

S100A4 Involvement in Metastasis: Deregulation of Matrix Metalloproteinases and Tissue Inhibitors of Matrix Metalloproteinases in Osteosarcoma Cells Transfected with an Anti-*S100A4* Ribozyme¹

Kristin Bjørnland, Jan Olof Winberg, Olav Tobias Ødegaard, Eivind Hovig, Thrina Loennechen, Ansgar O. Aasen, Øystein Fodstad, and Gunhild Mari Mælandsmo²

Institute for Surgical Research [K. B., A. O. A.] and Surgical Department B [K. B.], The National Hospital, Rikshospitalet, 0027 Oslo; Department of Biochemistry, Institute of Medical Biology [J. O. W., O. T. Ø.], and Department of Pharmacology, Institute of Pharmacy [T. L.], Faculty of Medicine, University of Tromsø, 9037 Tromsø; and Department of Tumor Biology, The Norwegian Radium Hospital [E. H., Ø. F., G. M. M.], 0310 Oslo, Norway

ABSTRACT

The biological function of the metastasis-associated gene *S100A4* is not fully understood, although there is evidence indicating interactions between the gene product and the cytoskeleton. We have examined whether an association could exist between *S100A4* and the regulation of matrix metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs). For these studies, three clones of a highly metastatic human osteosarcoma cell line (OHS) transfected with a hammerhead ribozyme directed against the *S100A4* gene transcript were used. The clones demonstrated different expression levels of *S100A4* and also different metastatic capacity. In the clone with the most prominent down-regulation of *S100A4*, the mRNA levels of MMP2, membrane type (MT) 1-MMP, and TIMP-1 were significantly reduced in exponentially growing cultures. Western blots, gelatin zymography, and ELISA showed similar expression patterns of MMPs and TIMPs at the protein level. In the clones with an intermediate expression of *S100A4*, reduced expression of MT1-MMP and TIMP-1 was detected, whereas the expression of MMP-2 was at the same level as in the control cells. In contrast to the other factors, TIMP-2 was up-regulated in all of the clones independent of the extent of ribozyme-induced down-regulation of *S100A4*. The transwell chamber assay demonstrated that the capacity of the ribozyme-transfected cells to cross uncoated filters was reduced, relative to control cells, according to the reduction in the *S100A4* expression level. The clone with the lowest reduction in *S100A4* did not demonstrate different motility compared with control cells, whereas transfectants with only 5% *S100A4* mRNA showed a 50% reduction in motility. Interestingly, this trend was even more striking when the capacity to cross Matrigel-coated filters was analyzed, as all the clones demonstrated between 40 and 75% reduced invasion. It is concluded that *S100A4* may exert its effect on metastasis formation not only by stimulating the motility of tumor cells but also by affecting their invasive properties through influencing the expression of MMPs and their endogenous inhibitors.

INTRODUCTION

Metastasis is a complex cascade of events involving a finely tuned interplay between the malignant cells and multiple host factors (1, 2). Thus, expression of the metastatic phenotype depends on a balance between regulatory elements that promote or inhibit the process at one or more of the sequential steps. A variety of molecules are involved in the different steps. Some of these have been identified directly related to their biological functions, and others have been identified after the cloning of the corresponding genes from cells with different metastatic potential.

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² To whom requests for reprints should be addressed, at Department of Tumor Biology, Norwegian Radium Hospital, 0310 Oslo, Norway. Phone: 47-22-93-46-44; Fax: 47-22-52-24-21; E-mail: g.m.malandsmo@labmed.uio.no.

Degradation and penetration of the ECM³ is a hallmark of tumor invasion and metastasis (2, 3). The tumor cells have to traverse the basement membranes and the underlying stroma both when they escape from the primary tumor and subsequently during intra- and extravasation. The invasion process is accomplished by the concerted action of multiple proteinases, particularly the MMPs and members of the urokinase plasminogen activator system (3). Both these systems are regulated by endogenous inhibitors, the TIMPs, and the plasminogen activator inhibitors, respectively. Several reports have implicated the proteinases and their endogenous inhibitors to be important in the metastatic spread of cancer cells (4–6).

It has been suggested that the *S100A4* gene product is involved in the formation of metastases. It was originally cloned from rodent mammary carcinoma cells, and the murine *S100A4* gene (*mts-1*) was found highly expressed in cell variants with metastatic capacity (7, 8). The involvement of *S100A4* in rodent cancer progression is documented by the transfection of the rat gene (*p9Ka*) into benign rat mammary epithelial cells that subsequently induced metastatic capacity in some syngeneic animals on injection into the mammary fat pad (9). Similarly, a high incidence of lung metastases of mammary carcinomas in *S100A4* transgenic mice has been observed (10, 11), and elevated p9Ka protein in rat prostate carcinoma cells was associated with a more malignant phenotype (12).

