Down-Regulation of MT1-MMP Expression by the α3 Chain of Type IV Collagen Inhibits Bronchial Tumor Cell Line Invasion

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SUMMARY: The basement membrane (BM) is the first barrier encountered by tumor cells when they become invasive. Moreover, some invasive tumor clusters are surrounded by a remnant or neosynthetized BM material. We have previously reported the presence of a particular α chain of type IV collagen, the α3(IV) chain, in bronchopulmonary carcinomas. This chain was not detected in the normal bronchial epithelium, but was found around some invasive tumor cluster BM. In the present study, we examined the effects of the α3(IV) chain on the invasive properties of bronchial tumor cell lines, with special emphasis on their expression of matrix metalloproteinase-2 (MMP-2) and its activator, membrane type 1-matrix metalloproteinase (MT1-MMP), which is largely involved in tumor progression. Two epithelial bronchial cell lines (16HBE14o− and BZR), showing different invasive abilities, were evaluated. Using the Boyden chamber invasion assay, we demonstrated that the α3(IV) chain inhibits the invasive properties of BZR cells and modifies their morphology by inducing an epithelial cell shape. In the presence of the recombinant NC1 domain of the α3(IV) chain, the expression of MMP-2 and tissue inhibitor of metalloproteinase-2 (TIMP-2) was not modified in either cell line. The NC1 α3(IV) domain did not modulate the MT1-MMP expression of noninvasive 16HBE14o− cells, whereas a 50% decrease of MT1-MMP mRNA was observed in invasive BZR cells. Accordingly, Western blot analyses showed a disappearance of the 45-kd MT1-MMP form when BZR cells were treated with the recombinant NC1 α3(IV) domain. These findings suggest that the α3 chain of type IV collagen may play a role in tumor invasion, at least by decreasing the expression and synthesis of MT1-MMP. (*Lab Invest 2001, 81:167–175*).

D uring tumor progression, basement membrane (BM) architecture and composition are dramatically modified. This remodeling is a key step that involves both de novo synthesis at the invasive front and extensive degradation of BM components, including type IV collagen and laminins. Type IV collagen, the major structural component of BM, is a family of proteins composed of three helical isoforms. Six genetically distinct subunits are now described (α1 through α6) (Butkowski et al, 1987; Hostikka et al, 1990, Zhou et al, 1993). Each chain is characterized by a long central Gly-X-Y domain, an amino-terminal 7 S domain, and a globular carboxy-terminal NC1 domain. Several studies have reported that biological activities triggered by the different α(IV) chains can be assigned to the specific NC1 domains of each chain (Chelberg et al, 1989; Monboisse et al, 1994). The most widely distributed form of collagen type IV is a heterotrimer composed of two α1(IV) chains and one α2(IV) chain (Kuhn, 1994; Yurchenco and Schittny, 1990). The other subunits (α3 through α6) have a more restricted tissue distribution, which emphasizes a functional specificity of these six chains (Kahsai et al, 1997; Miner and Saus, 1994; Ninomiya et al, 1995; Zhou et al, 1993). Likewise, the α3(IV) chain has been shown to be involved in several human pathologies, such as Goodpasture and Alport syndromes, in which mutations in the α3(IV) chain gene were associated with a progressive nephropathy. Furthermore, type IV collagen α3–deficient mice have auditory and renal deficits related to alteration in BM structure and function (Cosgrove et al, 1998; Miner and Saus, 1996). Moreover, Monboisse et al (1994) have shown that the α3(IV) chain prevents collagen type I–induced elastase and gelatinase secretion by human polymorphonuclear leukocytes (PMN), thereby decreasing their degradative properties. The α3(IV) chain has also been involved in cancer pathologies. Petitclerc et al (2000) have recently demonstrated that the α3(IV) domain inhibits tumor growth. Moreover, we have reported previously that the α3(IV) chain is present in some tumor BM lining well-differentiated tumor clusters in

bronchial cancers, whereas it is never detected in normal bronchi, suggesting a specific function of the $\alpha3(IV)$ chain in tumor progression (Catusse et al, 2000; Polette et al, 1997).

BM degradation involves activation of matrix metalloproteinases (MMP), a family of zinc-dependent proteases that regulate proteolysis of most ECM components. Among these MMP, MMP-2, which preferentially degrades type IV collagen, has been largely implicated in the BM degradation associated with tumor invasion. MMP-2 overexpression has indeed been correlated with an invasive phenotype in numerous cancer types and in many tumor cell lines (Stetler-Stevenson, 1994). As are most MMP, MMP-2 is secreted as an inactive proenzyme and needs a proteolytic cleavage of the N-terminal prodomain to become active. MMP-2 activation is a membrane-associated mechanism that requires the interaction of MMP-2 with TIMP-2 (tissue inhibitor of metalloproteinases-2) and MT1-MMP (membrane type 1-MMP) (Sato et al, 1994; Stetler-Stevenson, 1994). The MT1-MMP/TIMP 2 complex acts as a cell surface receptor for the C-terminal domain of MMP-2 (Strongin et al, 1995). Several reports have shown that overexpression of MT1-MMP, and thus an increase of MMP-2 activation, are correlated with an invasive phenotype (Gilles et al, 1996; Polette et al, 1998; Sato et al 1996). Although MT1-MMP is now considered as the main in vivo MMP-2 activator, many studies have demonstrated that MT1-MMP can also exhibit intrinsic matrix-degrading activities. Indeed, MT1-MMP has been shown to digest gelatin, β casein, $\varkappa$-elastin, fibronectin, vitronectin, β chain of laminin and dermatan sulfate proteoglycan, native fibrillar type I, II, and III collagen, and laminin-5 (Imai et al, 1996; Koshikawa et al, 2000; Ohuchi et al, 1997; Pei and Weiss, 1996; Sato et al, 1996).

