Functional Significance of Metastasis-inducing S100A4(Mts1) in Tumor-Stroma Interplay*

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Causal implication of S100A4 in inducing metastases was convincingly shown previously. However, the mechanisms that associate S100A4 with tumor progression are not well understood. S100A4 protein, as a typical member of the S100 family, exhibits dual, intracellular and extracellular, functions. This work is focused on the extracellular function of S100A4, in particular its involvement in tumor-stroma interplay in VMR (mouse adenocarcinoma cell line) tumor cells, which exhibit stroma-dependent metastatic phenotype. We demonstrated the reciprocal influence of tumor and stroma cells where tumor cells stimulate S100A4 secretion from fibroblasts in culture. In turn, extracellular S100A4 modifies the cytoskeleton and focal adhesions and triggers several other events in tumor cells. We found stabilization of the tumor suppressor protein p53 and modulation of its function. In particular, extracellular S100A4 down-regulates the pro-apoptotic bax and the angiogenesis inhibitor thrombospondin-1 genes. For the first time, we demonstrate here that the S100A4 protein added to the extracellular space strongly stimulates proteolytic activity of VMR cells. This activity most probably is associated with matrix metalloproteinases and, in particular, with matrix metalloproteinase-13. Finally, the application of the recombinant S100A4 protein confers stroma-independent metastatic phenotype on VMR tumor cells. In conclusion, our results indicate that metastasis-inducing S100A4 protein plays a pivotal role in the tumor-stroma environment. S100A4 released either by tumor or stroma cells triggers pro-metastatic cascades in tumor cells.

Metastatic dissemination of cancer cells results in incurable disease and becomes one of the leading causes of cancer patient deaths. Studies on genes and their protein products involved in this process are of great importance.

Causal implication of S100A4 (mts1, CAPL, peL98, Calvasculin, p9Ka, and FSP1) in metastatic tumor progression was demonstrated by several approaches. Transfection of rodent and human non-metastatic tumor cell lines with S100A4 converts their phenotype to metastatic cells, and conversely, the antisense- and ribozyme-mediated mts1-inactivation abolishes the metastatic potential of metastatic tumor cells (1–4). Tumors developed in transgenic mice bearing exogenous S100A4 gene acquired metastatic phenotype (5, 6). The tight association of S100A4 with metastasis allows us to rate it among the most reliable prognostic markers. A high level of S100A4 in various types of cancers (breast, esophageal, gastric, colorectal, bladder, gallbladder, and lung) correlates with unfavorable prognosis and lethality (7–11).

S100A4 belongs to the S100 family of Ca2+-binding proteins that comprises 20 members. They are involved in the regulation of various important cellular functions such as cell growth, cell-cell communication, energy metabolism, contraction, neurite outgrowth, and cell motility (for review see Refs. 12–14). S100A4 protein, as a typical member of the S100 family, exerts dual, intracellular and extracellular, functions. It was shown that S100A4 binds to several intracellular target proteins and modulates their function. The binding to the heavy chain of non-muscle myosin II (15, 16) and Liprinβ1 (17) associates S100A4 with cell motility and adhesion, which are determinant functions of the metastatic tumor cells. S100A4 interacts with the tumor suppressor protein p53 and regulates its transactivational function. As a consequence of this interaction, differential modulation of p53-target genes expression occurs in a cell-specific manner (18).

As an extracellularly active protein, S100A4 stimulates neurite outgrowth of primary hippocampal cells (19), angiogenesis (20), and migration of astrocytic tumor cells (21) and it is functionally essential in the periodontal ligament (22). It has been shown that the blood vessel network of S100A4-positive tumors is more pronounced as compared with S100A4-negative tumors. Purified S100A4 protein stimulates neovascularization of mouse cornea in vivo and increases endothelial cell motility in vitro. Secretion of S100A4 was demonstrated from tumor cells in vitro, and elevation of the protein level was detected in the blood of S100A4-transgenic mice (20). The activity of S100A4 has been associated with the multimeric conformational form of the protein but not with the dimeric one (19). These observations point out an important extracellular function of S100A4 and suggest a putative active role of S100A4 in the tumor-stroma interplay.

