ABSTRACT

We previously showed that human and murine 3T3-F442A preadipocytes produced and released matrix metalloproteinases (MMPs) 2 and 9 and that a treatment by MMP inhibitors resulted in the blockade of murine fat cell adipose conversion. In parallel, investigators reported that other protease inhibitors, the human immunodeficiency virus (HIV) protease inhibitors (PIs) involved in lipodystrophy in humans, also reduced the adipocyte differentiation process of several murine cell lines. The present work was performed to define the effects of MMP inhibitors and HIV-PIs on the human adipocyte differentiation process, to clarify the involvement of MMPs in the control of human adipogenesis, and to determine whether HIV-PIs interact with MMPs in the control of this process. The effect of two MMP inhibitor and four HIV-PI treatments on the differentiation of primary culture human preadipocytes, as well as the putative relationships between HIV-PIs and MMP-2 and -9 expression, release, or activity were investigated. We showed that MMP inhibitors and HIV-PIs reduced the human adipocyte differentiation process as assessed by the decrease of cell protein and/or triglyceride contents and expression of fatty acid binding protein and hormone-sensitive lipase, two adipocyte markers. Unlike MMP inhibitors, HIV-PIs were devoid of any effect per se on recombinant MMP-2 and 9 activities but reduced the expression and release of MMP-9 by human preadipocytes. Thus, the present study indicates that the modulation of the extracellular matrix components through the production and/or activity of MMPs, and, more precisely, MMP-9 might be a key factor in the regulation of human adipose tissue development.

The mechanisms responsible for the growth of adipose tissue (AT) are still not well defined. Adipocyte hypertrophy and hyperplasia, due to the recruitment and differentiation of adipocyte precursor cells, or preadipocytes, into adipocytes are major cellular events in the development of the fat mass. Together with the hypertrophy and the hyperplasia events, recent investigations have clearly demonstrated that angiogenesis and extracellular matrix (ECM) remodeling are required for coordinated growth of the fat depot (Lijnen et al., 2002; Rupnick et al., 2002). Among the enzymes involved in the degradation of the ECM components, the matrix metalloproteinase (MMP) family is considered to play a major role (Sternlicht and Werb, 2001). In a previous report, we demonstrated that mature human adipocytes and preadipocytes produce and release two members of the MMP family, MMP-2 and -9, the expression and activity of which were shown to be dependent on the adipocyte differentiation process (Bouloumí et al., 2001). Interestingly, we also reported that treatment of the murine 3T3-F442A preadipocytes, which also produced MMP-2 and -9 during adipose conversion, with MMP inhibitors decreased the rate of adipocyte differentiation, suggesting that MMP activities are required for adipogenesis in rodents. However, no data are available concerning the potential role of MMPs in the regulation of AT development in humans.

A lipodystrophy syndrome, characterized by body fat redistribution, hyperlipidemia, and insulin resistance, has been associated with the recent use of protease inhibitors (PIs) in the therapy of AIDS as consequence of human immunodeficiency virus (HIV) infection (Carr et al., 1998). The most prominent clinical sign of HIV-PI-associated lipodystrophy is a loss of subcutaneous fat (lipectrophy) in the face and the extremities that can be accompanied, in some cases, by fat
accumulation in the neck, back, and visceral depots, suggesting that it most likely involves HIV-PI-mediated dysregulation of the balance between the development and the regression of the AT depending on the anatomical location of the fat deposits. Numerous studies have examined the effect of HIV-PIs on adipocyte differentiation of murine cell lines and evidenced that they are potent inhibitors of adipogenesis in vitro (Zhang et al., 1999; Vernochet et al., 2003). Further analysis of the putative targets of HIV-PIs has focused on the transcription factors that regulate the expression of adipocyte-specific markers (Dowell et al., 2000; Caron et al., 2001).

