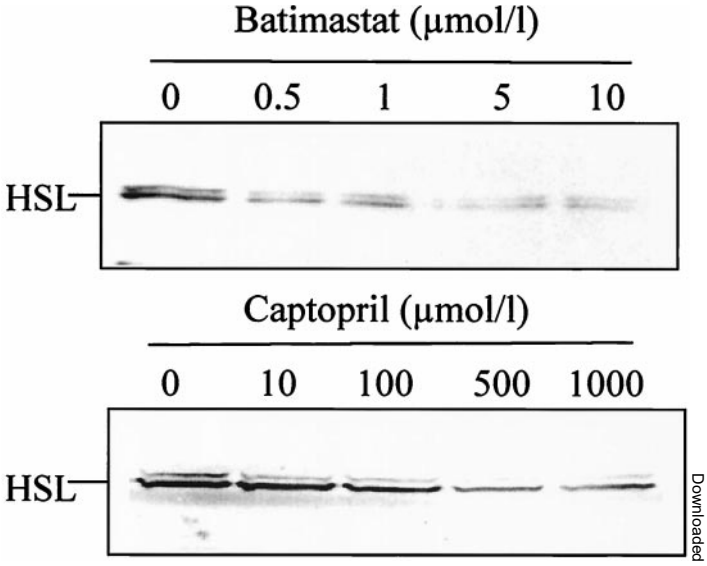

FIG. 6. Effect of MMP inhibitors on the expression of HSL, a marker of adipocyte differentiation. Once confluent, 3T3F442A preadipocytes were cultured in adipogenic medium in the presence of increasing concentrations of batimastat (from 0.5 to 10 μmol/l) and captopril (from 10 to 1,000 μmol/l). Medium was changed every 2 days. Western blot analysis using an anti-HSL antibody was performed on total protein extracts after 5 days of treatment on cells maintained for 24 h in serum-deprived medium. Representative autoradiographies are shown from three independent experiments.

1 mmol/l, $P < 0.05$, $n = 3$) and was not mimicked by losartan, an angiotensin receptor I antagonist (data not shown). After 10 days of treatment, 5 μmol/l batimastat and 1 mmol/l captopril inhibited markedly the amount of glycerol released in the culture medium (Table 1). The same inhibitory effect was observed when considering the cellular triglyceride and protein contents (Table 1). Moreover, because no lactate dehydrogenase could be detected in cell media, it was concluded that batimastat and captopril inhibited adipocyte differentiation without toxic or deleterious effects (Table 1). Finally, treatments with increasing doses of neutralizing antibodies against human MMP-2 and -9 for 5 days promoted a decrease in the cellular triglyceride contents, whereas no effect was observed in control assays using nonimmune mouse antibodies (Table 2), thus confirming that inhibition of MMP-2 and -9 led to a marked reduction in the adipocyte differentiation process.

TABLE 1
Effects of batimastat and captopril on lipogenic and lipolytic markers

	Control	Batimastat	Captopril
Glycerol (μmol/l)	445 ± 121	54 ± 19*	99 ± 18*
Triglycerides (mg/ml)	0.42 ± 0.03	0.21 ± 0.08*	0.17 ± 0.06*
Proteins (mg/ml)	2.7 ± 0.1	2.0 ± 0.2*	1.9 ± 0.4*
Lactate dehydrogenase	Undetectable	Undetectable	Undetectable

Once confluent, 3T3F442A preadipocytes were cultured in the adipogenic medium in the presence or absence of 5 μmol/l batimastat or 1 mmol/l captopril for 10 days. Glycerol and lactate dehydrogenase contents were measured in the media and cellular triglycerides and protein content were determined in cells maintained for 24 h in serum-deprived mdeium. Data are means ± SEM of three independent experiments. *$P < 0.01$ vs. control.

TABLE 2
Effects of neutralizing antibodies on cellular triglyceride contents

Concentration (ng/ml)	10	50	100
Anti–MMP-9	80 ± 11	67 ± 5*	66 ± 6*
Anti–MMP-2	78 ± 9	77 ± 8*	77 ± 8*
Nonimmune	110 ± 3	96 ± 11	92 ± 6

Once confluent, 3T3F442A preadipocytes were cultured in adipogenic medium in the presence or absence of increasing concentrations (10, 50, and 100 ng/ml) of anti–MMP-9, anti–MMP-2, or nonimmune mouse antibody for 5 days. Cellular triglyceride contents were determined on cells maintained for 24 h in serum-deprived medium. Data are means ± SEM expressed in percentage of the control for three independent experiments. *$P < 0.05$ vs. control.

DISCUSSION

The present study provides the first evidence that human adipose tissue and, more precisely, adipocytes and preadipocytes produce and release MMP-2 and -9. The further analysis of the adipocyte-derived secretion of MMPs performed on the murine 3T3F442A preadipocyte cell line demonstrates that MMP-2 and MMP-9 synthesis and release increased during adipocyte differentiation. Moreover, inhibition of MMP activities using batimastat, captopril, or neutralizing antibodies clearly reveals that MMPs play a role in adipocyte differentiation. These data suggest that the modulation of the ECM components through the production of MMPs might be an important key regulator of adipocyte differentiation and could contribute to the remodeling that occurs during the development of obesity.