The stroma is a supportive base of an epithelial layer and is composed of fibroblastic, smooth muscle, endothelial, inflammatory, and neural cells. It has been demonstrated that concomitant changes occur in the stroma surrounding the epithelial malignancy. These reciprocal molecular exchanges happen between tumor and stromal cells and facilitate tumor angiogenesis, invasion, and metastasis. Malignant tumor cells produce and secrete stimulatory growth factors and cytokines that locally activate the host microenvironment (cells and extracel-
lular matrix components). The activated cellular components of the stroma in turn produce biologically active molecules such as proteolytic enzymes, cytokines, and others that modify the proliferative and invasive behavior of the tumor cells. Fibroblasts are thought to be one of the main effector cells in the stroma compartment and an important source, along with tumor cells, of cancer-related matrix metalloproteinases (MMPs) \(^1\) (for review see Refs. 23–27).

One can hypothesize that S100A4 might be a part of the tumor-stroma interplay. The finding that growth factors such as epithelial growth factor, tumor growth factor-β1, and fibroblast growth factor-2 are able to activate S100A4 promoter and stimulate the expression of S100A4 (28) may support this idea. Moreover, a correlation of the expression of S100A4, enhanced MMP activity, and the metastatic potential of human tumor cells has been demonstrated (29, 30).

Here we present data on the influence of the S100A4 on the metastatic properties of the mouse adenocarcinoma cell line VMR whose metastatic behavior is stroma-dependent. This cell line develops metastases only upon subcutaneous injection but not upon intravenous injection where the tumor cells escape the contact with the host-derived stroma. We demonstrated that S100A4 confers metastatic potential on VMR cells. Moreover, VMR cells co-cultured with S100A4-positive fibroblasts induce the release of S100A4 from fibroblasts. In turn, remodeling of the actin cytoskeleton and adhesion contacts occurs in co-cultured tumor cells. The same type of alterations in cytoskeleton happens upon treatment of VMR cells with the recombinant S100A4 protein. Concomitantly, we observed stabilization and activation of tumor suppressor p53 protein, resulting in the modulation of p53-target gene expression. Using a zymography assay, we found high MMP-13 proteolytic activity in the conditioned medium (CM) of tumor cells after treatment with recombinant S100A4 protein. Data obtained indicate the reciprocal influence and cooperation between tumor cells and stroma-derived fibroblasts. We demonstrated the important role of S100A4 in this interplay needed for the acquisition of the metastatic phenotype.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Transfection**—VMR and CSML-0 mouse adenocarcinoma cell lines derived from two independent spontaneous tumors in A/Sn mice (31) were used. Wild type 4MEF/S100A4/H11001 and S100A4 knockout 3MEF/S100A4/−/− mouse embryonic fibroblasts were isolated from A/Sn mouse embryos of wild type and knock-out animals and immortalized by sequential passages in culture medium. Cells were cultured in DMEM supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 units/ml).

VMR cells were transfected with an S100A4 expression vector with constitutive hydroxymethyl glutaryl-CoA reductase promoter by electroporation. 1–2 \( \times \) 10^4 cells in 100 μl of phosphate-buffered saline were transferred into an electroporation cuvette, and a single pulse of 250 V and 250 microfarads was applied using a Bio-Rad electroporation system. Clones were selected in the presence of 400 mg/ml G-418.

**Metastasis Assay and Tissue Processing**—For experimental metastasis assay, 6-week-old A/Sn mice were injected intravenously with 10^6 tumor cells suspended in 0.2 ml of PBS. For the spontaneous metastasis assay, 10^5 cells were injected subcutaneously in 0.2 ml of PBS. Mice were examined and weighed every week. At the time of euthanasia, the visceral organs were removed by dissection, fixed in 4% formaldehyde, and examined grossly by visual counting of surface metastases.

