Involvement of matrix metalloproteinases in the adipose conversion of 3T3-L1 preadipocytes

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When mouse 3T3-L1 preadipocytes are induced to differentiate into adipocytes, they change from an extended fibroblast-like morphology to a rounded one. This change most likely occurs through extracellular matrix remodelling, a process known to be mediated in part by matrix metalloproteinases (MMPs). In this study, we have shown by semi-quantitative reverse transcriptase-PCR, zymographic and immunoblot analysis that MMP-2, MMP-9 and membrane type 1 (MT1)-MMP are regulated during adipose conversion. To assess the importance of MMPs for adipocytic differentiation we have used MMP-specific inhibitors as well as neutralizing antibodies. Treatment of 3T3-L1 preadipocytes with the broad MMP inhibitor Ilomastat or the more restricted MMP-2 Inhibitor I prevented their differentiation into adipocytes in a dose-dependent manner, as evidenced by absence of triglyceride accumulation. Inhibitor treatment prevented the fibronectin-network degradation, as well as the induction of

the genes for peroxisome-proliferator-activated receptor γ and adipisin, two adipocyte phenotype markers. Inhibitor treatment was effective when applied during the early stages of adipocytic conversion, whereas inhibitor treatment during later stages had little effect. Inhibitor treatment did not inhibit clonal mitotic expansion; nor did it affect the expression pattern of the adipogenic transcription factor CCAAT/enhancer-binding protein β (C/EBP β) or its nuclear translocation. It did, however, markedly reduce C/EBP β DNA-binding capacity. Taken together, these results suggest that MMPs, and notably MMP-2 and MMP-9, may be necessary mediators of adipocytic differentiation of 3T3-L1 cells.

Key words: CCAAT/enhancer-binding protein, extracellular matrix, fibronectin, peroxisome-proliferator-activated receptor gamma.

INTRODUCTION

Post-confluent, growth-arrested mouse 3T3-L1 preadipocytes differentiate into adipocytes when treated with an inducing mixture that includes insulin (Ins), dexamethasone (Dex) and isobutylmethylxanthine (IBMX) in the presence of fetal bovine serum (FBS) [1,2]. The sequence of events that leads to expression of adipocyte-specific genes in these cells involves activation of key transcriptional factors, notably peroxisome-proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer-binding proteins (C/EBPs) and sterol regulatory element binding protein 1 (ADD1/SREBP1) [3–5]. C/EBP β and C/EBP δ expression is induced early in the differentiation process in response to IBMX and Dex. These factors then trans-activate expression of PPAR γ and C/EBP α genes. PPAR γ and C/EBP α in turn activate transcription of adipocyte genes and thereby promote adipogenesis [6–9]. ADD1/SREBP1 also participates in the process by activating PPAR γ gene expression and promoting the production of an endogenous PPAR γ ligand [10,11].

As they differentiate, the fibroblastic preadipocytes change into rounded, lipid-filled mature adipocytes. These morphological changes result from alterations of expression and organization of extracellular matrix (ECM) and cytoskeleton components. Indeed, it has been shown that actin and tubulin gene expression decreases very early in the differentiation program [12,13]; that production of the basement membrane proteins laminin, entactin and collagen is modulated during differentiation [14–16]; and that fibronectin can down-regulate the expression of

genes for lipogenic proteins, and that its synthesis is diminished during adipose conversion [13,16–18]. Thus, diminished *de novo* expression and proteolytic degradation of pre-existing basement-membrane components of the ECM are critical events in adipocytic differentiation.

The balance between ECM deposition and degradation is orchestrated by a family of zinc-dependent proteases known as the matrix metalloproteinases (MMPs) [19–21]. Recently, it was shown that MMP-3/stromelysin-1 regulates adipogenesis during mammary gland involution [22]. The involvement of other MMPs in adipocyte differentiation has yet to be addressed. Among MMPs, the gelatinase subgroup, which comprises MMP-2 and MMP-9, are most likely to also be involved in such a process. Indeed, these enzymes preferentially degrade collagen IV, laminin and fibronectin [20]. In addition, MMP-2 has been found to be secreted by rat primary adipocytes and to be required for the formation of multicellular adipose clusters [23].