The aim of the present study is to examine the role of the $\alpha3(IV)$ chain in bronchial tumor progression. We thus investigated the influence of the $\alpha3(IV)$ chain on the migration and invasion of two bronchial carcinomas cell lines previously described as representing two different stages of bronchopulmonary tumor progression: 16HBE14o−, a noninvasive bronchial cell line, and BZR, an invasive bronchial cell line. We also explored the effects of the $\alpha3(IV)$ chain on degradative properties of both cell lines, focusing on the potential implication of MMP-2, MT1-MMP, and TIMP-2.

Results

Morphology and Invasive Capacity of Bronchial Carcinomas Cell on Type IV Collagen

To evaluate the influence of the $\alpha3$ chain of type IV collagen on tumor cell invasiveness, we analyzed the invasive capacities of two bronchial cell lines in a modified Boyden chamber. In this assay, the upper surfaces of the filters were coated with human placenta (HP) type IV collagen containing the $\alpha1(IV)$ and $\alpha2(IV)$ chains or bovine lens capsule (BLC) type IV collagen containing the $\alpha1(IV)$, $\alpha2(IV)$, and $\alpha3(IV)$ chains. 16HBE14o− cells did not display in vitro any invasive capacities, regardless of the coating used.

BZR cells demonstrated invasive behavior in Matrigel and HP (IV) collagen. In contrast, the BLC type collagen containing the $\alpha3(IV)$ chain reduced the invasive capacity of BZR cells by approximately 11-fold as compared with the rate of invasion through HP(IV) collagen, which does not contain the $\alpha3(IV)$ chain (Fig. 1A). We also compared the migration ability of BZR cells using peptides spanning different regions of the NC1 domain of the $\alpha3(IV)$ chain. We used the $\alpha3(IV)$ 185 to 203 and 185 to 191 peptides that contain the SNS triplet (residues 189 to 191) unique to the $\alpha3(IV)$ chain and which have been shown previously to have biologic properties (Han et al, 1997). The $\alpha3(IV)$ 194 to 203 peptide, which lacks the SNS triplet and has no described biologic effect, was used as peptide control in these migration experiments. When BZR cells were incubated with the $\alpha3(IV)$ 194 to 203 peptide, tumor cell invasion was not inhibited, whereas the addition of
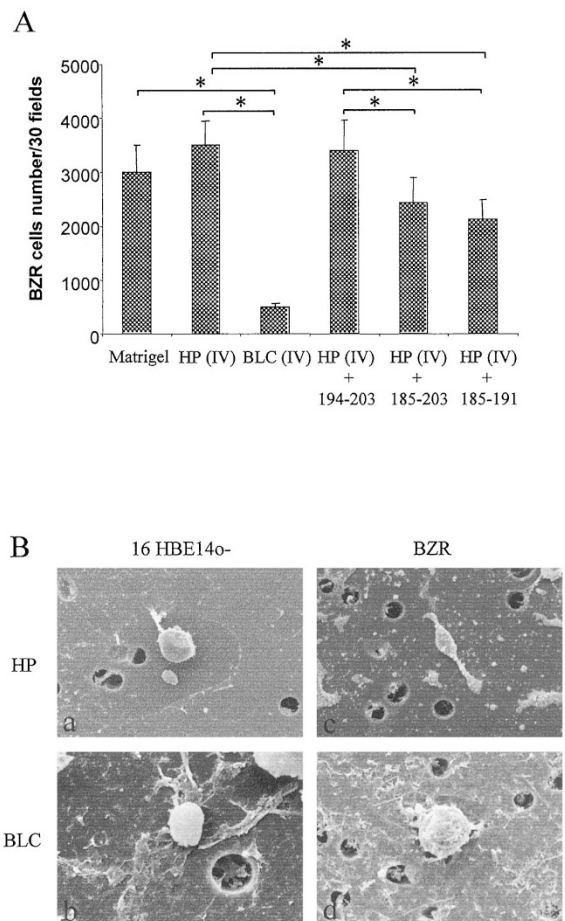
Figure 1.
A, Effects of bovine lens capsule (*BLC*) on BZR cell migration. BZR cells are layered on *Matrigel*, on human placenta (*HP*) type IV collagen, and on BLC type IV collagen. The $\alpha3(IV)$ 185 to 203, 185 to 191, and 194 to 203 peptides (10 μg/ml) were added to BZR cells layered on HP(IV). Each experiment was performed in triplicate and results are expressed as the mean of three different experiment $\pm$ SEM. Thirty fields (original magnification, $\times400$) were counted on each filter. * $p < 0.05$. B, Morphological analyses by SEM of 16HBE14o− and BZR cells in the modified chamber invasion assay: (a) 16HBE14o− cells layered on HP(IV) collagen; (b) 16HBE14o− cells layered on BLC(IV) collagen; (c) BZR cells layered on HP(IV) collagen; and (d) BZR cells layered on BLC(IV) collagen. a, c, d, original magnification, $\times1200$; b, original magnification, $\times3200$.

the α3(IV) 185 to 203 and 185 to 191 resulted in 31% and 37% inhibition of BZR cell invasion, respectively (Fig. 1A).