Role of the extracellular S100A4 was assessed by the following protocol. 5–6-week-old A/Sn mice were given an intravenous lateral tail vein injection with 10^6 tumor cells suspended in 0.2 ml of PBS along with 50 μg of recombinant S100A4 or control Myo117 protein (recombinant 117 as a fragment of heavy chain of non-muscle myosin) into tail vein. Mice were supplied with additional doses (1 μg/g) of the recombinant proteins by intravenous injection twice a week within 3 weeks. 6 weeks after the injection, mice were euthanized and the visceral organs were removed, fixed in 4% formaldehyde, and examined. Visual counting of metastatic nodules was carried out.

**Tumor Cell/Fibroblast Co-cultures**—Equal amount of tumor cells and fibroblasts were seeded at a density of 8–10 \( \times \) 10^4 cells/cm^2 either in T25 culture flasks (Nunc) for secretion analysis or in 6-well plates with round glass coverslips for immunofluorescence staining. Cells were maintained in DMEM, 10% FCS for 24 h for immunofluorescence. For the secretion analysis, co-cultured cells were incubated in 2 ml of fresh medium for 6 h. CM was collected, filtered through 0.45-μm membranes (Schleicher & Schuell), and subjected to immunoprecipitation. Cell viability upon the harvesting of CM was checked by counting cells in 0.25% trypan blue. Only the experiments with 98–99% alive cells were proceeded further.

**Immunoprecipitation and Western Blot**—0.5 ml of the CM were supplemented with a protease inhibitor mixture (1 mM dithiothreitol, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine) and 1% Nonidet P-40 and incubated with anti-S100A4 polyclonal antibodies for 2 h in ice. The immunocomplexes were collected using a protein G-Sepharose suspension (50% v/v), separated on gradient SDS-PAGE (4–20%), and transferred onto Immobilon transfer membrane (Millipore). The membrane was blocked for 20 min in 5% dried milk in Tris-buffered saline plus 5% FCS and incubated with anti-S100A4 polyclonal antibodies 1:1000 for 1 h followed by incubation with secondary goat anti-rabbit IgG horseradish peroxidase (Dako) at 1:2000. Positive bands were visualized with ECL plus Western blotting detection system (Amersham Biosciences).

**Immunofluorescence Analysis**—Cells were grown on 10-mm-diameter coverslips and fixed with freshly prepared 4% paraformaldehyde in PBS for 30 min. Incubation for 3 min in 0.2% Triton X-100 in PBS was used to permeabilize the cells. Primary and secondary antibodies were applied in PBS supplemented with 10% FCS, either for 1 h at room temperature or overnight at 4 °C. The antibodies used were rabbit anti-S100A4 (1:1,000), monoclonal anti-vinculin (1:600, Sigma), and monoclonal anti-p53 pAb421 (1:100) purified from ascite fluid on a protein G-Sepharose. Secondary Alexa Fluor antibodies were purchased from Molecular Probes and used in a dilution of 1:1,500. To visualize polymerized actin, TRITC-conjugated phalloidin was used. Coverslips were stained with 4',6-diamidino-2-phenylindole, mounted with Fluoromount-G (Southern Biotechnology Associates, Inc), and analyzed on a laser-scanning microscope LSM 510 (Zeiss).

**Northern Blot Analysis**—VMR cells grown at 80% confluence were treated with recombinant proteins for 24 h. Total RNAs were isolated as described previously (32). Gel electrophoresis and Northern blot analyses were performed as described previously (4). Filters were hybridized with murine p21waf1, bax, thrombospondin-1 (THBS1) and MMP-13 probes. The amount of mRNAs on the filters was calibrated by hybridization with \( [\gamma-^{32}P]ATP\)-labeled poly(U) probe. For quantification of the signal intensities, membranes were scanned using a Molecular Dynamics computing densitometer (Sunnyvale, CA) and analyzed with the ImageQuant software.

**Protease Activity Analysis of CM**—Cells were grown to 90% confluence in DMEM, 10% FCS. The medium was exchanged to a serum-free DME/F12 medium containing recombinant proteins of interest. The cultures were sustained for 24 h, and the harvested medium was filtered through 0.45-μm membrane filters and concentrated 20–50 times using Vivapore or Vivasin concentrators (Vivascience Ltd.). Subsequently, the concentrated CM were treated with trypsin and loaded onto SDS-PAGE gels. The cleared zones were assayed for protease activity using gelatin and casein zymography. Samples were separated in 10% SDS-PAGE containing either gelatin (0.5 mg/ml Sigma) or casein (0.5 mg/ml Sigma, and all of the procedures were performed as described previously (4). The cleared zones were quantified using LAS-1000 analyzer with subsequent quantification with Image Gauge computing program (Fuji film). The same concentrated CM was used for Western blot analysis with anti-MMP-13 antibodies (Neomarkers) as described above.