The aim of the present work was to analyse the importance of MMPs, and particularly of MMP-2 and MMP-9, in 3T3-L1 adipocytic differentiation. Here we show that inhibition of MMP activities during the initial stages of differentiation can block adipogenesis.

MATERIALS AND METHODS

Cell culture and differentiation

3T3-L1 preadipocytes were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's

Abbreviations used: ADD1/SREBP1, sterol regulatory element binding protein 1; C/EBP, CCAAT/enhancer-binding protein; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; IBMX, isobutylmethylxanthine; Ins, insulin; LAP, liver-enriched activating protein; LIP, liver-enriched inhibitory protein; MMP, matrix metalloproteinase; proMMP, precursor MMP; intMMP, intermediate MMP; MT1 MMP, membrane type 1 MMP; PPAR γ , peroxisome-proliferator-activated receptor gamma; RT-PCR, reverse transcriptase-PCR; TIMP-2, tissue inhibitor of metalloproteinase-2.

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medium (DMEM) containing 10 % (v/v) FBS. On experimental day 0 (2 days after they reached confluence), cells were induced to differentiate with 1.7 μ M Ins, 0.5 mM IBMX and 1 μ M Dex. On day 2 and every other day afterwards, fresh medium was substituted until day 7. Alternatively, post-confluent cells were incubated in medium containing varying concentrations of either Ionomycin (Chemicon International, Temecula, CA, U.S.A.) or MMP-2 Inhibitor I (Calbiochem, La Jolla, CA, U.S.A.). Both inhibitors were diluted in DMSO, and in all experiments cells were exposed to an identical concentration of vehicle. In some experiments, MMP-2 or MMP-9 neutralizing antibodies (Neomarkers) were used. Differentiation was assessed by microscopy and by Oil Red O staining. In the latter method, cells were washed twice with PBS, fixed in 3.7 % formaldehyde for 1 h and stained for 30 min with a 0.36 % (v/v) Oil Red O solution in 60 % (v/v) isopropanol. They were then washed several times with water [1]. For some experiments cell number was determined by trypsinizing cell monolayers from 6-well culture dishes and counting the suspended cells.

Semi-quantitative reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted using a guanidine isothiocyanate method described previously [24]. The RNA was treated with RNase-free DNaseI (Life Technologies, Burlington, ON, Canada). A 2 μ g aliquot of DNaseI-treated RNA was reverse-transcribed into cDNA using the SuperScript II Reverse Transcriptase (Life Technologies). A 3 μ l aliquot of the reverse transcription reaction was utilized for PCR amplification of cDNA fragments for MMP-2 (sense primer, 5'-TGT GTC TTC CCC TTC ACT TT-3'; antisense primer, 5'-GAT CTG AGC GAT GCC ATC AA-3'; 405 bp PCR product), MMP-9 (sense primer, 5'-GTA CCG CTA TGG TTA CAC-3'; antisense primer, 5'-CCG CGA CAC CAA ACT GGA T-3'; 372 bp PCR product), PPAR γ 1 and PPAR γ 2 (collectively called PPAR γ ; sense primer, 5'-GGA AAG ACA ACG GAC AAA TCA C-3'; antisense primer, 5'-ATC CTT GGC CCT CTG AGA TG-3'; 325 bp PCR product), adipon (sense primer, 5'-CCT GAA CCC TAC AAG CGA TG-3'; antisense primer, 5'-GGT TCC ACT TCT TTG TCC TCG-3'; 170 bp PCR product) and the ribosomal protein L30 (sense primer, 5'-AAG TGG GAA GTA CGT GCT GG-3'; antisense primer, 5'-CAC CAG TCT GTT CTG GCA TG-3'; 272 bp PCR product) as internal standard. The PCR reaction mixes contained 0.5 unit of rTaq DNA polymerase (Life Technologies), 1 $\times$ PCR buffer, 0.2 mM dNTP, 0.5 μ M of sense and antisense primers for L30 and adipon or PPAR γ in a total volume of 50 μ l. PCR was performed with MMP-2 or MMP-9 primers respectively for a total of 27 and 37 cycles, together with L30 primers for the last 22 cycles. For PPAR γ and adipon semi-quantification, the reaction was conducted for 22 cycles with their respective primers and L30 primers. The number of cycles was pre-determined to fall within the linear range of amplification of each PCR product. Each cycle involved a 94 °C for 1 min denaturation step, a 58 °C for 1 min (with MMP-2 and MMP-9) or 60 °C for 1 min (with PPAR γ and adipon) annealing step, and a 72 °C for 1 min polymerization step. The PCR products were electrophoresed on agarose gels, stained with ethidium bromide, revealed by UV irradiation and analysed by densitometry using the National Institutes of Health Image software. The authenticity of the amplified sequences was verified by restriction enzyme mapping.