The involvement of *S100A4* in human malignancies, however, has not been thoroughly investigated. We have previously transfected a ribozyme against *S100A4* into highly metastatic human osteosarcoma cells expressing high levels of the gene transcript (13). Using cell variants with ribozyme-induced reduction in *S100A4* expression, we observed a nearly complete reversal of the metastatic phenotype in animal models. Furthermore, an association between *S100A4* expression and the invasiveness of human breast (14, 15) and glioma cells (16), as well as the progression of human colorectal carcinomas (17), has been demonstrated. These results strongly support the hypothesis that *S100A4* is involved in the formation of human cancer metastases.

The biological functions of *S100A4* are incompletely understood, although a correlation between expression levels and cell motility has been observed (18–20). Furthermore, experimental evidence indicates that the gene product is associated with the cytoskeleton, and F-actin, nonmuscle tropomyosins, and nonmuscle myosin heavy chain have been suggested as interacting partners (21, 22). To study the biological factors by which *S100A4* influence the metastatic properties of cancer cells, we have taken advantage of the ribozyme-transfected cell clones demonstrating different *in vivo* metastatic potential (13). Previously, we found that *S100A4* influenced cytokine- and growth factor-induced MMP-1, MMP-9, and TIMP-1 expression in these cells (23). In the present work, we have extended our MMP and TIMP analyses and have examined the mRNA expression levels as well as the *in vitro* invasive capacity in the transwell chamber assay. The

³ The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-MMP; TIMP, tissues inhibitor of MMP.

analysis revealed that the invasive potential of low *S100A4* expressing cells was reduced, and corresponding alterations in both MMP and TIMP expression were observed. Our results suggest that *S100A4* may affect the metastatic potential of tumor cells by enhancing tumor cell motility and by influencing MMP and TIMP expression in a way that facilitates ECM destruction.

MATERIALS AND METHODS

Cells and Culture Conditions. The OHS cell line was established from a bone tumor biopsy from a patient treated at the Norwegian Radium Hospital and grown as monolayer cultures (24). The cells were transfected with a hammerhead ribozyme directed against the metastasis-promoting gene *S100A4* (*CAPL*) as described previously (13). The transfectants were grown in RPMI 1640 (Life Technologies, Inc., Middlesex, United Kingdom), supplemented with 10% FCS (Life Technologies, Inc.), L-glutamine (2.0 mM; Life Technologies, Inc.), and 400 $\mu\text{g/ml}$ Geneticin (Life Technologies, Inc.). 5'-rapid amplification of complementary DNA ends-PCR, detecting the 3'-product after ribozyme cleavage, was used to confirm ribozyme activity in the transfected clones. This assay also minimizes the possibility that the low *S100A4* expression in the resulting cell clones reflects clonal variations in the parental cell line. The pH β -1 cell clone was transfected with the vector alone, whereas the ribozyme-transfected clones were designated II-11a, II-11b, and III-14.

Preparation and Northern Blot Analysis of RNA. Total cellular RNA was prepared from cells at approximately 60% confluence (subconfluence growth conditions) or 100% confluence by extracting with Trizol (Life Technologies, Inc.). Samples of 5 μg were separated by agarose-formaldehyde gel electrophoresis, and the filters were hybridized with DNA probes labeled with ^{32}P by the random primer technique (25). The hybridizations were carried out in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM sodium EDTA at 65°C (26). The membranes were subsequently washed three times for 15 min in 40 mM sodium phosphate (pH 7.2) and 1% SDS at 65°C. To calibrate the filters and correct for uneven amounts of RNA loaded in each lane, the filters were rehybridized with a kinase-labeled (27) oligonucleotide probe complementary to nucleotides 287–305 of human 18S rRNA. Images obtained in a STORM Phospho Imager were used to determine the signal intensities. The mRNA expression levels were calculated relative to the expression of 18S rRNA in the respective sample. For each cell clone, three independent samples were analyzed, and the mean expression level of a particular mRNA was related to the mean expression level in the pH β -1 cells.

The following probes were used: (a) the cDNA probe for *S100A4* was kindly provided by Dr. E. Lukanidin, the Fibiger Institute, Copenhagen, Denmark (11); (b) the cDNA probes encoding TIMP-1 and TIMP-2 were kindly provided by Dr. W. G. Stetler-Stevenson (28); (c) the human cDNA for MT1-MMP was a generous gift from Dr. H. Sato (29); and (d) the cDNAs for MMP-1, MMP-2, MMP-9, and MMP-11 were kindly provided by British Biotech Pharmaceuticals (Oxford, United Kingdom).