**RESULTS**

**Intracellular and Extracellular S100A4 Promotes Tumor Progression**—The mouse mammary adenocarcinoma VMR cell line exhibits moderate metastatic capacity upon subcutaneous
administration with lung and liver specificity. We found that VMR cells injected directly into the blood (intravenous) do not develop detectable tumor lesions in visceral organs, whereas VMR cells obtained directly from a primary tumor injected intravenously into the blood stream developed vigorously multiple tumor nodules in various organs (lungs, liver, ovaries, and spleen) (Table I). These results suggest that VMR cells need to cooperate with the host stroma environment to obtain the full metastatic capacity. To assess a possible role of S100A4 in this process, we employed several approaches.

To study whether the exogenous expression of S100A4 in VMR cells (originally negative for S100A4) will influence their metastatic properties, we generated clones expressing S100A4. Two S100A4-expressing clones (BH5C3 and BH4D6), one vector-transfected cell line (BH-neo) and the original VMR cell line, were chosen. Their metastatic ability was checked in vivo in syngeneic mice. In spontaneous metastasis assay (subcutaneous inoculation of tumor cells), we did not find any visible difference between cell lines in the formation of metastatic lesions. However, upon intravenous injection (experimental metastases assay), the expression of S100A4 enabled VMR cells to colonize various visceral organs and develop tumors (Table II). These data indicate that the metastatic phenotype of VMR cells in spontaneous metastasis assay is strongly dependent on the expression of S100A4.

Previously, we have demonstrated that extracellular S100A4 protein stimulates angiogenesis in vitro (20) and promotes neurite outgrowth from hippocampal cells in vitro (19). To test whether extracellular S100A4 protein could influence tumor cell behavior in vitro, we injected mice with VMR cells along with recombinant S100A4 protein into the tail vein. Mice were supplied with additional doses of S100A4 protein by intravenous injection of the protein twice a week within period of 3 weeks. The animals were euthanized 4 weeks after the injections of the cells. Lungs and livers were removed and examined. Similar experiments were performed with VMR cells treated with the recombinant non-muscle myosin fragment (Myo117). We have chosen this recombinant protein as a negative control because it has both a size similar to S100A4 and a His tag. The data revealed a strong pro-metastatic effect of extracellular S100A4 on VMR cells (Table II). The number of metastatic nodules was >10 times higher in animals injected with S100A4-treated VMR cells compared with non-treated or treated cells with the Myo117 protein. Moreover, the size of the nodules in the VMR/S100A4 group was much larger compared with the control and Myo117-treated group. These data show that the extracellular S100A4 protein provides optimal conditions for tumor cell survival, homing and proliferating in visceral organs, and suggest an implication of S100A4 in tumor microenvironment.

Reciprocal Effects of Tumor Cells and Immortalized Fibroblasts—To assess the role of S100A4 in the tumor-stroma interplay, we analyzed cross-impact of tumor cells and fibroblasts in co-culture experiments. We used metastatic VMR and non-metastatic CSML-0 tumor cells. Neither of these tumor cell lines expresses S100A4. Conditioned medium from mono-cultures and co-cultures (tumor cells and fibroblasts) were collected and filtrated through a 0.45-µm filter. The proteins were immunoprecipitated with anti-S100A4 antibodies and analyzed by Western blot. The data in Fig. 1 show that co-culture of 4MEF/S100A4+/+ fibroblasts with VMR cells results in a significant increase of S100A4 in the CM (lane 5). Co-culture of the same fibroblasts (4MEF/S100A4+/+) with 3MEF/S100A4−/− fibroblasts or non-metastatic CSML-0 tumor cells does not influence the S100A4 release from 4MEF fibroblasts (Fig. 1, lanes 3 and 4). Moreover, CM from VMR cells collected after 24 h strongly stimulates the release of S100A4 protein from 4MEF/S100A4+/+ cells (Fig. 1, lane 6).