Zymographic analysis

3T3-L1 cell monolayers in 12-well plates were rinsed then incubated for 20 h in 1 ml of serum-free medium. The media

were collected and concentrated by centrifugation at 3000 *g* for 15 min through Centricon YM-30 membranes (Millipore, Bedford, MA, U.S.A.). Aliquots (normalized according to the corresponding initial volume) were electrophoresed under non-reducing conditions on 10 % (w/v) polyacrylamide gels containing 0.1 % (w/v) gelatin. The gels were washed in 2 % (v/v) Triton X-100 for 1 h and incubated at 37 °C for 16 h in an activation buffer (50 mM Tris/HCl, pH 7.4, 200 mM NaCl, 5 mM CaCl₂ and 0.02 % (w/v) NaN₃). They were then stained with Coomassie Brilliant Blue R-250.

Western-blot analysis

For membrane type 1 (MT1)-MMP experiments, the cells were washed with PBS and proteins were extracted in 1 ml of PBS/2 % (v/v) Nonidet P40. Proteins (50 μ g) were separated by SDS/PAGE on 10 % (w/v) polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). The membranes were incubated with an anti-(MT1-MMP) antibody (Chemicon International) and immunoreactive proteins were revealed by chemiluminescence (Amersham, Baie d'Urfé, QC, Canada). To equalize protein loadings, a preliminary protein gel was stained with Coomassie Brilliant Blue R-250 for a visual estimation of the quantity of proteins in the cellular extracts. For C/EBP β experiments, the cells were washed with PBS and scraped off the dishes in 1 ml of PBS. They were sedimented by centrifugation, lysed by the addition of 100 μ l of SDS-gel buffer, and heated to 100 °C for 5 min [6]. Extracted proteins were fractionated by SDS/PAGE on 12 % (w/v) polyacrylamide gels and analysed as described above with an anti-C/EBP β antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Nuclear extracts were prepared as described by Dent and Latchman [25] from cells treated for 24 h with the adipogenesis-inducing agents. Aliquots equivalent to 30 μ g of proteins were diluted in SDS-gel buffer, boiled for 5 min and analysed by Western blotting for C/EBP β proteins.

Electrophoretic mobility-shift assay (EMSA)

A double-stranded C/EBP consensus oligonucleotide (5'-GAT CGA TTG CGC AAT C-3'), end-labelled with ³²P [26] was used as probe. The binding mixture contained 5 μ g of nuclear extract, 2 μ g of poly (dI)-(dC), 20 mM Hepes, pH 7.9, 10 % glycerol, 0.5 mM DTT, 0.5 mM EDTA, 0.1 M KCl and 40 fmol of labelled probe (20000 c.p.m.). It was incubated for 10 min at 22 °C. In competition assays, prior to adding the labelled probe, the mixture was incubated for 10 min with 4 nmol (100-fold molar excess) of unlabelled double-stranded C/EBP oligonucleotide or of an unrelated double-stranded oligonucleotide (5'-GGT TCA GCT CCT CCT CGG CTC CAA TTC-3'). In supershift assays, nuclear extracts were incubated for 60 min with 1 μ g of an antibody directed against the C-terminus of C/EBP β prior to adding the labelled probe. The binding mixtures were electrophoresed at 250 V and 22 °C in a 4 % non-denaturing polyacrylamide gel with a 25 mM Tris/190 mM glycine/1 mM EDTA buffer. The gel was then dried and exposed to a phosphorimaging screen for 16 h. The pixel values in shifted complexes were quantified on a Typhoon phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Immunohistochemistry

Cells were grown to confluence on microscope slides, then induced to differentiate in the presence or absence of MMP inhibitors. On days 0 and 7 of the differentiation process, they were rinsed with cooled PBS and fixed in cooled (30:70, v/v)

methanol/acetone mix for 15 min. The cells were rehydrated in PBS for 15 min and treated with 2% (w/v) BSA in PBS for 30 min to block non-specific binding sites. They were then incubated with rat anti-fibronectin primary antibody (Calbiochem) for 1 h at 22 °C, then washed three times with PBS for 5 min each time. Finally the cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (Cedarlane laboratories limited, Hornby, Ontario, Canada) for 1 h at 22 °C before they were washed and examined by immunofluorescence microscopy.