Production of Conditioned Media for MMP and TIMP Determination. The cells were subcultivated in 10% FCS containing basal medium that contained the following components: Hams F12 medium, 20 mM HEPES buffer (pH 7.2–7.4), 400 $\mu\text{g/ml}$ Geneticin, nonessential amino acids (100 \times dilution), and 2.0 mM L-glutamine (all purchased from Life Technologies, Inc.). To determine the secretion of MMPs and TIMPs into the culture medium, triplicates of 0.5 and 0.7 million cells in 10% FCS containing basal medium were seeded in 25 cm² culture flasks. After overnight cultivation, the cells were washed three times with serum-free basal medium containing 1.0 mg/ml nonprotein serum replacement, RenCyte (SSR-1, MediCult AS, Copenhagen, Denmark). The cultures were subsequently maintained for 48 and 72 h in 2 ml of serum-free basal medium, containing RenCyte. Before freezing, 10 mM CaCl₂ and 100 mM HEPES (pH 7.5) were added to the harvested medium (30). Subsequently, the cells were trypsinated and counted. No significant differences between the two cell variants were observed (data not shown).

Gelatin and Casein Zymography. Harvested media from pH β -1 and II-11b cells were assayed for gelatinase activity as described previously, using 10% SDS-polyacrylamide gels (PAGE) containing gelatin (0.1% w/v; Sigma Chemical Co.; St. Louis, MO.) or casein (0.1%; Sigma; Refs. 31, 32). The gelatin zymograms were calibrated with human gelatinase standards from capillary whole blood (33). Gels were stained with 0.2% Coomassie brilliant

blue R-250 (Bio-Rad Laboratories, Hercules, CA) and destained in a solution containing 30% methanol and 10% acetic acid. Gelatinase activity was visualized as cleared regions in the blue gels. The area of the cleared zones was analyzed with the GelBase/GelBlot Pro computer program (UV Products, Cambridge, United Kingdom).

Western Immunoblotting Analysis. Conditioned media from pH β -1 and II-11b cells were electrophoresed on 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA) according to the manufacturer's manual. After blockage of nonspecific binding sites with nonfat milk (5% in Tris buffered saline), blots were incubated for 1 h at room temperature with primary mouse monoclonal antibodies against human MMP-1, MMP-2, MMP-3, TIMP-1, TIMP-2, and MT1-MMP respectively (diluted according to the manufacturer, Amersham, Buckinghamshire, United Kingdom). After washing, the blots were incubated for 1 h at room temperature with an alkaline phosphatase-conjugated secondary antibody (Sigma), diluted 1:2000 in blockage solution and developed with CDP-Star chemiluminescent substrate (New England Biolabs; Schwalbach/Taunus, Germany) according to the description of the manufacturer.

ELISAs. Levels of immunoreactive MMP-1, MMP-2, MMP-3, TIMP-1 and TIMP-2 were measured in unconcentrated media harvested from pH β -1 and II-11b cells by means of ELISAs (Amersham) following the manufacturer's protocols.

Evaluation of *In Vitro* Invasive Properties. The *in vitro* invasive properties of the parental OHS cells and the four clones pH β -1, II-11a, II-11b, and III-14 were evaluated in the transwell chamber assay (Costar, Cambridge, MA). Culture medium (150 μl) that contained 7.5×10^4 [³H]thymidine (Amersham)-labeled cells was applied in the upper compartments onto filters (6.5-mm diameter; 8- μm pore size) coated with Matrigel (50 $\mu\text{g/well}$; Collaborative Research, Bedford, MA). The lower compartments were filled with culture medium. After a 48-h incubation, the cells on the upper and lower parts of the filters were removed separately, and [³H]thymidine counted in a liquid scintillation analyzer (Packard, Illinois). Invasion was assessed as the ratio of the counts obtained from the lower compartment to total counts of both compartments. *In vitro* motility was performed similarly, except that the tumor cells were seeded onto uncoated filters. For cellular attachment studies, 7.5×10^3 cells were added to 96-well plates coated with Matrigel (30 $\mu\text{g/well}$). After incubation for 3.5 h, nonadherent cells were removed. Adherent cells were fixed in methanol, stained with 0.08% crystal violet, and repeatedly rinsed in water. Nuclear dye was eluted with 33% acetic acid, and absorbance was recorded at 550 nm in a ThermoMax ELISA reader (Molecular Devices Corp., Menlo Park, CA).

Statistical Analysis. Student's *t* test for independent analysis was applied to evaluate differences between the two cell clones with respect to *in vitro* invasive properties and expression of MMPs and TIMPs. Data are presented as mean values with 95% confidence interval (*in vitro* invasive properties and ELISA data), \pm SE (Northern blotting data) and \pm SD (gelatin zymography and Western blotting data). A *P* of less than 0.05 was considered as significant.