To explore the implication of S100A4 in tumor-stroma interplay, we compared the morphology of VMR cells co-cultured with S100A4-positive (4MEF/S100A4+/+) and S100A4-negative (3MEF/S100A4−/−) fibroblasts. Fibroblasts and tumor cells were distinguished by morphology or by immunostaining with anti-vimentin antibodies (data not shown).

Fig. 2A demonstrates that in the presence of 4MEF/S100A4+/+ fibroblasts, VMR tumor cells exhibit more flat and adherent morphology with prominent actin fibers, whereas VMR cells grown together with 3MEF/S100A4−/− fibroblasts were round-shaped with more subcortical distribution of actin. To associate the observed cytoskeleton remodeling with the extracellular S100A4, we incubated VMR cell culture with recombinant S100A4 protein. Similar to the previous experiment, we observed enhancement of actin cytoskeleton. Additionally, the staining for the adhesion marker vinculin revealed an appearance of adhesion contacts (Fig. 2B). The obtained data indicate that VMR cells may release factors that are able to enhance secretion of the S100A4 protein from fibroblasts. S100A4 protein in turn might influence VMR cells features, namely cytoskeleton and adhesion.

Extracellular S100A4 Stimulates Active Protease Secretion from VMR Cells—It is well established that expression level of MMPs correlates with metastatic potential in advanced cancer. In this work, we studied whether S100A4 added extracellularly may have influenced proteinase activity in VMR cells. VMR cells were cultured in the presence of various forms of the recombinant S100A4 protein. We used as a control the same non-muscle myosin fragment (Myo117) as in previous experiments. The CM from treated cells was harvested and concentrated, and protease activity was analyzed by zymography with β-casein and gelatin as substrates. The results obtained demonstrated that S100A4 strongly stimulates proteolytic activity in the conditioned medium of VMR cells after a 24-h incubation (Fig. 3A) but not after 6 h (data not shown). Treatment of VMR cells with recombinant S100A4 protein stimulates activity of an active 46–50-kDa proteinase with high affinity to β-casein and much less to gelatin substrates. To show that this proteinase belongs to the MMP family, proteolytic activity in the CM was blocked by EDTA (data not shown). The highest activity was associated with the multimeric form of S100A4. The activity of another high molecular mass (around 120 kDa) protease was exclusively detected in gelatin gels. Treatment of non-metastatic CSML-0 cells with different forms of S100A4 did not reveal increase of active proteinases in CM (Fig. 3B). To confirm the specificity of S100A4 on the proteinase activity, we used anti-S100A4 antibodies to block the S100A4 effect on VMR cells. As shown in Fig. 3C, the S100A4-mediated elevation of proteinase activity was inhibited by the S100A4-specific antibodies. Polyclonal antibodies revealed a 5-fold inhibition of proteinase activity, and a twice less effect was found with monoclonal antibody. The same antibodies did not influence the proteinase activity stimulation by phorbol 12-myristate

| Administration | No. of mice | Metastases in lungs | Metastases in liver | Metastases in visceral organs |
|----------------|-------------|---------------------|---------------------|-----------------------------|
| Subcutaneous   | 75/80       | ++                  | ++                  | ++                          |
| Intravenous a  | 2/40        | −/−                 | −/−                 | −/−                         |
| Intravenous b  | 10/10       | ++++                | ++++                | ++++                        |

a Intravenous injection of VMR cells from culture.
b Intravenous injection of cells directly from primary tumor. Primary tumors were disaggregated and single cell suspension was injected.

