Commentary

Protein S100A4: Too Long Overlooked by Pathologists?

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Invasion and metastasis are hallmarks of malignant tumors resulting from the interaction between tumor cells and the surrounding tissues. They include pathogenic steps, such as the proliferation and detachment of neoplastic cells, invasion of the extracellular matrix, angiogenesis, vascular dissemination, and eventually, homing of the tumor cells and proliferation at the new site. The activation of many genes and the expression of their products have been shown to be important in tumor progression. Throughout the past few years, the S100 family has emerged as an important group of proteins with the capacity to promote invasiveness and metastasis of many human neoplasms. In particular, recent studies shed light on the mechanisms of action of protein S100A4,¹⁻³ and indicate its possible prognostic role in human neoplasms. In the current issue of *The American Journal of Pathology*, Rosty and colleagues⁴ extend our knowledge on this protein by analyzing a subset of cell lines derived from pancreatic cancers and a series of primary adenocarcinomas of the pancreas. It is noteworthy that the attention of the authors was drawn on the putative significance of S100A4 in pancreatic cancer by an analysis of online serial analysis of gene expression (SAGE) database. Interestingly, the S100A4 gene has also been recently identified as being highly up-regulated in gastric adenocarcinoma using cDNA array technology.⁵ So far, studies on S100A4 were mainly restricted to research laboratories. It is likely that with the increasing amount of evidence for the involvement of S100A4 in human cancers, the results of this research area will soon gain significance in clinical practice.

The Calcium Binding Protein Family

Calcium binding proteins form a large family involved in numerous functions ranging from the control of cell-cycle progression and cell differentiation to enzyme activation and regulation of muscle contraction.^{6,7} They may be separated into two subfamilies, according to the presence or absence of a structural motif, the EF-hand, which

consists of a consensus sequence of generally 12 amino acids able to bind Ca^{2+} selectively and with high affinity.^{8,9} The number of EF-hand domains in calcium-binding proteins is variable and ranges from 2, as in S100 proteins, to 6, as for instance in calretinin. The S100 proteins represent one of the largest subfamilies of the EF-hand proteins with at least 19 different members; the degree of homology ranges from 25 to 65%.⁷ They were initially characterized as low-molecular weight (10 to 12 kd) acidic proteins and named by their solubility in 100% ammonium sulfate ("S100"). Characteristically, one of the EF-hand Ca^{2+} -binding loops of the S100 proteins contains 14 amino acids instead of 12. The functions of S100 proteins remained primarily unknown for several years. Recently, however, much interest has focused on S100A4 and some other S100 family members, such as S100A2,¹⁰ S100A6,¹¹ and S100B^{12,13} for their potential relevance in neoplastic diseases.

Biochemical Characteristics and Tissue Distribution of Protein S100A4

Protein S100A4 is a polypeptide of 101 amino acids with a molecular mass of ~11.5 kd.² It has been described under a variety of names including p9Ka,^{14,15} calvasculin,¹⁶ or CAPL.¹⁷ The corresponding gene, cloned by different groups, is known as *mts1* (metastasin),¹⁸ pEL98,¹⁹ 18A2,²⁰ 42A,²¹ and fsp (fibroblast-specific protein).²² Initial cloning experiments performed by screening cDNAs obtained from cultured cell lines before and after growth stimulation,^{19,20} as well as the gene isolation from metastatic tumor cell lines,¹⁸ already suggested a possible link between protein S100A4 expression, cell proliferation, and cancer progression. These findings have been subsequently supported by the demonstration of a marked up-regulation of S100A4 at the mRNA and protein level in murine NIH3T3 fibroblasts or normal rat kidney cells on transformation with oncogenes, such as v-K-ras, v-Ha-ras, or v-src,^{23,24} and by results obtained in