RESULTS

Regulation of MMP-2, MMP-9 and MT1-MMP expression during adipocyte differentiation

The gelatinase activities present in 3T3-L1 conditioned media, collected during the proliferation phase and from days 0 to 7 of the differentiation program were examined by zymography (Figure 1). Five gelatinase bands of 92, 68, 66, 62 and 59 kDa were detected. The 92 kDa band corresponds to precursor MMP-9 (proMMP-9), the 66, 62 and 59 kDa bands to the precursor (pro), intermediate (int) and mature forms of MMP-2, respectively. The 68 kDa band represents a yet unidentified gelatinase activity. During the proliferation phase, proMMP-2 was the more abundant band detected compared with proMMP-9, intMMP-2 and mature MMP-2 bands (Figure 1A). ProMMP-2 represented $71.62 \pm 4.89\%$ (mean $\pm$ S.E.M.) of MMP-2 activity (Figure 1B), the remaining 28% being equally represented by the intMMP-2 and MMP-2 forms. At cell confluency (day 0), the combined activities of these processed forms increased to 53% of MMP-2 activities ($P < 0.005$), they reached a maximum of 62% at days 3 and 4 of differentiation and then slightly decreased by day 7 ($P < 0.01$). ProMMP-9 activity remained unchanged from day 0 to day 4, but decreased three-fold by day 7 ($P < 0.005$, Figure 1A). To further confirm the synthesis of MMP-2 and MMP-9 in 3T3-L1 cells, we monitored the levels of their respective transcripts by semi-quantitative RT-PCR (Figure 1C). These levels increased 2–3-fold ($P < 0.005$) between days 0 and 7 of the adipogenic conversion.

Because proMMP-2 is known to be processed by MT1-MMP [27–29] we examined, by immunoblotting, the levels and molecular forms of the latter enzyme during adipocytic differentiation (Figure 1D). High levels of precursor (63 kDa), matured (60 kDa) and truncated (45 kDa) forms of MT1-MMP were observed in proliferating cells. During adipose conversion, the proMT1-MMP form gradually decreased from days 0 to 3 and became undetectable on days 5 and 7. The mature MT1-MMP form did not change significantly during the conversion. The truncated form, on the other hand, gradually increased, reached a maximum at days 2 and 3, and then decreasing afterwards, in a pattern similar to that of the processed forms of MMP-2. These results suggested that adipocytic differentiation of 3T3-L1 cells is accompanied by either increased truncation of proMT1-MMP to the 45 kDa form or its activation to the 60 kDa form which is then rapidly truncated to the 45 kDa form. These changes may contribute to the modulation of the relative amounts of MMP-2 activities observed during adipogenesis.

MMP inhibitors and neutralizing antibodies block adipocyte differentiation

To assess the importance of MMPs in adipogenesis, we examined whether two MMP inhibitors, Ilomastat and MMP-2 Inhibitor I, would impair this process. Ilomastat is a potent inhibitor of human MMP-1, -2, -3, -8 and -9. MMP-2 Inhibitor I is specific

Figure 1 MMP-2 and MMP-9 activities and MT1-MMP expression are regulated during adipocyte differentiation

(A) Zymography of conditioned culture medium collected from 3T3-L1 cells during proliferation phase (P) and adipose conversion (days 0 to 7). Non-identified gelatinase activity (★). (B) Quantification of proMMP-2 activation by scanning densitometry of the gel depicted in panel (A). The relative proportions of MMP-2 precursor (proMMP-2), intermediate (intMMP-2) and mature (MMP-2) forms is expressed as percentage of total MMP-2. (C) Total RNA isolated at days 0 and 7 of the adipose conversion was analysed for MMP-2 and MMP-9 gene expression by semi-quantitative RT-PCR. Ribosomal protein L30 was used as internal standard. (D) Whole proteins were extracted during the proliferation phase and at various times after adipogenic induction. Samples (50 μ g of proteins) were fractionated by SDS/PAGE and analysed by immunoblotting for MT1-MMP. The antibody recognized the MT1-MMP precursor (proMT1-MMP), mature and truncated forms. A portion of a blot stained with Coomassie Blue is shown in the bottom panel (Stain). Results are representative of four independent experiments.