RESULTS

S100A4, MMP, and TIMP mRNA Expression. To confirm ribozyme-induced reduction in *S100A4* expression, the mRNA level in all of the cell clones, in both confluent and subconfluent cell cultures, was determined by Northern blotting. The ribozyme-transfected clones (II-11a, II-11b, and III-14) displayed between 78 and 94% reduction in *S100A4* expression compared with the vector-alone-transfected cell clone pH β -1 (Fig. 1). In the cell clone with the most pronounced reduction in *S100A4* expression (II-11b), the mRNA level was reduced by 94% compared with pH β -1 cells (II-11b: 0.06 ± 0.01 ; pH β -1: 1 ± 0.05 ; *P* < 0.001). In the III-14 cell clone, showing the lowest down-regulation in *S100A4*, the expression level was 22% compared with that of control cells (III-14: 0.22 ± 0.06 ; *P* < 0.001). The pH β -1 cells had a level of *S100A4* mRNA comparable with that of the parental OHS cells.

The expression of several members of the MMP family was examined in the original OHS cell line, the vector-alone-transfected cell clone, and the three ribozyme-transfected cell clones (Fig. 1). In

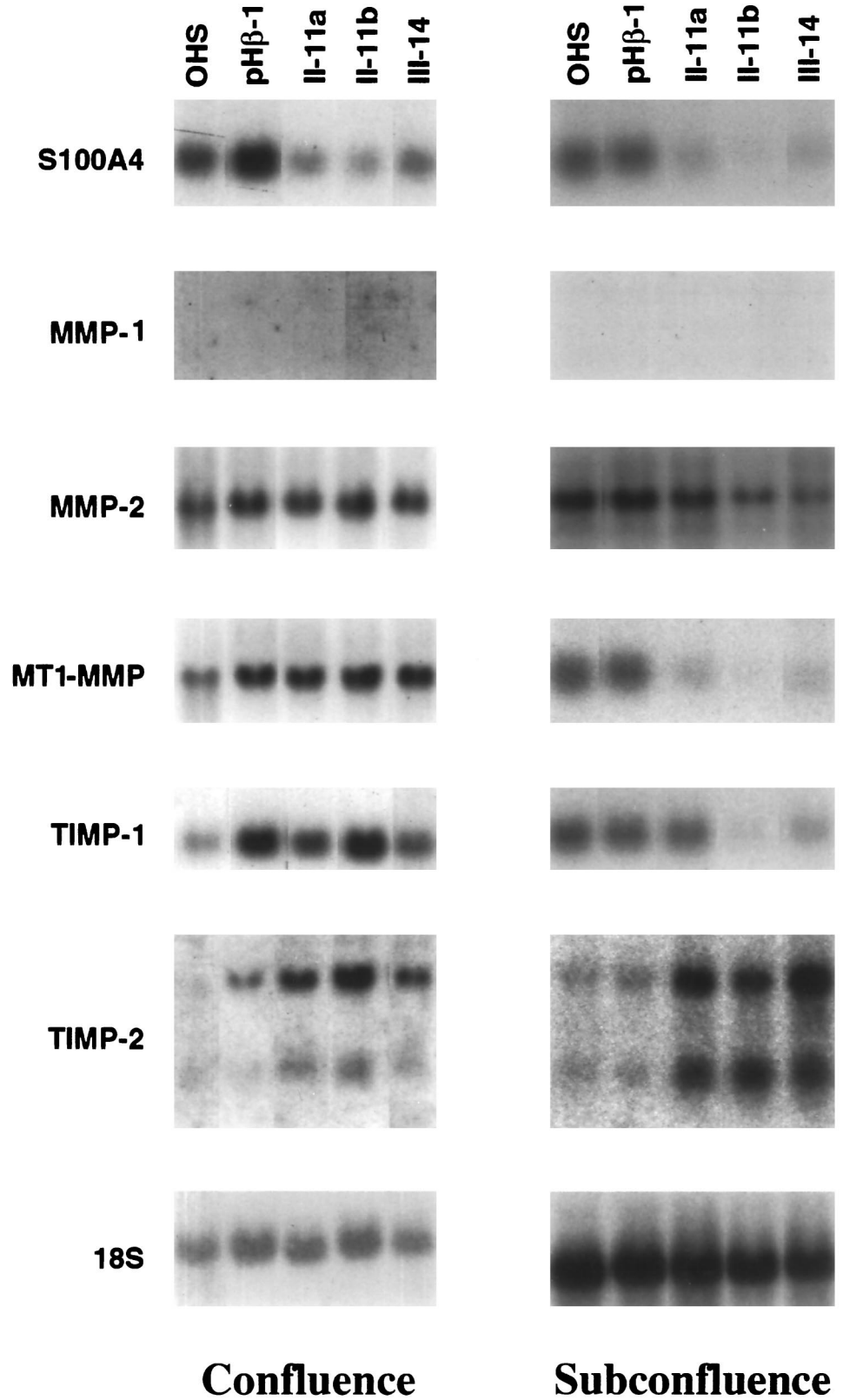


Fig. 1. Representative Northern blot analysis showing the expression levels of *S100A4* and factors involved in the MMP-dependent degradative pathway. The cells were grown according to standard incubation conditions, and total RNA was extracted from confluent and subconfluent cell cultures. The 18S rRNA was used as a loading control.

confluent cell cultures, no significant differences in the expression levels of the proteases could be observed between the control cells and the cells with ribozyme-induced reduction in *S100A4* expression. However, when exponentially growing cells were harvested, MMP-2 mRNA expression in the II-11b cells was significantly reduced compared with that of control cells (Fig. 1). The relative amount of

MMP-2 mRNA in II-11b cells was only 46% of that in the pHβ-1 cells (II-11b: 0.46 ± 0.07 ; pHβ-1: 1 ± 0.08 ; $P < 0.001$). Down-regulation, although not statistically significant, of MMP-2 mRNA in the II-11a cells was also observed, whereas the III-14 cells displayed equal amounts of MMP-2 mRNAs as the pHβ-1 cells. In line with the MMP-2 results, all of the ribozyme-transfected cell clones displayed

reduced levels of MT1-MMP mRNA. The most prominent reduction (97%) was found in the II-11b cells (II-11b: 0.03 ± 0.02 ; pH β -1: 1 ± 0.13 ; $P < 0.01$). The mRNA levels of MMP-1, MMP-9, and MMP-11 were below the level of detection in all of the clones, in both confluent and subconfluent cultures (Fig. 1 and data not shown).