Cell morphology was also assessed by scanning electronic microscopy analyses in this assay. Regardless of the coating used, 16HBE14o− cells displayed a rounded morphology characterizing an epithelioid-like phenotype (Fig. 1B). Invasive BZR cells, which progressively infiltrated the thin layer of type IV collagen, showed an elongated spindle cell morphology on HP(IV) collagen (Fig. 1B), but underwent important morphological changes when cultured on BLC collagen. Indeed, they lost their fibroblastic-like morphology and displayed the same rounded shape as noninvasive 16HBE14o− cells (Fig. 1B).

These data suggest that the α3(IV) chain could act on invasive cells by preventing the migration in a Boyden chamber, concomitant with important morphologic changes.

Effect of the Recombinant NC1 α3(IV) Domain on MMP mRNA Expression

Because the malignant phenotype and matrix degradation largely involve MMP production, we investigated MMP synthesis after exposure of the two cell lines to the α3(IV) chain. To clearly attribute the effect observed to the α3(IV) chain, we used a recombinant protein corresponding to the NC1 domain of the α1 or α3 chains of type IV collagen. Both bronchial tumor cell lines were cultured for 48 hours in serum-free DMEM with 100 μg/ml of either NC1 α1(IV) or NC1 α3(IV) or with control buffer. Then, the expression of MMP-2, TIMP-2, and MT1-MMP was analyzed by Northern blotting (Fig. 2A) and RT-PCR analyses (Fig. 2C).

We found that invasive BZR cells expressed TIMP-2, MMP-2, and MT1-MMP mRNA (Fig. 2, A and C). TIMP-2 and MMP-2 mRNA expression were not significantly modified by the culture conditions. MT1-MMP expression, in contrast, decreased significantly to approximately 50% when BZR cells were treated with the NC1 α3(IV) domain (Fig. 2, B and D). Noninvasive 16HBE14o− cells did not express any MMP-2 mRNA (Fig. 2, A and C). MT1-MMP and TIMP-2 mRNA expression were detected in 16HBE14o− but were not altered by the NC1 α3(IV) domain when compared with treatment with the NC1 α1(IV) domain or control buffer (Fig. 2, B and D).

Effect of the Recombinant NC1 α3(IV) Domain on MMP Protein Expression

The effect of the NC1 domains on MMP-2 secretion was analyzed by gelatin zymography. BZR cells secreted MMP-2 in its latent 72-kd form whereas 16HBE14o− cells did not produce any detectable MMP-2. These patterns were not modified by the culture conditions (NC1 α1[IV], NC1 α3[IV], or control) (Fig. 3A). The TIMP-2 levels were quantified by ELISA, which did not reveal any significant difference regardless of the culture conditions (Fig. 3B). MT1-MMP

contained in BZR and 16HBE14o− cell membrane extracts was analyzed by Western blot. Whatever the presence of exogenous MMP-2, we detected three major bands in control and NC1 α1(IV)–treated cells (a doublet at 63 kd and 60 kd and one band at 45 kd) in BZR cells. The treatment with the NC1 α3(IV) did not modify the level of the 63/60-kd doublet, but the 45-kd form was not detectable (Fig. 4A). MT1-MMP was found in its proenzyme 63-kd form in 16HBE14o− and this pattern was not modified by the culture conditions. To appreciate the consequences of the modified expression of MT1-MMP on the activation of MMP-2, exogenous pro-MMP-2 was added to the culture medium of both BZR and 16HBE14o− cell lines. The subsequent zymography analyses indicated no activation of exogenous MMP-2 in 16HBE14o− cells, whereas it revealed a decrease of the MMP-2 62-kd activated form, concomitant with the absence of MT1-MMP 45-kd form when BZR cells were cultured with the recombinant NC1 α3(IV) (Fig. 4B).

Discussion

In this study, we report that the α3(IV) chain modulates the invasive behavior of human bronchial cells by regulating both at the transcriptional and posttranscriptional levels the MT1-MMP.