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transgenic mice, mentioned later.^{25,26} Moreover a possible role of S100A4 in cell differentiation became soon apparent by the demonstration of S100A4 mRNA and protein up-regulation in human promyelocytic leukemia HL-60 cells during macrophagic or granulocytic differentiation in response to phorbol 12-myristate 13-acetate or dimethylsulfoxide,²⁷ by findings indicating gene and protein overexpression during the conversion from murine mesenchymal to epithelial cells, or in rat pheochromocytoma cells after induction of cell elongation.^{21,22} Studies on the distribution of protein S100A4 in normal tissues have been hampered for a long time by technical problems related to the cross-reactivity of the available antibodies. Most of our current knowledge is derived from immunohistochemical analysis performed in rat tissues.²⁸ Here, intracellular S100A4 was expressed in smooth muscle cells; brown adipose tissue; liver; some absorptive and keratinized epithelia; acid-secreting parietal cells of the stomach; neuronal cells within plexus of the autonomic nervous system; and in a subset of cells of the immune system in spleen, lymph nodes, bone marrow, and blood. In particular, protein S100A4 was highly expressed by smooth muscle and endothelial cells of both arteries and veins. In humans, S100A4 protein expression has so far been demonstrated in monocytes, macrophages, and polymorphonuclear granulocytes.²⁷ Faint expression has been described in keratinocytes, melanocytes, Langerhans' cells, and sweat glands,²⁹ findings not confirmed by others.³⁰ Conversely, protein S100A4 has been detected only in a subset of cells of the normal ovary and prostate, and it has not been detected in normal tissues obtained from the breast, colon, thyroid, lung, kidney, and pancreas.³⁰ An increasing body of evidence clearly indicates that, in addition to its intracellular location, protein S100A4 may be secreted into the extracellular space. For instance, studies on the rat mammary gland suggested an extracellular location of S100A4 around the ductuli.²⁸ Further, release of protein S100A4 has been reported to occur in intact periodontal cultured cells³¹ and mammary carcinoma cells.³² These findings are in agreement with the notion that many other S100 proteins can be secreted. For example, human monocytes secrete protein S100A8 and protein S100A9 after activation by protein kinase C.³³ The data collected so far indicate that S100 proteins form noncovalent dimer inside the cell and covalently linked dimers in the extracellular space.³⁴ Presumably, calcium binding to these proteins induces conformational changes resulting in exposure of new binding sites at their surface, and, consequently, allows for the interaction with novel target proteins. Recent studies also demonstrate that, in solution, S100A4 exists in a monomer-dimer equilibrium influenced by the binding of calcium,³⁵ and that protein S100A4 homo- and heterodimerization may occur *in vivo*.³⁶ In fact, the ability of protein S100A4 to form homodimers, heterodimers, and even oligomers³⁷ reflects the structural plasticity of this protein, and may provide the structural basis for the diversity of its biological functions *in vivo*.

Protein S100A4 Promotes Cancer Progression

Several observations support a role of protein S100A4 in invasive growth and metastasis of cancers. As mentioned above, the initial findings of elevated S100A4 in transformed murine fibroblasts, and in metastatic mouse cell lines suggested an association between this protein and molecular mechanisms involved in tumor progression.^{18,19,38} Transfection experiments showed later that rodent or human S100A4 can induce a metastatic phenotype in previously nonmetastatic rat mammary cells.^{39,40} Similarly, transfection of the rodent S100A4 gene into the B16 murine melanoma⁴¹ and into human breast cancer MCF-7 cells⁴² increased the capability to metastasize to the lungs. Conversely, antisense S100A4 RNA or anti-S100A4 ribozyme suppressed the metastatic potential of highly metastatic cell lines.^{43,44} Transgenic mouse studies demonstrated that protein S100A4 by itself was not able to initiate tumors. However, it induced metastatic disease of cells that had been initiated by other oncogenes. In fact, transgenic mice with additional copies of the S100A4 gene developed normally and, compared to control mice, did not show an elevated tumor incidence.⁴⁵ However, when S100A4 transgenic mice were mated with *neu* transgenic mice, known for developing mammary cancer after multiple pregnancies, offspring that inherited both genes developed mammary neoplasms with significantly more lung metastases compared to mice that inherited only the *neu* oncogene.²⁶ In addition mating GRS/A mice, which spontaneously develop mammary tumors, with S100A4 transgenic mice showed that mice bearing GRS/A protein S100 hybrids form aggressive mammary carcinomas able to metastasize, compared to GRS/A mice alone.²⁵