As can be seen from Fig. 1, TIMP-1 was down-regulated at subconfluent growth conditions in all of the cell clones with reduced *S100A4* expression. The II-11a cell clone expressed only 25% less TIMP-1 than the control cells (II-11a: 0.75 ± 0.10 ; pH β -1: 1 ± 0.19 ; not significant), whereas II-11b expressed as little as 5% of the levels in the control cells (II-11b: 0.05 ± 0.01 ; pH β -1: 1 ± 0.19 ; $P < 0.01$). In contrast to what was the case for TIMP-1, the TIMP-2 mRNA levels were significantly up-regulated in all of the ribozyme-transfected clones (Fig. 1), at both confluence and subconfluence. In subconfluent cell cultures, the different ribozyme-transfected cell clones expressed between 2.5 and 4 times more TIMP-2 than the pH β -1 cells.

Gelatin Zymography. Protein data on MMPs and TIMPs were obtained from the two clones with the most prominent difference in *S100A4* expression. The typical zymogram in Fig. 2A shows that the pH β -1 cells produced larger amounts of a M_r 72,000 gelatinase than the II-11b cells. The quantitative evaluation of the different zymograms is shown in Table 1, indicating that the M_r 72,000 gelatinase produced by the II-11b cells at average was reduced to approximately 70% to that of the pH β -1 cells. This difference is probably somewhat larger, as it previously has been shown that this assay is linear over a limited range (34). Interestingly, in most zymograms it appeared that a band with an approximate M_r of 64,000–67,000 was more pronounced in the medium from the pH β -1 cells than from the II-11b cells. This band probably represents an active or intermediate form of MMP-2 since the activation of MMP-2 is thought to occur through an intermediate (35, 36). The zymograms also showed a faint band with M_r approximately 55,000 in the media from the control cells, probably representing MMP-1 (Fig. 2A).

To verify whether the bands detected in the gelatin zymography gels were due to only MMPs, one control gel was washed and incubated in buffers containing 10 mM EDTA. No bands were detected after this treatment, which indicated that the cleared zones obtained in gelatin zymography were entirely due to MMP activity.

Serum-free media from the pH β -1 and II-11b cells were incubated at different cell densities, and incubation times were also analyzed by means of casein zymography to examine MMP-3, MMP-10, and MMP-11 activities. None of the cultures showed activity against casein.

Western Blot and ELISA Analyses. The Western blot and ELISA analysis showed that the amounts of immunoreactive MMP-2 in conditioned media from the II-11b cells were approximately 20 and 50%, respectively, of the values for the pH β -1 cells (Fig. 2, B and C). When analyzed by ELISA, conditioned media from nontransfected parental OHS cells secreted similar amounts of immunoreactive MMP-2 as the pH β -1 cells (data not shown). Although no MMP-1 mRNA signals could be demonstrated, ELISA analysis demonstrated that both pH β -1 and II-11b cells secreted small amounts of immunoreactive MMP-1. The II-11b cells secreted 2.6 ng MMP-1/million cells, and the corresponding values for the control cells were 7.1 ng MMP-1/million cells (mean values, $P = 0.03$).