Using modified Boyden chambers, we showed that the α3(IV) chain inhibited the invasive abilities of BZR cells. These observations could be correlated with those obtained by scanning electron microscopy. Indeed, when layered on HP type IV collagen, BZR cells displayed fibroblastic-like features, as well as filipodia and lamellipodia, attributes that have been largely associated with epithelial cell migration and/or invasion. In contrast, BZR cells became rounded when they were cultured in the presence of BLC type IV collagen and lost their migration-associated features. Furthermore, no effect of the α3(IV) chain was observed on noninvasive 16HBE14o− cells strongly suggesting that the α3(IV) chain has specific interactions with invasive tumor cells. These in vitro results may be related to our previous in vivo observations describing the presence of the α3(IV) chain around the well differentiated clusters of squamous cell carcinomas and adenocarcinomas (Catusse et al, 2000). All of these data taken together suggest that the α3(IV) chain has a limiting effect on tumor cell invasion. In this study, we also clearly showed that type IV collagen extracted from human placentas, containing exclusively the α1 and α2 chains, allowed the tumor cells to migrate. This observation was in agreement with other studies that have demonstrated that metastatic tumor cells adhere and exhibit motile behavior on the triple helical domain within α1(IV) chain (Chelberg et al, 1989; Li et al, 1997). In the same way Matrigel, principally containing laminin, permitted the BZR cell invasion. These results are in agreement with many in vitro and in vivo observations reporting that laminin promotes the malignant phenotype (Fridman et al, 1991; Kim et al, 1994; Terranova et al, 1984). In light of these data, it can be postulated that interactions
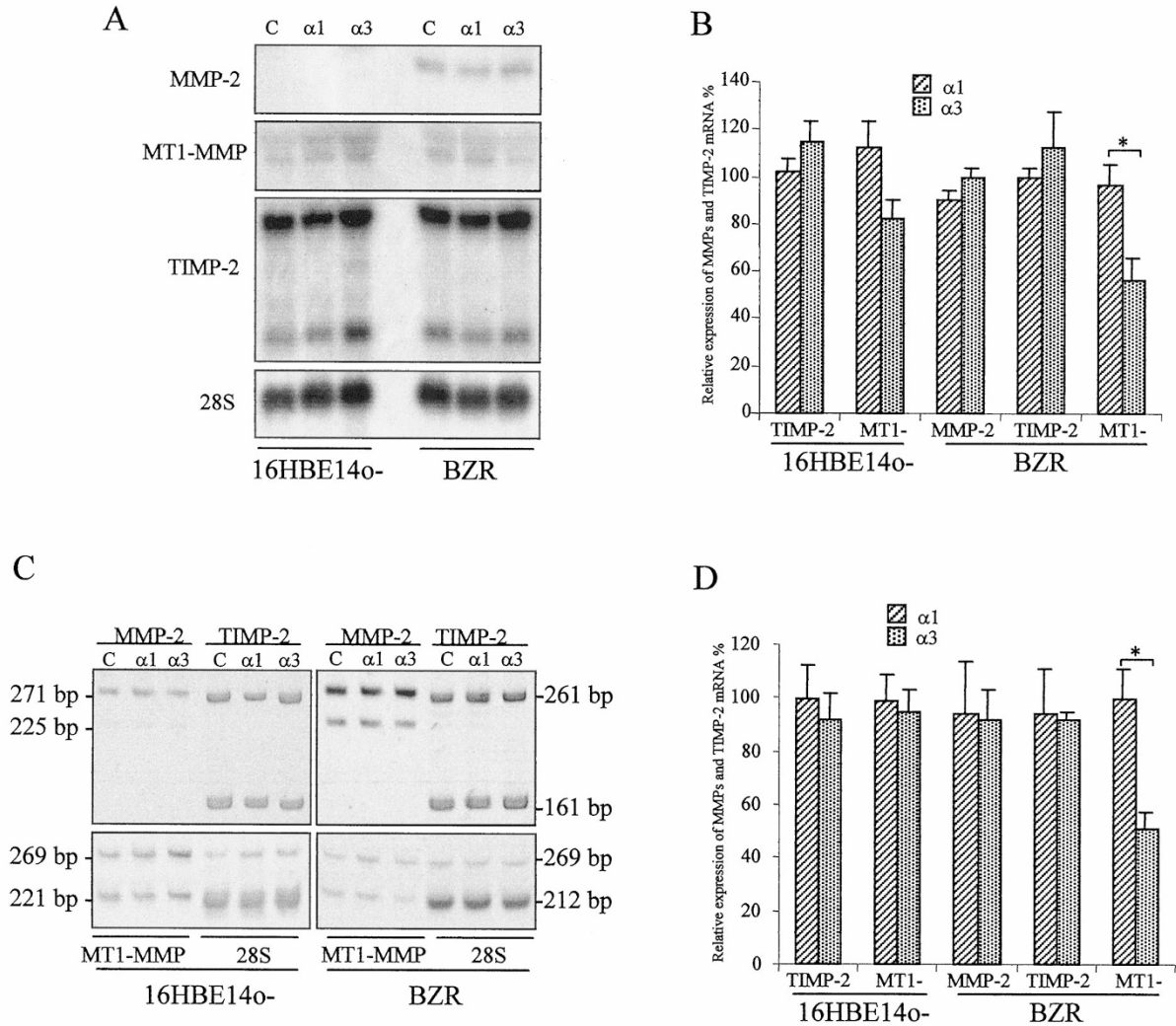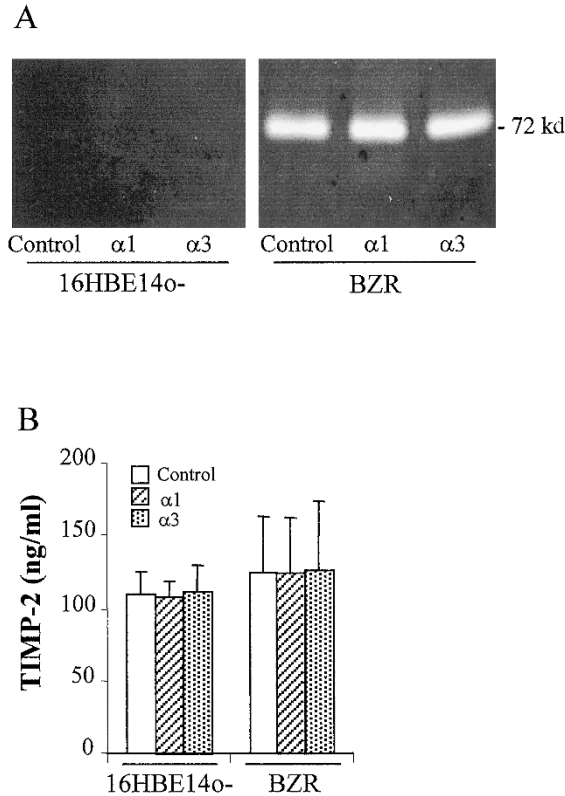
Figure 2.