Protein S100A4 Is a Prognostic Marker for Many Cancer Types

The association between protein S100A4 expression and tumor progression obviously raises the question whether this protein represents a useful prognostic marker in clinical practice. In a first attempt to address this question, the expression of several members of the S100 protein family was investigated by Western blot techniques in a panel of human breast-cancer cell lines and in breast cancer tissues.⁴⁶ The results obtained with cell lines, however, did not strictly correlate with the tumorigenicity of the cells and with the expression of other prognostic factors, such as estrogen and progesterone receptors. On the other hand, protein S100A4 expression could be demonstrated in most breast carcinomas, whereas it was very low or absent in control tissues. Further, the finding of a correlation between the expression level of protein S100A4 and the presence of the urokinase-type plasminogen activator, a well-known marker for cancer invasion, suggested a possible prognostic role of S100A4 in human breast cancers. Similar conclusions were supported by a study performed on a small series of breast carcinoma patients that showed a correlation between S100A4 gene expression and aggressive disease.⁴⁷ Re-

cently, two retrospective studies, based on the same well-characterized group of 349 patients with a follow-up period of 19 years,^{48,49} analyzed the prognostic significance of protein S100A4 in breast cancer, and evaluated the association between protein expression, as detected by immunohistochemical staining, and variables with potential prognostic value for patient outcome. The anti-serum stained 56% of the carcinomas either strongly or at a borderline level, whereas 44% of the carcinomas remained unstained. The overall survival for patients with carcinomas expressing S100A4 was significantly worse than for those patients considered negative for S100A4. The results also suggested that not only the presence of protein S100A4 but also the percentage of expressing cells could correlate with the clinical course. Further, a weak but statistically significant association of S100A4 staining could be demonstrated with the presence of nodal metastasis, positive staining for *c-erbB3*, cathepsin D, and *c-erbB2*. Conversely, there was no obvious association between protein S100A4 expression and other variables, such as tumor size, histological grade, menopausal status, and staining for hormone receptors. Nevertheless, despite a limited patient population, the studies suggested that the presence of S100A4 protein in breast cancer is a more valuable factor at predicting patient outcome than the extent of lymph node involvement by cancer.

In analogy to studies performed on breast cancer, the prognostic significance of protein S100A4 expression has recently been evaluated in a series of esophageal-squamous carcinomas, non-small lung cancers, and primary gastric cancers.^{50–52} Patients with S100A4-positive esophageal carcinomas [13 of 52 (25%)] had a significantly poorer prognosis than patients with S100A4-negative carcinomas; the protein S100A4 status in cancer specimens remained the only independent prognostic parameter in a multivariate analysis.⁵⁰ Immunohistochemically S100A4 was detectable in 81 of 135 (60%) lung cancers. S100A4 was found to be useful to identify patients with poor prognosis, as its tissue expression was correlated with progression of the tumor size as well as nodal status.⁵¹ Finally, protein S100A4 was found to be significantly more expressed in poorly than in well-differentiated gastric adenocarcinomas, and was correlated with nodal metastatic disease and peritoneal dissemination.⁵²

The significance of S100A4 in colorectal tumors remains more controversial. Initial studies demonstrated the presence of substantial amounts of S100A4 mRNA in a subset of human colorectal adenocarcinoma cell lines as well as in tissue specimens containing adenocarcinomas. Immunohistochemical studies revealed no staining for protein S100A4 in the epithelial cells of normal colonic mucosa and in colonic adenomas, whereas carcinomas arising in adenomas and invasive carcinomas showed S100A4-expressing cells in 44% and 94% of cases, respectively.⁵³ In contrast, subsequent studies based on Western blot techniques could not demonstrate a significant increase of S100A4 proteins in colorectal carcinomas versus normal colonic mucosa.⁵⁴ More recently it was observed that the percentage of connective and

epithelial cells immunohistochemically positive for S100A4 in colonic neoplasms significantly decreased with increasing grade of malignancy.⁵⁵ The reasons for these discrepancies remain unclear. Different investigative approaches, the use of different antibodies and staining procedures, as well as tumor heterogeneity may at least partially explain the divergent findings. In this context, it is important to note that some members of the S100 protein family, such as S100A2, were found to be down-regulated in neoplastic breast cells compared to normal cells and that, the expression of S100A4 protein decreases quantitatively from low-grade human astrocytomas to high-grade anaplastic astrocytomas and to glioblastomas.⁵⁶ These results highlight the complexity of the biological functions of the S100 protein family members, which presumably have reciprocal regulation mechanisms and may influence cell behavior in opposite directions. Taken together, the data collected so far indicate that, in particular tumor subsets, protein S100A4 may indeed represent an important prognostic factor. Larger studies with longer follow-up are clearly needed to further clarify the prognostic significance of this protein. It would be also of great interest to know whether the detection of S100A4 protein in biopsy specimens of primary tumors has a predictive role, ie, whether it may help to select patients necessitating more extensive diagnostic investigations to rule out metastatic disease or more aggressive treatments.