To detect TIMP-2 protein in the culture media by Western blots, it was necessary to concentrate the media harvested after 72 h ten times. The medium from II-11b cells contained approximately twice as much of TIMP-2 as the medium from the pH β -1 cells (data not shown). ELISA analysis on unconcentrated media did not detect immunoreactive TIMP-2 protein, which also showed that the secreted amounts of TIMP-2 are very small. In accordance with the Northern blot

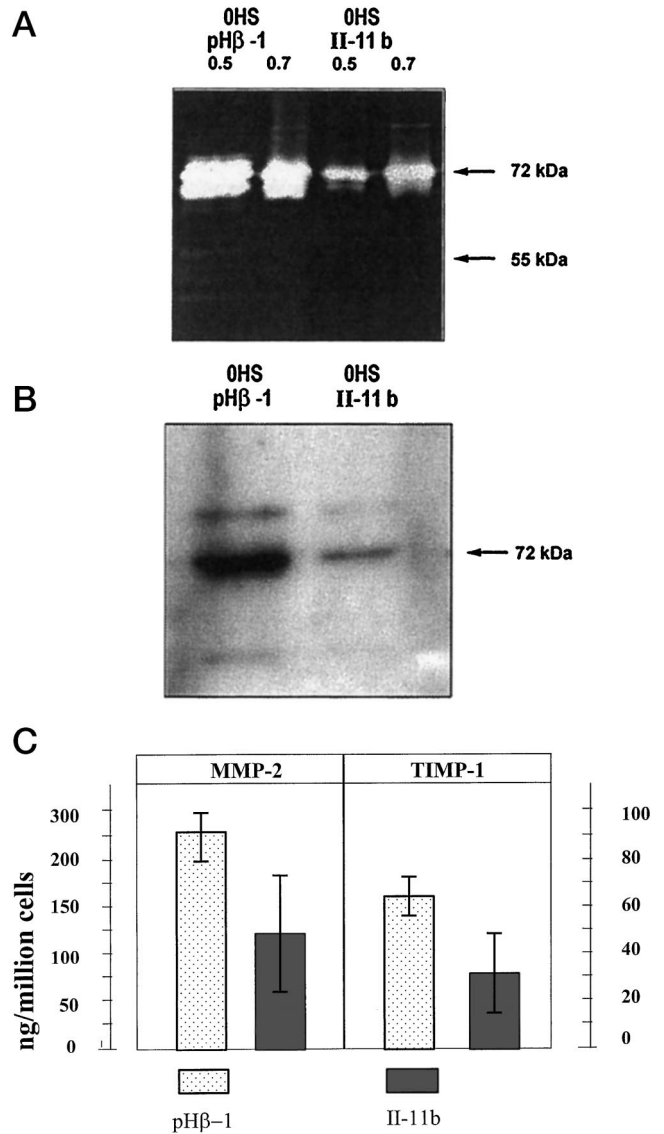


Fig. 2. Gelatin zymogram (A) and Western blotting (B) of harvested media from the two cell clones pH β -1 and II-11b performed as described in "Materials and Methods." C, levels of MMP-2 and TIMP-1 immunoreactivity in pH β -1 and II-11b cells. Subconfluent cells were cultured in serum free medium for 2 days, and conditioned media were collected and assayed by ELISA. Levels of immunoreactive MMP-2 and TIMP-1 are presented as mean of six and four independent determinations, respectively. Bars indicate 95% confidence interval for each sample.

analyses, ELISA analysis on unconcentrated media harvested from exponentially growing cells demonstrated that the TIMP-1 protein level was down-regulated in cells with reduced *S100A4* expression (Fig. 2C). Lastly, no immunoreactive MMP-3 was found by either ELISA or Western blot analysis.

In Vitro Invasive Properties. There was no significant difference between the parental OHS and the vector-alone-transfected cell clone pH β -1 with respect to attachment, motility, and invasion (data not shown). We were also not able to demonstrate any significant different ability to attach to Matrigel between the four transfected cell lines (data not shown). As shown in Fig. 3, the ribozyme-transfected clones II-11a and II-11b demonstrated 20 and 50% reduction in motility, respectively, as compared with the pH β -1 cells. However, no significant difference between the ability of the III-14 and the pH β -1 cells to cross uncoated filters were found. In the invasion assay, all of the clones with ribozyme-induced reduction in *S100A4* mRNA levels

Table 1 Relative amounts of the M_r 72,000 band (MMP-2) from gelatin zymography

Cell-conditioned medium from the OHS clones pH β -1 and II-11b have been analyzed as described in "Materials and Methods." Mean values and SDs are presented.