Effect of the NC1 α3(IV) on the expression of MMP-2, TIMP-2, and MT1-MMP by 16HBE14o− and BZR cells. Northern blotting and quantitative RT-PCR analyses of MMP-2, TIMP-2, and MT1-MMP mRNA were performed on total RNA extracted from BZR and 16HBE14o− cells cultured with either the control buffer (*C*), the recombinant NC1 α1(IV) domain *(α1)*, or the recombinant NC1 α3(IV) domain *(α3)*. A, MT1-MMP mRNA of 4.5 kb and TIMP-2 mRNA of 3.8 kb and 1.2 kb were clearly detected in 16HBE14o− and BZR cells. MMP-2 (3.2 kb) was only found in BZR cell lines. B, The relative expression of MMP-2, MT1-MMP, and TIMP-2 mRNA were normalized with 36B4 level. C, Endogenous MMP-2, TIMP-2, MT1-MMP, and 28S bands were detected at the expected sizes (225 bp, 161 bp, 221 bp, and 212 bp, respectively). pCTR01 control bands for MMP-2, TIMP-2, MT1-MMP, and 28S were obtained at the expected sizes (271 bp, 261 bp, 269 bp, and 269 bp, respectively). D, MMP-2, TIMP-2, MT1-MMP, and 28S were expressed as a ratio to its specific internal control. The levels of mRNA were normalized with 28S level. The results are expressed as a percentage of control. * $p < 0.05$.

between tumor cells and BM components, such as the ubiquitous α1(IV) chain of type IV collagen or laminin, favor the tridimensional migration of invasive cells. On the other hand, the α3 chain of type IV collagen significantly impedes the invasive capacities of the tumor cells.

Because MMP-2 expression has been reported to be involved in tumor cell invasion in most bronchopulmonary cancers (Nawrocki et al, 1997), we studied the regulation of MMP-2, MT1-MMP (its activator), and TIMP-2 (its specific inhibitor) by the NC1 domain of the α3(IV) chain in two bronchial cell lines. We observed that the recombinant NC1 α3(IV) domain decreased MT1-MMP mRNA and protein expression in BZR cells, but did not modify the mRNA and protein expression of TIMP-2 and MMP-2. Accordingly, Pasco et al (2000) have recently shown a down-regulation of MMP ex-
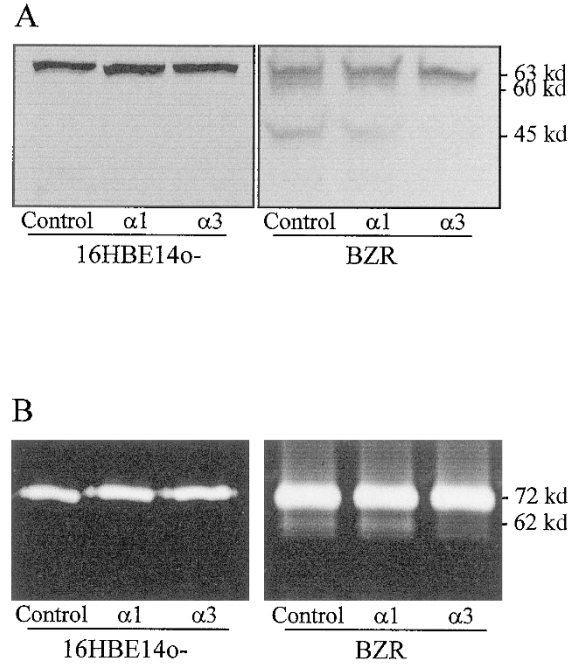
pression by the α3(IV) chain in sarcomatous cells. In agreement with our data, modulation of MMP expression by ECM components has been previously documented. Indeed, Gilles et al (1998) have reported an increase of MMP-2 activation in response to secreted protein, acidic, rich in cysteine (SPARC). Similarly, fibrillar collagen type I, when embedded in three-dimensional gel, has been shown to modulate the synthesis and activation of MMP-2 by enhancing MT1-MMP expression (Gilles et al, 1997; Kurschat et al, 1999). Moreover, Gilles et al (1997) have reported that collagen type I induces MT1-MMP expression in invasive breast carcinoma cell lines but not in the noninvasive counterparts. In contrast to the ECM molecules cited above, we demonstrated here that the α3(IV) chain has a negative effect on MT1-MMP mRNA expression in invasive bronchial BZR cells. Moreover,

A

Control   α1   α3   Control   α1   α3

16HBE14o-   BZR

- 72 kd

A

- 63 kd
- 60 kd

- 45 kd

Control   α1   α3   Control   α1   α3

16HBE14o-   BZR

B

TIMP-2 (ng/ml)

200

150

100

50

0

☐ Control
▨ α1
▦ α3

16HBE14o-   BZR

Figure 3.
MMP-2 and TIMP-2 protein expression by BZR and 16HBE14o− cell lines. A, Analyses of gelatinolytic activities in the conditioned media of BZR and 16HBE14o− cells cultured with either the control buffer, the recombinant NC1 α1(IV) domain *(α1)*, or the recombinant NC1 α3(IV) domain *(α3)*. Results showed the 72-kd latent form of MMP-2 in BZR cells. B, Quantification of TIMP-2 released in culture media of BZR and 16HBE14o− cells cultured in presence of either the control buffer, the recombinant NC1 α1(IV) protein *(α1)*, or the recombinant NC1 α3(IV) protein *(α3)*. TIMP-2 concentrations were determined by ELISA.