The detection of S100A4 in 83% of high-grade pancreatic intraepithelial lesions (PanIN) by Rosty and colleagues⁴ deserves particular attention. In fact, the identification of predictive molecular markers in general may have a decisive impact on the clinical management of patients with precancerous lesions. Conversely, the putative role of protein S100A4 as a diagnostic marker as suggested by Rosty and colleagues⁴ remains in our view primarily speculative. Several studies have detected this protein in a subset of nonneoplastic cells, and so far, knowledge on protein S100A4 expression in epithelial cells with reactive changes is not available. Although the findings reported in this issue of *The American Journal of Pathology* are intriguing and deserve further investigation, it is unlikely that S100A4 expression will in the future, act as an unequivocal biomarker able to accurately discriminate between neoplastic and nonneoplastic cells.

Mechanisms of S100A4 Effects in Tumors

Tumor progression is characterized by complex processes such as cell motility and invasiveness, as well as cell proliferation. Evidence is accumulating for an important role of members of the protein S100 family in these processes. Possible mechanisms are summarized below and in Figure 1.

Cell Motility

A possible role of S100A4 in cell motility was suggested by initial studies performed with human promyelocytic leukemia HL-60 cells²⁷ and a mouse mammary

Figure 1. Possible effects of S100A4 in tumors on gene up-regulation and/or protein overexpression. Nonmuscle tropomyosin, nonmuscle myosin, and wild-type p53 are intracellular binding targets. The expression of E-cadherin, metalloproteinases, and thrombospondin 1 has been found to be modulated by S100A4 gene up-regulation.

adenocarcinoma cell line.⁵⁷ Protein S100A4 has been shown to interact with components of the cytoskeleton, such as the heavy chain of nonmuscle myosin,⁵⁸ and nonmuscle tropomyosin.⁵⁹ It has been suggested that S100A4 protein affects the cytoskeleton of metastatic cells through modulation of the myosin phosphorylation by protein kinase C in a calcium-dependent manner.⁵⁸ Further, protein S100A4 may increase myosin solubility and therefore suppress its assembly,⁶⁰ or it may directly destabilize myosin filaments.⁶¹ Similarly, S100A4 binding to nonmuscle tropomyosin is also thought to be responsible for the disorganization of actin filaments.⁵⁹ Taken together, these findings indicate that S100A4 alters the cytoskeletal organization of cells, which is essential for facilitating cell motility and diapedesis.

Cell Adhesion and Detachment

Interestingly, cytoskeletal dysregulation induced by S100A4 seems to be linked to a redistribution of the membrane-associated adhesive glycoprotein CD44, thus creating patchy and strongly adhesive CD44 expression patterns on the cell surface. This possibly enables neoplastic cells to acquire an invasive behavior.⁶² Along this line of thought, studies were designed to investigate the interaction between S100A4 and cadherins, a family of transmembrane glycoproteins that mediate Ca^{2+} -dependent cell-cell adhesion, and suppress invasion.⁶³ The expression of E-cadherin and S100A4 was monitored in two mouse tumor cell families and found to be inversely regulated. Transfection experiments showed a reciprocal down-regulation of both molecules and suggested that the invasiveness of tumors expressing S100A4 may be at least partially induced by the abrogation of E-cadherin expression.⁶⁴ Similar mechanisms have been postulated in humans on the basis of immunohistochemical analysis of both proteins in a series of non-small cell lung cancers; an inverse correlation of E-cadherin and S100A4 expression was demonstrated.⁵¹

Remodeling of the Extracellular Matrix

An additional step forward in understanding the mechanisms linking S100A4 to cancer invasion came from studies analyzing possible interactions of this protein with matrix metalloproteinases.⁶⁵ Dysregulation of metalloproteinases is essential for the remodeling of extracellular matrix proteins and for tumor cell migration and invasion. In recent studies, down-regulation of S100A4 expression on transfection of highly metastatic osteosarcoma cell lines with a hammerhead ribozyme directed against the S100A4 gene transcript resulted in a reduction of the mRNA levels of MMP2, membrane-type 1-MMP, and of the endogenous tissue inhibitor TIMP-1. Consequently, inhibition of S100A4 expression resulted in a marked reduction of the capacity of transfected cells to migrate through Matrigel-coated filters.⁶⁶ However, it remains unknown how S100A4 is involved in this apparent regulation of metalloproteinases and their inhibitors.