The present work was performed to define the effects of MMP inhibitors and HIV-PIs on the differentiation of preadipocytes isolated from the stroma vascular fraction of human AT. It was also expected to clarify the involvement of MMPs in the control of the adipocyte differentiation process in human AT and to determine whether HIV-PIs interact with MMPs in the control of this process. We evidenced that the broad-spectrum MMP inhibitor batimastat strongly inhibited human adipocyte differentiation. Moreover, HIV-PIs such as indinavir (IDV), ritonavir (RTV), saquinavir (SQV), and nelfinavir (NFV) also lead to a strong reduction of the human adipocyte differentiation process by a mechanism that may involve their inhibitory effect on MMP-9 expression and release by treated preadipocytes. Finally, we showed that MMP-9 inhibitor also reduced the differentiation of human preadipocytes, suggesting that MMP-9 is specifically involved in the batimastat-mediated inhibition of human adipogenesis. These data suggest that the modulation of the ECM components through the production and/or activity of MMPs and, more precisely, MMP-9 might be a key regulator of human AT development.

**Materials and Methods**

**Cell Culture and Treatment.** Chemicals were from Sigma-Aldrich (Saint-Quentin Fallavier, France), and cell culture reagents were from Invitrogen (Cergy Pontoise, France), Roche Diagnostics (Meylan, France), or Cambrex Bio Science (Verviers, Belgium).

Human subcutaneous abdominal white AT was obtained from moderately overweight women undergoing plastic surgery (mean age 39 ± 2 years, mean body mass index 26.5 ± 0.8 kg/m²). The isolation of human AT-derived stromal cells and the culture of stromal preadipocytes (i.e., fat cell precursors) differentiated into adipocytes were performed as previously described (10). Briefly, sterile AT was cut into small pieces and digested under agitation with collagenase (300 U/ml) for 1 h 30 min at 37°C. After centrifugation, washing, and filtration steps, the stromal cells were suspended in Dulbecco’s Modified Eagle’s medium F-12 supplemented with 10% fetal calf serum and plated at 60,000 cells/cm². After 24 h, the medium was changed for medium consisting of Dulbecco’s Modified Eagle’s medium F-12 supplemented with 33 μM biotin, 17 μM pantothenate, and 50 μg/ml gentamicin (basal medium) in the presence of 66 nM insulin, 1 nM triiodothyronine, 100 nM cortisol, 10 μg/ml human transferrin (adirogenetic medium), and, for the first 3 days, 1 μg/ml ciglitazone. After the 3-day priming period, the cells were cultured in the adipogenic medium, which contained 1 mg/ml gelatin. Briefly, culture medium batches (20 μl) were directly loaded onto gels; after electrophoresis, proteins were renatured by exchanging SDS with 2.5% Triton X-100 (20-min incubation repeated twice). The gels were then incubated for 1 h at 37°C in 50 mM Tris-HCl, pH 8.8, 5 mM CaCl₂, and 0.02% NaN₃ and stained with Coomassie Blue. The presence of gelatinolytic activity in the culture medium batches was visualized as uncolored areas on an otherwise blue gel. Migration of proteins was compared with that of prestained molecular weight markers.

**Gelatin Zymography.** Proteins with gelatinolytic activity were identified by electrophoresis in the presence of SDS in 8% polyacrylamide gels containing 1 mg/ml gelatin. Briefly, culture medium batches were loaded directly onto gels; after electrophoresis, proteins were renatured by exchanging SDS with 2.5% Triton X-100 (20-min incubation repeated twice). The gels were then incubated for 1 h at 37°C in 50 mM Tris-HCl, pH 8.8, 5 mM CaCl₂, and 0.02% NaN₃ and stained with Coomassie Blue. The presence of gelatinolytic activity in the culture medium batches was visualized as uncolored areas on an otherwise blue gel. Migration of proteins was compared with that of prestained molecular weight markers. The gels were scanned by an imaging densitometer and quantified using the NIH image program (developed at the U.S. National Institutes of Health).

**Fluorometric Activity Assay on Recombinant MMP-2 and -9.** In vitro assays were performed on 96-well plates. Human recombinant activated MMP-2 (2 nM) or MMP-9 (4 nM) (Calbiochem) were preincubated in the presence or absence of increasing concentrations of batimastat (from 0.01 nM to 0.01 μM) or HIV-PIs (from 1 nM to 100 μM) for 30 min at 37°C. Twenty micromolar of the fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dnp-Ala-Arg (Bachem, Bubendorf, Switzerland) were then added to each well, and fluorescence (excitation, 325 nm; emission, 390 nm) was recorded every 2 min for 60 min. MMP activity was evaluated by the maximum rate per minute on five separate measurements in each well.

**Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).** Changes in mRNA levels from specific genes were quantified by real-time RT-PCR. Total RNAs were extracted using Qiang Rneasy Minikit according to the manufacturer’s instructions, and RNA concentrations were determined using a fluorometric assay (Ribogreen; Molecular Probes, Eugene, OR). RNA (0.5 μg) was reverse-transcribed using the ThermoScript RT system (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was also performed without ThermoScript enzyme on RNA samples to check for any genomic DNA contamination. PCR primers were designed using Primer Express software according to the recommendations of Applied Biosystems (Foster City, CA). The forward and reverse primer sequences for fatty acid binding protein (aP2), hormone-sensitive lipase (HSL), and MMP-9, respectively, were as follows: aP2, GCATGGCCAAACCTAACATGA (forward) and CCTGGCCATGTATGAAGGAAA (reverse); HSL, GTGCAAAGACGGAGGACATG (forward) and CCTGTTGCATATGGAAG (reverse); and MMP-9, CCCTGGAGACCTGAGAACCA (forward) and CCAACCCGATTTGTAACCATGC (reverse).

Each amplification reaction was performed with 15 ng of cDNA sample in duplicate in 96-well optical reaction plates with a GeneAmp 5700 sequence detection system. The PCR mixture contained forward and reverse primer mix (final concentration, 900 nM for HSL or MMP-9 and 300 nM for aP2) and SYBR Green PCR Master Mix. For ribosomal RNA control (18S rRNA), a mixture containing primers and fluorogenic probe mix, TaqMan Universal PCR Master Mix (Applied Biosystems) was used. All reactions were performed under the same conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were analyzed with the GeneAmp 5700 software, and all values were normalized to the levels of 18S rRNA.
Statistical Analysis. Values are expressed as means ± S.E.M. Data were analyzed with one-way analysis of variance coupled with post hoc Dunnett’s multiple comparison test. Statistical significance was set at *p < 0.05.

Results

MMP Inhibitor Batimastat Treatment Decreases the Differentiation of Human Preadipocytes. To determine whether the broad spectrum MMP inhibitor batimastat could affect the human adipocyte differentiation process, primary cultures of human preadipocytes were treated with 1 or 5 μM batimastat for 10 days. Total protein and cell triglyceride contents were quantified, and the expression of two adipocyte differentiation markers, aP2 and HSL, was analyzed by real-time RT-PCR.

As is clearly shown in Fig. 1A, 5 μM batimastat led to a strong inhibition of the adipocyte differentiation process since treated cells exhibited no changes in shape and less cytoplasmatic accumulation of lipid droplets. Quantification of total protein and triglyceride contents, used as lipogenic indices, demonstrated that batimastat treatment statistically decreased adipogenesis (23 and 44% decrease in protein and triglyceride concentrations, respectively) (Fig. 1B). Moreover, the analysis of aP2 and HSL mRNA levels showed that batimastat led to a reduction of the expression of both adipocyte differentiation markers (29 and 33% decrease in aP2 and HSL mRNA expression, respectively) (Fig. 1B). Batimastat at 1 μM had no effect on the differentiation process (data not shown).

HIV-PI Treatments Decrease the Differentiation of Human Preadipocytes. We then investigated the effect of four HIV-PIs on the human adipocyte differentiation process. Thus, primary cultures of human preadipocytes were treated for 10 days with increasing concentrations of HIV-PIs: 5, 10, 25, or 50 μM for SQV and IDV; 1, 5, and 10 μM for NFV; and 5, 10, and 25 μM for RTV. These concentrations were chosen according to those usually found in the plasma of treated patients and proved to be effective on murine adipocyte differentiation (Lenhard et al., 2000; Roche et al., 2002). Total protein and cell triglyceride contents were quantified, and the expression of aP2 and HSL was analyzed by real-time RT-PCR. Only the concentrations for which a maximal effect on the differentiation process was observed, without detected toxicity (measured by the Toxilight kit, Cambrex Bio Sciences; data not shown), are presented.