B

Control   α1   α3   Control   α1   α3

16HBE14o-   BZR

- 72 kd
- 62 kd

Figure 4.
Processing of MMP-2 to its active form is correlated with presence of the 45-kd form of MT1-MMP. A, Detection of MT1-MMP in BZR and 16HBE14o− cells extracts by Western blotting analyses. The anti-MT1-MMP antibody recognized three major bands of 63 kd, 60 kd, and 45 kd in the NC1 α1(IV) (α1)- or the buffer control-treated BZR cells. The anti-MT1-MMP antibody detected only two bands of 63 kd and 60 kd in the NC1 α3(IV) (α3)-treated BZR cells. 16HBE14o− cells only expressed the 63-kd form of MT1-MMP. B, Zymography analyses of exogenous MMP-2 activation. Results showed two forms (the latent 72-kd form and the active 62-kd form) of MMP-2 when BZR cells were cultured with the recombinant NC1 α1(IV) domain (α1) or the buffer control. One form of MMP-2 (72 kd) was detected when BZR cells were cultured with the recombinant NC1 α3(IV) (α3). 16HBE14o− were not able to activate pro-MMP2, regardless of the culture conditions. The results shown are representative of three experiments.

the regulation of MT1-MMP mRNA expression by the recombinant NC1 α3(IV) domain seems to be specific to invasive cells because we did not observe any modification of MT1-MMP expression and activation in noninvasive 16HBE14o− cells treated with the recombinant NC1 α3(IV) domain. Taken together, these results suggest that the NC1 α3(IV) domain has specific interactions with invasive tumor cells leading to a decrease of MT1-MMP expression.

We also observed a potential post-transcriptional regulation of MT1-MMP by the NC1 α3(IV) domain in invasive BZR cells. Indeed, MT1-MMP Western blot analysis showed that treatment with the NC1 α3(IV) domain did not modify the level of the 63/60-kd doublet, whereas it abolished the 45-kd form. This MT1-MMP 45-kd form has been previously characterized as a N-terminal–truncated MT1-MMP lacking its active site and has been reported as a byproduct of MMP-dependent proteolysis of the 60-kd active form of MT1-MMP (Lohi et al, 1996; Stanton et al, 1998). Although the exact role of the 45-kd processed form of MT1-MMP is not known, several studies have shown that the MT1-MMP 45-kd form is correlated to MMP-2 activation (Lohi et al, 1996; Stanton et al, 1998). Accordingly, we also

showed here that the absence of 45-kd form was associated with a decrease in the MMP-2 activation. Previous reports have demonstrated that MT1-MMP-mediated pro-MMP-2 activation at the cell surface depends on the TIMP-2 concentration (Butler et al, 1998). Indeed, at low concentrations, TIMP-2 increases MMP-2 activation by forming an MT1-MMP/TIMP-2 complex available for MMP-2 binding, whereas at high concentrations, TIMP-2 binds all free active MT1-MMP molecules needed to activate pro-MMP-2. Thus, the balance between TIMP-2 and MT1-MMP determines the activation status of MMP-2. In this study, we showed that the NC1 α3(IV) reduced MT1-MMP expression and did not modify TIMP-2 expression in invasive BZR cells. A relative excess of TIMP-2 could reduce the amount of free active MT1-MMP present at the cell surface of invasive BZR cells, thereby preventing activation of pro-MMP-2. These results suggest that the NC1 domain of the α3(IV) chain could decrease the tumor cell invasive behavior by regulating MMP-2 activation via MT1-MMP synthesis and processing. In addition to being implicated in metastatic progression via its role in MMP-2 activation, recent observations have described that MT1-MMP itself displays the ability to degrade several matrix components (Imai et al, 1996; Koshikawa et al, 2000; Ohuchi

et al, 1997; Pei and Weiss, 1996; Sato et al, 1996). Because the amount of pro-MMP-2 in its active 62-kd form was not impressive in invasive BZR cells, we cannot exclude the possibility that MT1-MMP is directly responsible for BZR cell migration independently of MMP-2 activity. Thus, the down-regulation of MT1-MMP by the α3(IV) chain could also have a direct effect on the tumor invasion process by decreasing ECM degradation mediated by MT1-MMP.

In conclusion, these results demonstrate that the α3 chain of type IV collagen, present in tumor neosynthesized BM, by acting on MT1-MMP expression could limit cancer progression.

Materials and Methods

Cell Lines and Culture

The human bronchial cells lines, 16HBE14o– and BZR, were derived from normal bronchial cells immortalized after transfection with the SV40 large T-antigen. BZR cell line was also infected with the *v-Ha ras* oncogene. The cells were cultured at 37° C and 5% CO_2 in DMEM (Gibco BRL, Paisley, Scotland) supplemented with penicillin streptomycin (50 ng/ml) and 10% FCS (Gibco BRL).