No. of cells $\times 10^6$	Incubation time (h)	Relative amounts of MMP-2 ^a Mean \pm SD (%)				$P < ^c$
		pH β -1	N^b	II-11b	N^b	
0.5	48	100 \pm 7	3	46 \pm 19	6	0.002
0.7	48	100 \pm 0.3	3	68 \pm 12	6	0.013
0.5	72	100 \pm 2	3	76 \pm 9	4	0.012
0.7	72	100 \pm 5	3	80 \pm 13	4	NS ^d

^a The intensity of the M_r 72,000 band including the M_r 64,000–67,000 band for the pH β -1 cells was quantitated, and the volume of the cleared zones for the II-11b cells is reported relative to that of pH β -1 cells.

^b Number of cell cultures analyzed.

^c Calculated by Student's t test for independent analysis.

^d NS, not significant.

demonstrated significantly lower capacity to pass Matrigel-coated filters than the control cells (Fig. 3).

DISCUSSION

Several investigators have suggested that the *S100A4* gene product may be of importance for the metastatic capacity of cancer cells, but the mechanisms by which *S100A4* enhances the metastatic potential are largely unknown. In the present study, we have investigated whether *S100A4* is involved in the metastatic process by regulating important events such as tumor cell attachment, motility, and invasion. We found that human osteosarcoma cells transfected with a hammerhead ribozyme against the *S100A4* transcript, displayed reduced *in vitro* invasive properties and alterations in the expression levels of MMPs and TIMPs. To our knowledge, this report is the first indicating that altered *S100A4* expression induces changes in the expression of MMPs and their endogenous inhibitors.

Attachment to basement membrane components is important in the metastatic process. In the present study, we could not demonstrate that cells with reduced expression of *S100A4* had altered the capacity to attach to Matrigel compared with control cells. In line with this, the expression of several adhesion molecules, such as N-CAM, N-cadherin, α_3 , α_5 , $\alpha_v\beta_5$, and β_1 integrins was unchanged in the cell clones when analyzed by immunocytochemistry.⁴ As far as we know, there are no other reports on a possible association between *S100A4* and tumor cell attachment.

In the transwell chamber assay, the control cells were significantly more motile than the II-11a and II-11b ribozyme transfected cells. However, no significant difference in motility was observed between the control cells and the ribozyme transfected clone III-14. In this latter cell clone, the ribozyme-induced down-regulation of *S100A4* was not as efficient as in the two other clones, indicating that large differences in *S100A4* expression are necessary before any effects on motility can be observed. Together the data support previous reports which hypothesize that *S100A4* influences the motile properties of transformed (18, 19) as well as normal cells (20) and suggest that increasing the motility of tumor cells may be one of the mechanisms by which *S100A4* promotes tumor dissemination (9, 18–21, 37).

All of the ribozyme transfected clones showed significantly lower invasion than the control cells. This finding suggests that *S100A4*, in addition to influence motility, also affects the capacity of tumor cells to degrade basement membrane components. In line with our observations, *S100A4* antisense RNA suppressed invasiveness more than motility in transfected Lewis lung carcinoma cells (38). It has, however, been reported that a mouse mammary carcinoma cell line trans-

fectured with *S100A4* demonstrated increased motility, but that no changes in *in vitro* invasiveness and *in vivo* metastasis compared with the parental cell line could be observed (19). These conflicting results may relate to differences between the human and murine system or to characteristics of the individual cell lines.

Several reports have shown that MMP-2 is up-regulated in malignant tissues and cells (5, 39, 40), and high levels of MMP-2 in tumor biopsies and serum from cancer patients have even been found to correlate with poor clinical outcome (5, 41, 42). In line with this, we found that the less invasive cells displayed reduced levels of MMP-2 mRNA. However, only the II-11b cells that express 5% *S100A4* mRNA compared with control cells, showed significantly reduced MMP-2 mRNA levels. Of the clones with intermediate *S100A4* expression, the II-11a cells had a nonsignificant 20% reduction of MMP-2 mRNA, whereas the III-14 cells showed equal levels of MMP-2 mRNAs compared with control cells. As for any motility effects to be observed, it is possible that a threshold down-regulation of *S100A4* is necessary before any effects in MMP-2 expression can be observed by Northern blot analyses.