### Table I

Metastatic capacity of VMR cells at various routes of administration

| Administration | No. of mice | Metastases in lungs | Metastases in liver | Metastases in visceral organs |
|----------------|-------------|---------------------|---------------------|-----------------------------|
| Subcutaneous   | 75/80       | ++                  | ++                  | ++                          |
| Intravenous a  | 2/40        | −/−                 | −/−                 | −/−                         |
| Intravenous b  | 10/10       | ++++                | ++++                | ++++                        |

a Intravenous injection of VMR cells from culture.
b Intravenous injection of cells directly from primary tumor. Primary tumors were disaggregated and single cell suspension was injected.
abolished by anti-S100A4 antibodies but not by control IgG. Twice the expression of MMP-13 mRNA, and this effect could be demonstrated that extracellular S100A4 stimulates more than the control and S100A4-treated VMR cells. Data in Fig. 4B employed Northern blot hybridization with RNAs isolated from MMP-13 expression occurs at a transcriptional level, we estimated whether the activation of induction of MMP-13 (37). To check whether the activation of MMP-13 expression occurs at a transcriptional level, we employed Northern blot hybridization with RNAs isolated from the control and S100A4-treated VMR cells. Data in Fig. 4B demonstrate that extracellular S100A4 protein stimulates secretion of MMP-13 and a weak lower band possibly corresponding to the modified pro-form or the active form of the enzyme. CM from cells treated with phorbol 12-myristate 13-acetate and tumor necrosis factor α (data not shown), the known potent activators of MMPs (35). Recently, we have demonstrated that extracellular S100A4 protein stimulates secretion of MMP-13 from endothelial cells. \(^2\) To check whether the observed 46–50-kDa cleared band detected in the casein-zymography experiments corresponds to MMP-13, we performed Western blot analysis using anti-MMP-13 antibody (Fig. 4A). The antibody detected two bands, a 60-kDa strong upper band corresponding to an inactive form of MMP-13 and a weak lower band possibly corresponding to the modified pro-form or the active form of the enzyme. CM from cells treated with phorbol 12-myristate 13-acetate + tumor necrosis factor α was used as a positive control for the induction of MMP-13 (37). To check whether the activation of MMP-13 expression occurs at a transcriptional level, we employed Northern blot hybridization with RNAs isolated from the control and S100A4-treated VMR cells. Data in Fig. 4B demonstrate that extracellular S100A4 protein stimulates more than twice the expression of MMP-13 mRNA, and this effect could be abolished by anti-S100A4 antibodies but not by control IgG.

**Extracellular S100A4 Modulates Transcription of p53-dependent Genes**—Previously, we demonstrated that intracellular S100A4 binds and regulates p53 function (18). Here we tested whether the recombinant S100A4 protein added to cultured VMR cells may influence activity of wild type p53. We analyzed p53 protein in VMR cells incubated with recombinant S100A4 protein. The data obtained showed stabilization of the p53 protein that resulted in an increase in the amount of p53 protein that was easily detectable by immunohistostaining and Western blot analyses (Fig. 5A). To analyze whether the stabilization of p53 protein has any functional significance, we checked the expression of p53 target genes after 24 h of S100A4 treatment (Fig. 5B). Northern blot analyses showed a strong (>2-fold) reduction in the level of thrombospondin-1 expression in VMR cells treated with the multimeric form of S100A4 in comparison with the dimeric form where only a 1.2-fold reduction was observed. Both forms of S100A4 revealed equal (2-fold) inhibitory effect on the transcription of bax. The RNA levels of thrombospondin-1 and bax in cells incubated with the control protein Myo117 were equal to the control, non-treated probes. We observed no prominent effect on p21waf expression after treatment of VMR cells with extracellular S100A4 protein. These data demonstrate the ability of extracellular S100A4 to alter the transcriptional activation function of p53 in VMR cells.

**DISCUSSION**

S100A4 is a typical member of the S100 family of Ca\(^{2+}\)-binding proteins with dual, intracellular and extracellular, functions. Metastasis-inducing effect of S100A4 has been demonstratively proven (1–11). However, the mechanism of the metastasis-promoting function of S100A4 is not clear ad extremum. It was shown that the induction of S100A4 expression

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\(^2\) B. Schmidt-Hansen, D. Ornas, M. Grigorian, E. Tulchinsky, E. Lukanimid, and N. Ambartsumian. (2004) Oncogene, in press.