As shown in Fig. 2, A and B, the protein and/or triglyceride contents of the cells were decreased under HIV-PIs: 25% decrease in triglyceride concentration for IDV (50 μM), 28% decrease in triglyceride concentration for RTV (10 μM), and 27 and 46% decrease in protein and triglyceride concentrations, respectively, with SQV (10 μM); and 34 and 56% decrease in protein and triglyceride concentrations, respectively, with NFV (5 μM). The expressions of aP2 and HSL mRNAs were also decreased, respectively, by: 28 and 42% with IDV (50 μM), 38 and 47% with RTV (10 μM), 72 and 82% with SQV (10 μM), and 76 and 87% with NFV (5 μM) (Fig. 2, C and D). These results indicate an alteration of the differentiation process with the four HIV-PIs used. However, it is noticeable, as shown for the protein and triglyceride contents, that SQV and NFV exhibited stronger effects on aP2 and HSL expression than IDV or RTV. It should be noted that higher concentrations of SQV (25 and 50 μM), RTV (25 μM), and NFV (10 μM) lead to cell death within a few days in our model.

HIV-PIs Do Not Modify the Activity of Human Recombinant MMP-9 or MMP-2. To establish whether HIV-PIs could affect the human adipocyte differentiation process by interacting with the MMP-dependent pathways, we first analyzed the capacity of HIV-PIs to inhibit MMP activities. The MMP inhibitor batimastat was used as control. MMP-2 and -9 activities were determined by in vitro fluorescence assays using human recombinant activated MMP-2 and -9.
together with a fluorogenic MMP substrate, in the presence of increasing concentrations of HIV-PIs (from 1 nM to 100 μM) or batimastat (from 0.01 nM to 0.01 μM). As expected, batimastat reduced, in a concentration-dependent manner, the fluorescence emission induced by the cleavage of the MMP substrate. However, none of the four HIV-PIs tested had an effect per se on the basal fluorescence detected in the MMP-9 or -2 activity assays (Fig. 3).

**HIV-PI Treatments Reduce MMP-9 Gelatinase Activity Released by Human Preadipocytes.** To determine whether HIV-PIs could affect the gelatinase activities released by 10-day-differentiated human preadipocytes, medium conditioned overnight by preadipocytes treated with IDV (50 μM), RTV (5 μM), SQV (10 μM), or NFV (5 μM) was collected and analyzed by gelatin zymography.

As observed in Fig. 4, MMP-9 (92 kDa) and MMP-2 (72 kDa) proforms (artificially activated by the gelatin zymography technique) were the major forms detectable in preadipocyte-conditioned media. An active form was visible for MMP-2 (62 kDa) but not for MMP-9 (82 kDa). Densitometric analysis of the lytic areas showed that HIV-PI treatments reduced the MMP-9 gelatinase activity (proform) released by treated preadipocytes (53, 40, 74, and 91% decrease with IDV, RTV, SQV, and NFV, respectively). Interestingly, this decrease of MMP-9 gelatinase activity in the medium, corresponding to the proform, was not accompanied by an increase of the active MMP-9 form (still not detectable), suggesting that this reduction was due to a decrease in the secretion of the enzyme rather than to an increase in the maturation.

Unlike for MMP-9, the MMP-2 gelatinase activity (pro + active forms) released into the medium by treated preadipocytes did not present a common pattern for the four HIV-PIs used. Indeed, IDV treatment enhanced the MMP-2 activity released into the medium (27% increase), RTV and SQV were devoid of effect, and NFV reduced it (20% decrease) (Fig. 4).

**HIV-PI Treatments Reduce MMP-9 Expression by Human Preadipocytes.** Considering the results obtained with the gelatin zymography technique, real-time RT-PCR analysis was performed on mRNAs extracted from the same 10-day-treated preadipocytes to investigate any effect of HIV-PIs on MMP-9 expression.

As depicted in Fig. 5, all HIV-PI treatments led to a sta-
A statistically significant reduction of MMP-9 expression: 64% decrease with IDV (50 μM), 75% decrease with RTV (10 μM), 82% decrease with SQV (10 μM), and 96% decrease with NFV (5 μM). These data are in accordance with those obtained by gelatin zymography, suggesting that the observed decrease of MMP-9 gelatinase activity released into the medium under HIV-PI treatments is probably due to a reduction of MMP-9 expression.