for MMP-2. Post-confluent 3T3-L1 preadipocytes were incubated in medium containing different concentrations of each inhibitor (0 to 50 μ M) for the 48 h of adipogenic stimulation, and then in inhibitor-free medium until day 7. Accumulation of cytoplasmic triglycerides was assessed by Oil Red O staining as a measure of adipogenic conversion. Incubation with either inhibitor blocked the conversion in a concentration-dependent manner (Figures 2A and 2B). The blockage was partial at 10 μ M and complete at 25 μ M. Microscopic examination and Trypan Blue exclusion tests performed on inhibitor-treated cells did not reveal any sign of cytotoxicity, at any of the inhibitor concentrations used or at

Figure 2 Effect of MMP inhibitors on adipocyte differentiation

Post-confluent 3T3-L1 cells were incubated for 48 h during the adipogenic induction in culture medium containing Ilomastat (**A**) or MMP-2 Inhibitor I (**B**) at the indicated concentrations. The cells were subsequently cultured for 5 days in inhibitor-free medium and stained with Oil Red O on day 7. (**C**) 3T3-L1 cells were treated with Ilomastat (25 μM) or MMP-2 Inhibitor I (20 μM) for various time periods (as indicated; P, proliferation phase) before, during or after induction to differentiate. Cells were then stained with Oil Red O on day 7. These experiments were performed on three separate occasions with similar results.

any stage during the differentiation process (results not shown). All subsequent experiments were performed using Ilomastat at 25 μM and MMP-2 Inhibitor I at 20 μM.

We then examined whether there was a time window in the differentiation program when MMP activities were most required. The results shown in Figure 2(C) indicate that treating the cells with either inhibitor on days 0 and 1 only (0–1) of the differentiation protocol blocked adipocytic conversion as effectively as exposing them to the inhibitor for the whole length of the protocol (0–7). Exposing them from day 2 or 3 (days 2–7 or 3–7) had a slight inhibitory effect. In contrast, the inhibitors had no visible effect when added to the cells during the proliferation phase (P) or later in the differentiation protocol (days 5–7). We also observed a decrease in lipid accumulation when preadipocytes were treated with MMP-2 and MMP-9 neutralizing antibodies during the 2-day stimulation of the adipose conversion, whereas incubation with normal rabbit serum had no effect (Figure 3). These results suggest that MMP activities are required for adipocytic conversion, but mainly in the early phases of the process.

Soon after differentiation induction, 3T3-L1 preadipocytes undergo approximately two rounds of mitotic clonal expansion as they express the early adipogenic genes [30]. We examined whether this event was altered by treatment with MMP inhibitors. We induced post-confluent 3T3-L1 preadipocytes to differentiate in the absence or presence of Ilomastat or MMP-2 Inhibitor I and determined their number after 3 days. For both vehicle- and inhibitor-treated cells, the number increased almost 4-fold (results not shown), indicating that the MMP inhibition did not affect mitotic clonal expansion step.

MMP inhibitors prevented fibronectin network degradation

Fibronectin is one of the MMP-2 substrates in the ECM [31]. It is known to inhibit preadipocyte differentiation [17] and its expression is down-regulated during adipogenesis [13,16]. We verified by immunocytochemistry the effect of Ilomastat and MMP-2 Inhibitor I on the fibronectin network on day 7 of the differentiation protocol. This network diminished noticeably in

Figure 3 Effect of neutralizing antibodies on adipocyte differentiation

Post-confluent 3T3-L1 cells were incubated for 48 h during the adipogenic induction in culture medium containing 1 µg/ml normal rabbit serum (NRS), anti-MMP-2 or anti-MMP-9 antibody. The cells were subsequently cultured for 5 days in inhibitor-free medium and stained with Oil Red O on day 7. Lipid accumulation was quantified by measuring absorbance at 510 nm. *, $P < 0.05$. These experiments were performed on three separate occasions with the same results.

control differentiated cells, but was preserved in inhibitor-treated undifferentiated cells (Figure 4). This suggests that the prevention of fibronectin degradation is one mechanism through which these inhibitors block adipocytic differentiation. This was confirmed in immunoblotting experiments (results not shown).