A reduction in MT1-MMP expression in all of the ribozyme-transfected cells was observed. As was found for MMP-2 expression, the clone with the lowest *S100A4* mRNA levels displayed the least amounts of MT1-MMP mRNA. MT1-MMP is the biological activator of MMP-2 (36). Because it is the active form of MMP-2 that degrades basement membrane components, lowering MT1-MMP levels would be effective in reducing MMP-2 activity. In line with this, we found by zymography that the most invasive pH β -1 cells had a larger fraction of a M_r 64,000–67,000 form of gelatinase than the less invasive II-11b cells. Thus, it is tempting to speculate that the metastasis promoting *S100A4* gene product can participate in the regulation of expression and/or the activity of MMP-2.

The TIMP-2 mRNA levels were up-regulated in all of the cell clones with ribozyme-induced reduction of *S100A4* expression. These results indicate that the expression of TIMP-2 is influenced by smaller changes in *S100A4* expression than MMP-2. The deregulation of TIMP-2 in the ribozyme-transfected clones is in accordance with several reports showing that TIMP-2 attenuates tumor invasion both *in vitro* and *in vivo* (43–46). On the other hand, reduced *S100A4* expression was associated with down-regulation of TIMP-1 mRNA and protein. It may seem contradictory that down-regulation of a metastasis-associated gene is correlated with decreased levels of an MMP-inhibitor. However, many recent reports have shown that MMP inhibitors are multifunctional proteins. Enhanced TIMP-1 expression has been demonstrated in several cancer forms (47), and in human

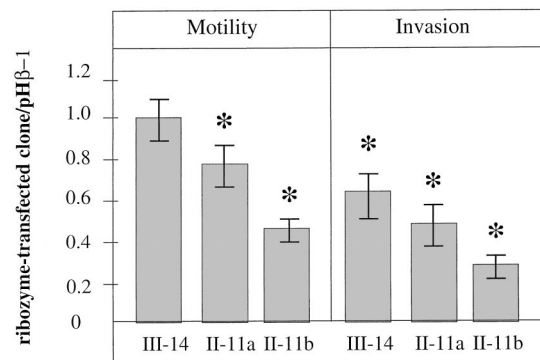


Fig. 3. *In vitro* motility and invasion of anti-*S100A4* ribozyme-transfected osteosarcoma cells (III-14, II-11a, II-11b). The cells were grown as described in "Materials and Methods." The ratios between control cells (pH β -1) and ribozyme-transfected cells are given as mean values of $n = 3$ experiments, each with six parallel measurements. Bars indicate 95% confidence intervals. *, $P < 0.05$.

⁴ Unpublished results.

hepatoma cells, we have previously shown that the more invasive cells expressed the highest levels of TIMP-1 (48). Various mechanisms have been suggested to explain how high levels of a proteinase inhibitor can be associated with a more aggressive phenotype. It is conceivable that proteinase inhibitors are produced by the tumor cells to protect themselves from the ongoing proteolysis (49), and it has also been suggested that TIMP-1 acts as a growth factor (49, 50). Thus, when studying the association between *S100A4* expression and individual MMPs and TIMPs, it is important to evaluate the balance between enzymes and their inhibitors because it is the net proteolytic activity that determines the capacity to degrade ECM. On the basis of our *in vitro* and *in vivo* results, *S100A4* down-regulation seems to influence the MMP/TIMP balance in a way that reduces ECM degradation.

Few previous studies have reported on the relationship between *S100A4* and the expression of proteinases (19, 38). In human glioma cells, only the noninvasive and non-*S100A4* expressing cells displayed detectable levels TIMP-2 (16), and a significant correlation between enhanced expression of *S100A4* and the invasive marker urokinase plasminogen activator-receptor has been demonstrated in human breast cancers (14). Furthermore, cathepsin D, an aspartic proteinase that in many studies is associated with poor clinical outcome in breast cancer patients was observed in some MCF-7 mammary carcinoma cells transfected with *S100A4* (15). Altogether, these data support our findings and suggest that the *S100A4* gene may induce changes in the expression of proteolytic enzymes and their inhibitors that result in a more malignant phenotype.

Moreover, we observed that differences in expression of both MMPs and TIMP-1 were influenced by cell density. Similar observations have also been reported by others (51, 52), and cell density has been shown to influence both *in vitro* invasion and malignancy (52–56). It is conceivable that effects of *S100A4* may be influenced by cell density since the protein has been found to interact with cytoskeletal components.

In conclusion, by using a human osteosarcoma cell line transfected with a specific ribozyme against *S100A4*, we obtained data suggesting that the *S100A4* gene product may be involved in promoting tumor invasion and metastasis via two pathways: (a) increasing the motility of the tumor cells; and (b) deregulating MMP and TIMP expression. The results are encouraging for further work on the exact mechanisms through which these effects are mediated.

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