The cellular processes that lead to the growth of adipose tissue are still not well defined. However, it is now well recognized that excessive adipose tissue development is an independent risk factor for diabetes, hypertension, atherosclerosis, and cardiac dysfunctions (18). Increased energy intake together with decreased energy expenditure will provide increased substrate availability for the adipocytes, leading to hypertrophy. The maintenance of the energy imbalance will also lead to the recruitment of new fat cells within the adipose tissue, explaining late-onset hyperplasia. The late appearance of these new fat cells will further increase the adipose mass and restrain more dramatically the capacity of adipose tissue to reduce its mass under energy restriction or increased energy expenditure (3). Moreover, although these processes are still poorly understood, the capillary networks of the adipose tissue will extend, allowing sufficient oxygen and metabolic supply to the expanding tissue (2). Thus, development of obesity is characterized by an extensive adipose tissue remodeling including hypertrophy, hyperplasia, and angiogenesis.

Remodeling is associated with changes in the ECM components. ECM, long considered as a passive structure, is now recognized as a surface able to control activity and presentation of several growth factors, but also as a partner of plasma membrane receptors (19). Thus, changes in ECM components have profound effects on the behavior of cells residing on ECM. A large number of enzymes, among them the family of MMPs, contribute to ECM remodeling. These enzymes act as broad-spectrum proteases for major ECM degradation events that occur during tissue remodeling. In the present study, we provide evidence that human adipose tissue produces and releases MMP-2 and -9. Indeed, although the MMP-2 activity was significantly higher than that of MMP-9, both activities were detectable in the media conditioned for 24 h by explants of human subcutaneous adipose tissues. Moreover, transcripts for MMP-2 and -9 were detected in mature isolated human adipocytes by RT-PCR analysis. Furthermore, media conditioned by human preadipocytes in primary culture exhibited both gelatinase activities, the amount of which was dependent on the differentiation stage, a finding that further strengthened the adipocyte/preadipocyte origin of MMPs. The gelatinase levels detected in human subcutaneous adipose tissue–conditioned media as well as the amounts of MMP transcripts in freshly isolated human adipocyte showed high interindividual variations, suggesting that adipocyte-derived MMP synthesis, secretion, and/or activity might be under the control of still-unknown modulating factors. After the first step of our studies demonstrating the production of MMP-2 and -9 by human adipocytes and preadipocytes, we shifted toward a murine cell line, the murine 3T3F442A preadipocyte cell line, to facilitate further exploration.

Analysis of the preadipocyte-conditioned media clearly showed a differentiation-dependent increase in the activity and protein secretion of MMP-2 and -9. This differentiation-dependent increase was related to enhanced MMP-2 and -9 expression as observed by RT-PCR analysis. Interestingly, MMP-2 mRNA expression increased in a constant manner during the differentiation, as also observed with VEGF expression, whereas MMP-9 expression showed a different profile, with a strong downregulation at the end of the differentiation period, suggesting that MMP-9 expression is probably induced during a narrower window than MMP-2 along the differentiation process. Since the balance between MMPs and their natural inhibitors, the TIMPs, is determinant for the net proteinase activity, we studied the expression of both TIMP-1 and -2, preferential inhibitors of MMP-9 and -2, respectively (20). Western blot analysis showed a complete inhibition of TIMP-1 expression when preadipocytes entered differentiation, whereas TIMP-2 expression was not significantly altered all along the process. Although more investigations have to be performed to determine the mechanisms of such an expression regulation, these data clearly show a specific pattern of MMP/TIMP expression during the adipocyte differentiation, in favor of an increased adipocyte MMP activity.

In light of the particular induction profiles of MMPs, we tested the hypothesis that MMPs might play a role in the control of adipose differentiation. Preadipocytes, when entering in the differentiation process, secrete ECM components, the nature of which changes during the differentiation time course (9–11). The extracellular network of fibronectin—the first to develop—as well as the network structures of type IV collagen and laminin are degraded during the process of adipocyte differentiation, whereas the type I collagen network—the last to develop—remained organized over the course of the late stage.

Since transforming growth factor–β has been described to inhibit adipocyte differentiation through the stimulation of preadipocyte-secreted fibronectin (21,22), it is suggested that ECM organization into a suitable structure is required for adipocyte differentiation. To investigate the potential role of MMPs in adipose differentiation, MMP inhibitors such as batimastat (23) and captopril, described

to act as an inhibitor of MMP activities at high concentrations (24–26), as well as neutralizing antibodies were used to treat confluent preadipocytes. No toxic effects were observed whatever compound was used, but a clear concentration-dependent inhibition of adipocyte differentiation was evidenced with the three MMP inhibitors. Indeed, the expression of adipocyte differentiation markers, such as HSL, as well as the adipocyte specific metabolic activities, i.e., release of glycerol originating from the basal lipolyis and intracellular accumulation of triglycerides through lipogenesis, were strongly inhibited. The inhibitory effect of captopril, though less potent than batimastat, was not related to an angiotensin II effect since it was not mimicked by losartan, an AT1 antagonist. Taken together, the present data suggest that changes in ECM through MMP-mediated degradation might play a critical role in the adipocyte differentiation process. Moreover, since it has been described that angiogenesis stimulated by adipocyte-conditioned medium was inhibited by TIMP (27), it is tempting to speculate that MMPs may represent new, interesting therapeutical targets to monitor adipose tissue growth by reducing adipose differentiation on the one hand and inhibiting the angiogenic processes on the other.

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