Boyden Chamber Invasion Assay

The in vitro invasiveness of cell lines was assessed by using a modified chamber assay. Cells (10^5) were suspended in 800 μl of DMEM supplemented with 0.2% BSA (Sigma, St Louis, Missouri) and layered in the upper compartment of the chamber (Nucleopore, Pleasanton, California). The lower compartment of the chamber was filled with 200 μl of DMEM supplemented with 10% FCS and 2% BSA. The two compartments were separated with a porous filter (8 μm pore, Nucleopore) coated with either 25 μg/filter of type IV collagen extracted from human placenta (Sigma) that contains the α1 and α2 chains only, or 25 μg/filter of type IV collagen extracted from BLC that contains the α1, α2, and α3 chains, prepared as previously described (Monboisse et al, 1994), or coated with 25 μg/filter Matrigel obtained from the Engelbrecht-Holm-Swarm (EHS) tumor. Moreover, to assess the specific effect of the α3(IV) chain on BZR migration, BZR tumor cells deposited onto membranes coated with HP(IV), were incubated with peptides corresponding to the residues 185 to 203, 185 to 191, and 194 to 203 of the NC1 domain of the α3(IV) chain (Cybergene, St Malo, France). The chambers were incubated for 5 hours at 37° C. The filters were then fixed in cold methanol at −20° C for 10 minutes and stained with hematoxylin for 2 minutes. The cells on top of the filter were wiped away with a cotton swab. Quantification of the invasion was performed by counting the number of the cells at the lower surface of the filters (30 fields at 400× magnification). Each cell line was tested three times and each experiment was performed in triplicate. Scanning electron microscopic observation was also performed on the different coated filters. For this purpose, 10^4 cells were incubated in the upper compartment of the chamber for 2 hours. The filters were fixed in 2.5% glutaraldehyde, dehydrated in a gradient of ethanol, critical point-dried, and observed with an XL 30 Philips scanning electron microscope.

Purification of the Recombinant NC1 Domain-GST Fusion Proteins

The construction of pGEX 2T expression vectors carrying cDNA encoding the α1(IV) or α3(IV) NC1 domains-GST fusion proteins was described by Dehan et al (1996). Expression in *Escherichia coli* JM1 01 cells were carried out as described by Smith and Johnson (1988). Fusion proteins were present in inclusion bodies. The insoluble cell pellet from bacterial cultures were sonicated three times for 2 minutes and centrifuged (8000 ×g, 10 minutes, 4° C) in 40 m$_M$ Tris, pH 8.0. The final pellet was solubilized in 50 m$_M$ Tris, pH 8.0, containing 8 $_M$ urea and 1% β-mercaptoethanol. The mixture was then fractionated by column chromatography (PD 10 Sephadex G25, Pharmacia, Uppsala, Sweden) previously equilibrated in 50 m$_M$ Tris, pH 8.8, at 4° C. After overnight storage at 4° C, the fusion proteins were affinity-purified on glutathione-Sepharose (Pharmacia) and eluted with an excess of reduced glutathione (Boehringer Mannheim, Germany). The free glutathione was removed on a PD 10 Sephadex G25 column equilibrated in PBS. Aliquots of fusion proteins taken at all steps of the purification process were reduced and subjected to a 10% SDS polyacrylamide gel electrophoresis. The gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid, then destained in 10% acetic acid and 20% methanol.

Preparation of Serum-Free Conditioned Media

Cells were plated in 6-well plates (400,000 cells/well) for 48 hours in DMEM/FCS. The cells were next washed twice with serum-free DMEM and were incubated for 48 hours in serum-free medium (SFM) containing the recombinant NC1 domains (100 μg/ml) or control buffer (Tris, pH 8.8, 40 m$_M$). For MMP-2 activation analysis, the culture media were incubated in SFM/MMP-2 (SFM and SFM conditioned for 48 hours by MMP-2-transfected CHO as a source of exogenous MMP-2) mixture containing the recombinant NC1 domains (100 μg/ml) or control buffer. The conditioned media were collected for zymography and/or TIMP-2 ELISA analyses (see below). Cultures were set up in triplicate and experiments were repeated at least three times. The number of cells per flask was determined by cell counting.

Northern Blotting Analyses

Total RNA was extracted from both cell lines cultivated in SFM containing the recombinant NC1 (α1 or α3) domains or control buffer as described above. After 48 hours, the conditioned media were collected for zymographic and/or for TIMP-2 analyses, and the cells were collected for Northern blotting and RT-PCR

analyses. RNA extraction was performed with RNA Miniprep kits used as recommended by the manufacturer (Qiagen, Hilden, Germany). Six-microgram samples of each RNA were analyzed by electrophoresis in 1% agarose gels containing 10% formaldehyde and transferred onto nylon membranes (Hybond-N; Amersham, Buckinghamshire, United Kingdom). The membranes were hybridized with cDNA probes for MMP-2 (1600 bp, kindly provided by Dr. G. Murphy, Cambridge, United Kingdom), MT1-MMP (1700 bp, kindly provided by Dr. Seiki, Kanazawa University, Japan), TIMP-2 (1035 bp, kindly provided by Dr. Y. De Clerck, Los Angeles, California) labeled with ^{32}P using random labeling kit (Amersham). The RNA loading was normalized using the 36B4 probe. The signals were quantified by fluorometric scanning (LAS-1000, Fuji).