MMP inhibitor-induced adipogenic block is associated with reduced expression of PPAR γ and adipsin, but normal expression of C/EBP β

To determine whether the differentiation block was due to lack of induction of adipogenic genes, we measured by semi-quantitative RT-PCR the relative levels of transcripts for PPAR γ and adipsin (an early and a late marker of the adipocyte differentiation, respectively) in vehicle- and MMP inhibitor-treated cells. The results are illustrated in Figure 5. As expected, the levels of PPAR γ and adipsin mRNA transcripts markedly increased in control cells after adipogenic induction. In contrast, in cells incubated with Ilomastat or MMP-2 Inhibitor I, a similar

Figure 4 MMP inhibitor treatment prevented fibronectin network degradation

Post-confluent 3T3-L1 cells were subjected to adipogenic induction in the presence or absence of Ilomastat (25 µM) or MMP-2 Inhibitor I (20 µM). On days 0 and 7 of the adipose program, immunocytochemical analyses were performed with an anti-fibronectin antibody (Ab). Magnification, 20 ×.

Figure 5 Effect of MMP inhibitors on PPAR γ and adipsin gene expression following induction of adipocyte differentiation

Total RNA isolated at various times after adipogenic induction, in the presence or absence of Ilomastat (25 µM) or MMP-2 Inhibitor I (20 µM), was analysed for PPAR γ and adipsin gene expression by semi-quantitative RT-PCR. Ribosomal protein L30 was used as an internal standard. Results are representative of four independent experiments.

Figure 6 Effect of MMP inhibitors on C/EBP β expression and activity

Preadipocytes were subjected to an adipogenic treatment in the presence or absence of Ilomastat (25 μ M) or MMP-2 Inhibitor I (20 μ M). (A) Whole cell extracts were prepared at

treatment produced only a slight and belated induction in the level of adiponin and PPAR γ transcripts.

Since transcription of the PPAR γ gene during adipogenesis is controlled by the C/EBP β factor [9,32], we investigated whether treatment with either inhibitor altered C/EBP β protein expression during adipose conversion. We analysed, by immunoblotting, the level of this protein in total extracts prepared from vehicle- and inhibitor-treated cells at different points in the differentiation protocol. The results showed that the inhibitors had no effect on either the levels or the pattern of expression of the LIP (liver-enriched inhibitory protein) and LAP (liver-enriched activating protein) C/EBP β isoforms (Figure 6A). We performed the same analysis on nuclear proteins extracted 24 h after induction of differentiation. No significant difference in the levels of C/EBP β was observed between vehicle- and inhibitor-treated cells (Figure 6B), an indication that the treatment did not affect C/EBP β translocation into the nucleus. Analysis of cytosolic extracts (results not shown) also supported this conclusion. However, when the nuclear extracts were analysed by C/EBP β -specific EMSA, the radiolabelled complex observed with extracts from cells treated with Ilomastat inhibitor was about half as intense as that observed with extracts from control cells, as determined by densitometry (Figure 6C, compare lane 2 with lane 6). Some reduction in complex intensity was also observed with extracts from cells treated with MMP2 Inhibitor I, but it was less consistent. In all cases, the oligonucleotide electrophoretic shift was specifically due to C/EBP β binding since it could be completely abrogated in the presence of a 200-fold molar excess of unlabelled C/EBP oligonucleotide (Figure 6C, lanes 3, 7 and 11) whereas a 200-fold molar excess of the unrelated RE1-lk oligonucleotide had no effect (Figure 6C, lanes 4, 8 and 12). The complexes were further retarded in the presence of an anti-C/EBP β antibody (Figure 6C, lanes 5, 9 and 13), confirming that they contained this factor. These results suggest that diminished binding of C/EBP β to its cognate element in the PPAR γ gene promoter may be one of the reasons why adipogenic induction failed to increase the level of PPAR γ transcripts in cells treated with the MMP inhibitors, particularly the broader inhibitor Ilomastat.