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**TABLE II**

| Groups          | No. of mice with metastases | Average number of metastases in lungs and liver | Average number of metastases in other organs |
|-----------------|-----------------------------|-------------------------------------------------|---------------------------------------------|
| VMR             | 1/12                        | 0.5 ± 0.6                                       | 0                                           |
| BH-neo\(^a\)    | 0/7                         | 0                                                | 0                                           |
| BH5C3\(^a\)     | 7/7                         | 166 ± 40.0                                      | 6                                           |
| BH4D6\(^a\)     | 7/7                         | 53 ± 8.0                                        | 2                                           |
| VMR/S100A4\(^a\)| 5/7                         | 15.4 ± 6.55                                     | 0                                           |
| VMR/S100A4/Myo\(^a\)| 2/7                     | 1.28 ± 2.63                                     | 0                                           |

\(^a\) VMR cells expressing S100A4.

\(^b\) VMR cells treated with the recombinant S100A4 protein or fragment of non-muscle myosin heavy chain.

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**FIG. 1. Immunoprecipitation of the S100A4 protein from CM.** CM from equal amount of tumor cells, fibroblasts, or co-cultured cells was incubated with anti-S100A4-polyclonal antibodies. Immunocomplexes were collected using protein G-Sepharose and analyzed in Western blot using anti-S100A4-polyclonal antibodies.

**FIG. 2. The effect of S100A4 on the remodeling of actin cytoskeleton and focal adhesions in VMR cells.** A, co-culture of VMR cells with 3MEF/S100A4\(^{-/-}\) or 4MEF/S100A4\(^{+/+}\) fibroblasts. Double staining with antibodies against actin (red) and S100A4 (green) is shown. B, extracellular recombinant S100A4 modulates cytoskeletal organization and focal adhesions in VMR cells. VMR cells were cultured without (control VMR) or with recombinant S100A4 protein (VMR+S100A4) and stained with antibodies against actin (red) or vinculin (green). Vinculin staining revealed cellular adhesion contacts.
leads to an increased cell motility and metastasis of tumor cells (36). Interaction of S100A4 with intracellular target proteins, non-muscle myosin heavy chain and liprin \( ^{1} \) might be responsible for tumor cell adhesion and motility (15–17). The tumor suppressor p53, another S100A4 target protein (18), is deeply involved in various pathways implicated in tumor progression (apoptosis, angiogenesis, cell differentiation, and motility).

Simultaneously, these data were accumulated indicating an important extracellular function of S100A4, mostly attributed to its multimeric form. Thus, extracellularly added recombinant S100A4 strongly stimulates neuronal differentiation (19), increases motility of endothelial cells (20), stimulates motility of tumor cells (21), and inhibits mineralized nodule formation in a rat osteogenic cell culture (22). Based on this knowledge, we assumed that S100A4 might have an important role in tumor-stroma interaction by promoting tumor vascularization and acquisition of metastatic properties of tumor cells. As it was shown previously, cell lines positive for S100A4 exhibit high metastatic potential, whereas S100A4-negative cell lines as a rule are non-metastatic or less metastatic (37).

Here we use a S100A4-negative mouse mammary adenocarcinoma cell line VMR that reveals a metastatic phenotype only when injected subcutaneously but not directly into the blood stream. However, when tumor suspension that contains a mixture of tumor and stroma cells was injected intravenously, the cells efficiently colonized visceral organs. These data propose that cells need to cooperate with a host stroma environment to display a fully metastatic phenotype. Induction of intracellular S100A4 alters VMR cell phenotype toward metastatic in experimental (intravenous) metastasis assay. We have demonstrated before that the expression of S100A4 in VMR cells was followed by an immense S100A4 secretion into extracellular space (20). Interestingly, exposure of VMR cells to the recombinant S100A4 protein by co-injection of cells and the protein into mice tail vein followed by several administrations of S100A4 into the bloodstream results in enhanced metastatic colonization of lung and liver compared with non-treated and control protein-treated animals. These data suggest that S100A4 might replace the stromal signals and advance tumor cell progression. Moreover, the protein by itself could originate from both tumor and tumor environment, i.e. stroma cells.