RT-PCR Analyses

RT-PCR was performed using 10 ng of total RNA. An internal control RNA template, pCTR01, was introduced in each sample to monitor the assays of MMP-2, MT1-MMP, TIMP-2, and 28S (5.10^4, 2.10^4, 2.10^4, and 4.10^6 copies, respectively). RT-PCR was performed using the GeneAmp Thermostable RNA PCR Kit (Perkin-Elmer, Foster City, California) and for pairs of oligonucleotides (Eurogentec, Seraing, Belgium). Forward and reverse primers for human MMP-2, MT1-MMP, TIMP-2, and 28S were designed as follows: MMP-2 primers (forward 5'-GGCTGGTCA-GTGGCTTGGGGTA-3', reverse 5'-AGATCTTCTTCTT-CAAGGACCGGTT-3'), MT1-MMP primers (forward 5'-CCATTGGGCATCCAGAAGAGAGC3', reverse 5'-GG-ATACCCAATGCCCATTGGCCA-3'), TIMP-2 primers (forward 5'-GTCATCTTGATCTCAT-AACGCTGG-3', reverse 5'-AGCCCATCTGTACCTGTGGTTCA-3'), and 28S primers (forward 5'-GTTCACCCACTAATAGGGAA-CGTGA-3', reverse 5'-GGATTCTGACTTAGAGGCGT TCAGT-3'). Reverse transcription was performed at 70° C for 15 minutes, followed by a 2-minute incubation at 95° C for denaturation of RNA-DNA heteroduplexes. Amplification was started by 15 seconds at 94° C, 15 seconds at 68° C, 10 seconds at 72° C (30 cycles for MMP-2, MT1-MMP, and TIMP-2, and 17 cycles for 28S), and terminated by 2 minutes at 72° C. RT-PCR products were separated by acrylamide gel electrophoresis, stained with Gelstar (FMC, Bioproducts, Rockland, Maine), and quantified by fluorometric scanning (LAS-1000, Fuji). The expected sizes for MMP-2, MT1-MMP, and TIMP-2 are 271 bp, 269 bp, and 261 bp, respectively, for the pCTR01; and 225 bp, 221 bp, and 161 bp, respectively, for the endogenous mRNA. The expected size for the 28S is 269 bp for the pCRT01 and 212 bp for the endogenous rRNA.

Preparation of Cell Lysates

Confluent BZR cells were cultured in six-well plates with either 100 μg/ml recombinant NC1 α1(IV) domain, 100 μg/ml recombinant NC1 α3(IV) domain, or the control buffer in SFM or in SFM/MMP-2 for 48 hours. The conditioned media were stored at −20° C and were analyzed by gelatin zymography and the cells were reserved for protein extraction. The cells were washed twice with PBS (Gibco BRL) and lysed with lysis buffer (0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 0.1 M Tris-HCl pH 7.4, 1% NP 40, 5 mM Iodoacetamide, 1 mM PMSF) for 30 minutes at 4° C. Cell lysates were aspirated with a 21-gauge needle and centrifuged at 12,500 ×*g* for 10 minutes at 4° C. The supernatant was stored at −20° C until used. Sample were analyzed by Western blot. Amounts of proteins were evaluated with the DC Protein Assay kit (BioRad, Hercules, California).

Gelatin Zymography Assays

Samples were diluted 1:2 in a Laemmli buffer (BioRad) and were separated on a 10% SDS polyacrylamide gel containing 1 mg/ml gelatin. SDS was removed from the gel with 2% triton X-100 washes. The gels were incubated overnight at 37° C in 50 mM Tris pH 7.6, 5 mM $CaCl_2$, and 0.1% Triton X-100, and stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid, then destained in 10% acetic acid and 20% methanol. The proteolytic activities were quantified from gels by densitometric scanning (LAS-1000, Fuji).

TIMP-2 ELISA

After overnight coating with rabbit-anti-TIMP-2 antibody (1:750) (Gilles et al, 1998) at 4° C, 96-well ELISA plates were washed with 0.1 M PBS, 3% BSA, 0.05% Tween 20, and incubated with 50 μl of conditioned media samples or pure TIMP-2 (0.31 to 20 ng/ml) for 2 hours at 37° C. After repeated washes with 0.1 M PBS, and 0.01% Tween 20, 96-well ELISA plates were incubated with chicken anti-TIMP-2 antibody (1:200) (Gilles et al, 1998) for 2 hours at room temperature, washed, and incubated with peroxidase-coupled swine antichicken antibody (1:10,000) in PBS-BSA-Tween 20 for 1 hour at room temperature. Absorbance was read at 405 nm after incubation of 96-well ELISA plates with 2,2'-azino-bis-[ethylbenthiazoline-6-sulfonic acid] (ABTS) (Sigma) solution supplemented with H_2O_2.

Western Blotting Analyses

Twenty micrograms of proteins from cells lysates were mixed 1:2 with Laemmli buffer (BioRad) containing 5% β-mercaptoethanol (v/v), boiled for 5 minutes, separated on a 10% SDS-PAGE gel, and transferred to a nitrocellulose filter (Hybond-C Extra, Amersham, Buckinghamshire, United Kingdom). Transfer was performed at 120V for 1 hour. The membrane was blocked with 0.1% Tween 20 and 5% milk in PBS 2 hours at room temperature before exposure with the 4MMP-3H3 antibody (1:10, a generous gift from Dr. M. C. Rio, Strasbourg, France) overnight at 4° C. The membranes were incubated with a secondary biotinylated sheep-antimouse antibody (1:1500; Amersham, Arlington Heights, Illinois) for 1 hour at room temperature and then with the streptavidin-peroxidase complex (1:1500; Amersham) for 30 minutes. Immunoreactive protein bands were detected with ECL Plus Western blotting reagents (Amersham).

Statistical Analyses

Data are expressed as means $\pm$ SD. Student's *t* test was used to compare the invasive ability of the cells and both expression and synthesis levels of MMP-2, MT1-MMP, and TIMP-2 under various experimental conditions. $p < 0.05$ was considered significant.

Acknowledgements

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