DISCUSSION

The mouse 3T3-L1 preadipocyte cell line is a good model for studying adipogenesis because its adipose conversion program is well documented [3–5]. Using inhibitor-based approaches, many recent studies have demonstrated that aspartyl or serine endoproteases are involved in the differentiation of 3T3-L1 cells [33–36]. The goal of this study was to examine the importance of members of the matrix metalloproteinase family of endoproteases in this process. These extracellular proteinases degrade components of the extracellular matrix. They have been shown to be essential for embryonic development, morphogenesis and reproduction [19–21]. They are also involved in cellular remodelling during adipogenesis [4,12–18]. Among them, MMP-

various times after adipogenic induction and aliquots were fractionated by SDS/PAGE then analysed by immunoblotting for C/EBP β . The antibody recognized the LAP (liver-enriched activating protein, 32 kDa) and the LIP (liver-enriched inhibitory protein, 18 kDa) C/EBP β isoforms. (B) Nuclear extracts (30 μ g) were prepared from vehicle- or inhibitor-treated preadipocytes exposed for 24 h with adipogenesis-inducing agents and analysed by immunoblotting for C/EBP β . (C) To examine C/EBP β DNA-binding capacity, an EMSA of a radiolabelled C/EBP consensus oligonucleotide probe was conducted using 5 μ g of the nuclear extract proteins examined in (B). For supershift assays, extracts were preincubated with an antibody directed against the C-terminus of C/EBP β . In competition assays, a 200-fold molar excess of unlabelled C/EBP or of an unrelated oligonucleotide was added to the binding mixture. The vertical bar identifies the position of the specific complexes.

2 seemed of particular interest, as it reportedly plays a role in the destruction of the original basement membrane matrix during the multicellular clustering of rat adipocytes [23]. Our RT-PCR and zymographic analyses show that expression and secretion of MMP-2 and MMP-9 by 3T3-L1 cells are regulated during adipocytic differentiation. Since membrane-bound MT1-MMP is known to initiate proMMP-2 activation [27–29], we also examined the expression of immunoreactive MT1-MMP. The level of the 60 kDa mature form did not significantly vary during differentiation. In contrast, the level of the 63 kDa precursor form diminished during the process while that of the 45 kDa truncated form increased. This increase was concomitant with the elevation of gelatinase activities associated with processed MMP-2, in agreement with previous reports [29,37]. The mechanism leading to the production of the truncated form of MT1-MMP is unknown. This form may result from direct cleavage of the proMT1-MMP or from secondary cleavage of the mature MT1-MMP. In the latter case, differentiation may be associated with a transient increase of the mature form and its rapid conversion into the truncated form. The mature form may be primarily responsible for proMMP-2 activation. Whether the truncated form also participates in this activation is still to be determined. Recent studies propose that proMMP-2 activation requires also the presence of the tissue inhibitor of metalloproteinase-2 (TIMP-2) which constitutes a ternary complex in association with MMP-2 and MT1-MMP [27–29]. It has been reported that expression of TIMP-2 was unaffected by adipocyte differentiation [22].

To assess the importance of MMPs in adipocytic differentiation, we exposed 3T3-L1 cells to either Ilomastat or MMP-2 Inhibitor I as well as MMP-2 and MMP-9 neutralizing antibodies. Neither synthetic inhibitor affected cell growth when added to proliferating preadipocytes, or expression of differentiation markers when added to fully adipocytes for a period of 6 days (results not shown). The inhibitors did block adipocytic conversion provided that they were present in the cell culture medium during the initial stages of the differentiation program, suggesting that this is when MMP activities play a critical role. The fact that the more specific MMP-2 Inhibitor I, as well as the MMP-2 and MMP-9 neutralizing antibodies were able to affect differentiation may be explained by three non mutually exclusive hypotheses: (i) MMP-2 and MMP-9 have a predominant function in preadipocyte membrane remodelling during adipogenesis; (ii) MMP-2 and MMP-9 have a distinct function which converges with the functions of other equally critical MMPs to promote membrane remodelling and adipogenesis; and (iii) MMP-2 and MMP-9 act at a downstream position in a cascade of events involving other MMPs. The use of other specific MMP inhibitors should allow experimental verification of these hypotheses.