Co-culture experiments demonstrated a mutual influence of tumor cells and immortalized fibroblasts by remodeling the cytoskeleton and the adhesions plaques of tumor cells. Our data suggest the existence of factor(s) released by tumor cells into the CM, which can activate the release of S100A4 from co-cultured fibroblasts. In turn, the S100A4 protein exerts a profound influence on tumor cells. Thus, the treatment of VMR cells with the S100A4 protein results in the stabilization of p53 protein and modulation of expression of p53-regulated genes. Interestingly, both intracellular (18) and extracellular S100A4 exhibit high metastatic potential, whereas S100A4-negative cell lines as a rule are non-metastatic or less metastatic (37).
protein inhibits the expression of the thrombospondin-1 gene, which encodes an inhibitor of angiogenesis (38). This observation fits well with a pro-angiogenic function of S100A4 (20). Additionally, we found that extracellular S100A4 down-regulates another p53-regulated pro-apoptotic gene, bax, which might influence tumor cell survival.

It is well established that remodeling of extracellular matrix and activation of MMPs are immanent features of tumor metastasis. Here we demonstrate the induction of active MMPs from the VMR cells as a direct response to S100A4 protein treatment. The major protease activity was identified by molecular size (46–50 kDa) and antibody reactivity in Western blot as MMP-13. MMP-13 expression is tightly associated with various human tumors such as breast carcinomas, squamous cell carcinomas, and head and neck carcinomas (39–43). By having a broad proteolytic capacity, MMP-13 degrades very efficiently native fibrillar collagen and several other extracellular matrix components, allowing MMP-13 to serve its role as a potent extracellular matrix-destructing tool facilitating invasion and metastasis (39, 44). Based on the data obtained here, we propose an important role of the extracellular S100A4 protein in tumor progression by remodeling tumor cell cytoskeleton, promoting cell motility and adhesion, and facilitating the degradation of extracellular matrix via induction of proteolytically active metalloproteinases. S100A4 protein might be produced and secreted either by tumor or stroma cells or both. The scenario of the process is highly dependent on the properties of the tumor cells. Tumor cells must be able to produce factor(s) to activate secretion of S100A4 from the stromal cells and respond to the extracellular S100A4, presumably via a receptor activation mechanism. We may assume that S100A4 interacts with a putative membrane receptor and triggers downstream events either via signal transduction or protein internalization or both. Our preliminary results revealed intracellular distribution of the recombinant S100A4 added in extracellular space. Internalization of other S100 proteins was demonstrated earlier (45, 46). In addition, the activation of phospholipase C and protein kinase C and the activation of the extracellular signal-regulated kinase 1 and 2 signaling cascade by extracellular S100A4 were demonstrated in primary hippocampal neurons (19). We assume that the transcriptional factors p53 and probably NF-κB might be involved in S100A4-driven MMP activation in VMR cells. Recently, we have shown that extracellular S100A4-mediated stimulation of MMP-13 trans-activation is synchronous with an activation of NF-κB in endothelial cells. Additionally, simultaneous activation/stabilization of p53 and increase of the proteolytic activity are demonstrated here. Together, these results support our assumption of the possible implication of these transcription factors in S100A4-mediated proteinase activation, which is in good agreement with the data on their tight implication in the transcriptional regulation of MMPs (47–49).

Based on the data presented here, we propose that S100A4 is an active metastasis-regulating component secreted by either tumor or tumor-activated stromal cells or both. The extracellular S100A4 might target tumor or vasculature (endothelial) cells, probably by binding to a yet unknown receptor and triggering a cascade of events either through signal transduction or internalization followed by interaction with the intracellular target proteins (tumor suppressor p53, non-muscle myosin, and others) or both. As a result, tumor cells acquire a more progressed metastatic phenotype with altered cell adhesion, motility, and high proteolytic activity. Additional targeting of endothelial cells stimulates their motility, enhancing tumor angiogenesis that provides routes for metastatic dissemination. Altogether, these observations allow us to conclude that S100A4 is a pro-metastatic key player in tumor-stroma interaction that finally determines the fate of neoplastic disease. Moreover, S100A4, as an extracellular component, might be considered as a molecular target for cancer therapy.

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Functional Significance of Metastasis-inducing S100A4(Mts1) in Tumor-Stroma Interplay
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