MMP-9 Inhibitor Treatment Decreases the Differentiation of Human Preadipocytes. Following the previous results with batimastat and HIV-PI treatments, suggesting a particular involvement of MMP-9 subtype in human adipogenesis, primary cultures of human preadipocytes were treated or not with 1 or 5 μM MMP-9 inhibitor for 10 days. Total protein and cell triglyceride contents were assayed, and the expression of aP2 and HSL was analyzed by real-time RT-PCR.

As observed in Table 1, protein and triglyceride contents were significantly decreased (44 and 52% decrease in protein and triglyceride concentrations, respectively) as well as mRNA expression of aP2 and HSL (67 and 73% decrease in aP2 and HSL, respectively) in cultures receiving 5 μM MMP-9 inhibitor, indicating an alteration of the differentiation process of treated preadipocytes. As for batimastat, MMP-9 inhibitor at 1 μM had no effect on human adipocyte differentiation (data not shown).

MMP-9 Inhibitor Treatment but Not Batimastat Treatment Decreases MMP-9 Gelatinase Activity Released by Human Preadipocytes. To determine whether the two MMP inhibitors could modify the release of the native preadipocyte-derived MMP-9 and -2, we performed gelatin zymography on medium conditioned overnight by preadipocytes treated for 10 days with 5 μM batimastat or MMP-9 inhibitor.

As previously reported (Fig. 4), MMP-9 (92 kDa) and MMP-2 (72 kDa) proforms (artificially activated by the gelatin zymography technique) were the major forms detectable in preadipocyte-conditioned media. Surprisingly, densitometric analysis of the lytic areas showed that a 10-day treatment with MMP-9 inhibitor reduced MMP-9 gelatinase activity released into the medium by preadipocytes (84% decrease), without affecting that of MMP-2 (pro + active forms) (Fig. 6). This decrease of MMP-9 gelatinase activity in the medium, corresponding to the proform, was not accompanied by an increase of the active-MMP-9 form (still not detectable), suggesting that this reduction was due to a decrease in the secretion of the enzyme rather than an increase in the maturation. Real-time RT-PCR analysis performed on mRNAs extracted from the same 10-day-treated preadipocytes revealed a decrease of MMP-9 expression (data not shown), confirming the results obtained with gelatin zymography. Batimastat treatment had no effect on MMP-9 or MMP-2 gelatinase activities released into the medium by treated human preadipocytes (Fig. 6).

Discussion
We previously reported that human mature adipocytes and preadipocytes as well as murine preadipocytes produced and released two key enzymes of ECM remodeling, the matrix metalloproteinases MMP-2 and -9 (Bouloumié et al., 2001). Moreover, we showed that treatment of murine 3T3-F442A preadipocytes with MMP inhibitors decreased the rate of adipocyte differentiation. These data were recently confirmed by others, suggesting that MMP activities were required for adipogenesis in rodents (Croissandeau et al., 2002; Chavey et al., 2003). In the present work, we found that batimastat, a broad-spectrum MMP inhibitor, also reduced the differentiation of human preadipocytes in primary cultures, demonstrating for the first time that MMP activities are required for adipogenesis in humans too. This effect was strictly mediated by its action per se on MMP activities since batimastat strongly inhibited the activity of human recombinant MMP-2 and -9 without affecting their secretion by treated preadipocytes.

Other protease inhibitors, i.e., HIV-PIs, involved in lipo-
dystrophy seen in HIV-patients receiving PI therapy (Carr et al., 1998), have been recently linked to alterations of adipocyte differentiation in murine cell lines in vitro (Zhang et al., 1999; Lenhard et al., 2000; Mondal et al., 2001). We reported here that treatments with HIV-PIs IDV, RTV, SQV, and NFV reduced the differentiation of human preadipocytes in primary cultures. The effectiveness of these products on the human differentiation process, NFV, SQV > RTV, and IDV, was comparable with that described in other in vitro studies (Lenhard et al., 2000; Jain and Lenhard, 2002).