The inhibitors did not inhibit the mitotic clonal expansion preceding differentiation, in accordance with our observation that they do not affect cell proliferation. Although this expansion is a consistently observed step in the differentiation program of 3T3-L1 cells, a recent study suggests that it is not a prerequisite for the differentiation [38]. At the molecular level, the block was associated with diminished expression of PPAR γ and adipsin, two markers of adipose conversion. Since PPAR γ expression is controlled by members of the C/EBP [32] and SREBP [39] families, induced expression of C/EBPs leads to increased binding of the factor to its recognition element in the PPAR γ gene promoter and to transactivation of this gene [9,40]. Cell treatment with Ilomastat or MMP2 Inhibitor I did not prevent induction of C/EBP β expression or its translocation into the nucleus but it was associated with a decrease of DNA-binding capacity of C/EBP β . We hypothesized that this diminished binding may be

due to a change in expression of the dominant-negative C/EBP homologous protein (CHOP-10) [41], but the expected down-regulation was also observed in MMP inhibitor-treated cells (results not shown). The diminished DNA-binding capacity of C/EBP β may partly explain why the cascade of C/EBP β -promoted transcription of adipogenic genes such PPAR γ and adipsin is impaired in MMP inhibitor-treated cells. This explanation is consistent with the finding that MMP inhibitors are effective in blocking adipose conversion only if present during the early stages of differentiation, when C/EBP β induction is maximal.

In a recent study, Alexander et al. [22] found that Ilomastat promotes 3T3-L1 adipocytic differentiation. The reasons for the contradiction between their results and ours are unclear. Their experimental protocol differed from ours on two major aspects: (i) they cultured the cells in DME-H21 medium containing fetal calf serum whereas we cultured the cells in DMEM medium supplemented with fetal bovine serum; and (ii) to induce adipogenesis, they used insulin at a concentrations 100-fold higher than we did. It is difficult to rationalize how these differences could lead to such conflicting results. To reconcile these different results, and the fact that ECM remodelling is necessary for adipocyte differentiation, one has to assume that under certain conditions inhibition of Ilomastat-sensitive MMPs causes compensatory up-regulation of other MMPs that are insensitive to the inhibitor and that can also promote the remodelling. Discordant effects of protease inhibitors on 3T3-L1 cell differentiation have been observed before. For example, the HIV protease inhibitor ritonavir was found to increase lipogenesis in one study [36] and to decrease it in another [42]. It is possible that alternative pathways to adipocytic conversion exist, each characterized by qualitatively and quantitatively distinct protease repertoires. The culture conditions may influence which pathway is determining for differentiation.

In a recently published report, Bouloumié et al. [43] have examined the effect of other MMP inhibitors (batimastat and captopril) on the adipocyte differentiation of 3T3F442A cells. They also observed a significant inhibition, corroborating our findings and reinforcing the view that MMPs, in particular MMP-2 and MMP-9, are important for adipocyte conversion. How does inhibition of MMPs, MMP-2 in particular, lead to inhibition of adipocyte differentiation? We have shown that this inhibition leads to the maintenance of the fibronectin network, the destruction of which always accompanies adipocytic differentiation. Bortell et al. [18] have previously shown that inhibition of 3T3-L1 adipocytic conversion by transforming growth factor β 1 is associated with lack of fibronectin down-regulation, suggesting that this down-regulation is an integral part of the differentiation process. Consistent with this notion are the results of studies showing that addition of fibronectin to the culture medium or cultures of cells on fibronectin matrices prevented preadipocyte morphological shape change and adipogenesis [17,44]. Both reports suggest that modified ECM components, acting via their association with cytoskeleton and cell-surface receptors, may initiate mechanisms leading to adipocytic differentiation and the transactivation of adipocyte-specific genes. One of the mechanisms initiated in this way may involve the down-regulation of C/EBP β DNA-binding activity. Our findings that MMP inhibitors did not change the expression of PPAR γ and adipsin in fully differentiated adipocytes also agree with the fact that MMP-2, and possibly other MMPs, are crucial for the initiation of adipose conversion.

In conclusion we have demonstrated that MMPs (MMP-2 and MMP-9 in particular) promote the early steps of 3T3-L1 preadipocyte differentiation into adipocytes.

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