According to the inhibitory action of both batimastat and HIV-PIs on the human differentiation process, we then investigated the potential relationships between MMPs and, more particularly, MMP-2 and -9 and HIV-PIs. We did not find any direct effect of HIV-PIs on human recombinant MMP-2 and -9 activities. However, we found that all HIV-PI treatments reduced the release of MMP-9 by treated preadipocytes. This effect was specific to the MMP-9 gelatinase subtype since MMP-2 release did not present the same profile of changes as MMP-9 but depended on which HIV-PI was used as treatment. The decrease of MMP-9 secretion might be due to the reduction of MMP-9 mRNA expression observed with preadipocytes under HIV-PI treatments. Interestingly, the effectiveness of HIV-PIs (NFV and SQV versus RTV and IDV) at ASPET Journals on April 13, 2017 jpet.aspetjournals.org Downloaded from

![Fig. 4. Effect of HIV-PIs on MMP-2 or MMP-9 gelatinase activities released into culture medium by human preadipocytes. Stromal preadipocytes from human subcutaneous AT were cultured in an adipogenic medium with vehicle (control) or in the presence of 50 μM IDV, 10 μM RTV, 10 μM SQV, or 5 μM NFV. After 10 days of culture, the cells were washed and maintained overnight in basal medium with treatments. Media were then collected and analyzed by gelatin zymography. Densitometric analysis of the lytic areas was performed, and results are expressed as a percentage of the control. Data are means ± S.E.M. for four to seven independent experiments. *, p < 0.05 versus control; **, p < 0.01 versus control.](image)

![Fig. 5. Effect of HIV-PIs on MMP-9 expression by human preadipocytes. Stromal preadipocytes from human subcutaneous AT were cultured in an adipogenic medium with vehicle (control) or in the presence of 50 μM IDV, 10 μM RTV, 10 μM SQV, or 5 μM NFV. After 10 days of culture, the cells were washed and maintained overnight in basal medium with treatments. Total RNAs were extracted from cells, and real-time RT-PCR analysis was performed using specific primers for MMP-9 cDNAs. The results obtained were normalized to the levels of 18S rRNA. Data are means ± S.E.M. expressed as a percentage of the control for five independent experiments.](image)
results are expressed as a percentage of the control. Data are means ± S.E.M. for five to seven independent experiments.

Effect of batimastat and MMP-9 inhibitor on MMP-2 or MMP-9 gelatinase activities released into culture medium by human preadipocytes. Stromal preadipocytes from human subcutaneous AT were cultured in an adipogenic medium in the presence of 5 μM batimastat, 5 μM MMP-9 inhibitor (MMP-9 Inh), or its vehicle (control). After 10 days of culture, the cells were washed and maintained overnight in basal medium with treatments. Media were then collected and analyzed by gelatin zymography. Densitometric analysis of the lytic area was performed, and results are expressed as a percentage of the control. Data are means ± S.E.M. for five to seven independent experiments. **, p < 0.01 versus control.

IDV) on MMP-9 secretion and/or expression was similar to that observed on the differentiation process.

Although few data are available in the literature concerning the potential relationships between MMPs and HIV-Pis, Mondal et al. (2001) have recently reported a strong increase in the release of active MMP-2 and a slight increase in the release of pro and active MMP-9 by differentiating 3T3-L1 cells under IDV and RTV treatment. Paradoxically, the same authors described inhibition of 3T3-L1 adipocyte differentiation with both HIV-Pis. These results are unexpected since other studies have reported an increase of secreted MMP-2 and/or -9 in murine preadipocyte models in vitro, including that observed on the differentiation process.

In conclusion, our data reveal the specificity of impact of MMP inhibitors and HIV-Pis on the human fat cell precursors, the preadipocytes. They show that the modulation of the extracellular matrix components through the production of MMPs and, more precisely, MMP-9 might be a key factor in the regulation of human adipose tissue development. They also suggest that, by affecting the production of MMP-9, HIV-Pis promote human adipose tissue atrophy by preventing replacement of lost adipocytes.

Acknowledgments

We thank Marie-Thérèse Canal for technical assistance.

References


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