Cell Proliferation and Apoptosis

An association between S100A4 and cell proliferation has been postulated after the initial cloning experiments that isolated the S100A4 gene from growth-stimulated cells.²⁰ Recently, it became evident that, as demonstrated for other S100 proteins such as S100B,⁶⁷ a possible mechanism of action may imply binding of S100A4 to the tumor-suppressor protein p53. Using a dexamethasone-inducible clone of B16 murine melanoma transfected with MMTV-S100A4(*mts1*), it was shown that S100A4 expression is associated with elevated levels of wild-type p53.⁶⁸ These results, however, may be biased by the formation of p53 and glucocorticoid receptor complexes, resulting in cytoplasmic sequestration of both, or by dexamethasone-regulated pleiotropic effects on gene regulation. Nevertheless, it was suggested that a complex of S100A4 with p53 and the sequestration of p53 may result in a stimulation of the cells to enter the S phase by abrogating the control functions of p53 at the G_1 -S checkpoint.^{3,68}

The physical interaction between wild-type p53 and S100A4, as well as the possible biological significance of this interaction, has been analyzed in elegant experiments using a wide array of investigative approaches.⁶⁹ First, transfection of S100A4(*mts1*)-negative cells with S100A4(*mts1*) constructs led to clonal death, and this death could be prevented by co-transfection with the anti-apoptotic gene *bcl-2*. Second, the binding of S100A4 to the extreme end of the C-terminal regulatory domain of wild-type p53 was demonstrated by co-immunoprecipitation, affinity chromatography, and Western blot analysis. Finally, it was shown that, via interaction with p53, S100A4 differentially modulates the transcription of p53-regulated genes, such as *p21/WAF* and *bax*. It was concluded that S100A4 cooperates with wild-type p53 to stimulate apoptosis, and that this process, at an early stage of tumor development may accelerate the loss of wild-type p53 functions, and consequently lead to the selection of more aggressive cell clones. Preliminary data suggest that the interaction between S100A4 may also

modulate the functions of at least some p53 mutants and therefore play important roles in advanced cancer stages.⁷⁰

Angiogenesis

Angiogenesis is critical for tumor growth and cancer metastasis. Interestingly, experiments with S100A4-inducible cell lines grown at high density suggest that S100A4 strongly down-regulates the thrombospondin 1 (THBS1) gene,⁷¹ another p53 target, which is known to repress tumor progression by inhibition of angiogenesis.⁶⁹ Thus, it is conceivable that S100A4 also promotes angiogenesis *in vivo* by preventing the anti-angiogenic effect of THBS1. Further, preliminary experiments suggest that S100A4 protein may act directly as an angiogenic factor.⁷² Tumors developing in *S100A4(mts-1)* transgenic mice revealed an increased vascular density. S100A4 oligomers were capable of stimulating motility, but not proliferation of endothelial cells *in vitro*, and inducing corneal neovascularization *in vivo*. Further studies, however, will be necessary to better clarify this apparent co-stimulatory angiogenic effect of protein S100A4 and to identify putative cell membrane receptors for extracellular forms of this protein.

Regulation of S100A4 Gene Transcription and mRNA Translation

The human S100A4 gene has been mapped on chromosome 1. It is clustered with 12 other genes, belonging to the S100 family, on the 1q21 region that is altered in several cancer types.^{73,74} In contrast to other gene clusters, however, the S100 family genes retain a specific pattern of expression, and they are most likely characterized by independent regulation mechanisms. Interestingly, Northern blot analysis of normal mouse organs revealed S100A4 mRNA in organs without protein expression,¹ suggesting therefore translational down-regulation and/or posttranslational degradation.³² Two splice variants of the human S100A4 mRNA with some tissue specificity of expression exist.^{75,76} The significance of these variants with respect to gene activity in different organs and/or cancer progression remains unclear.

S100A4 gene transcription seems to depend on growth-modulatory conditions of the cells. For instance, it was found that its expression in macrophages may be affected by molecules involved in the functional modulation of these cells in inflammation, such as lipopolysaccharides, tumor necrosis factor- γ , concanavalin A, and by modulation of the cytosolic Ca^{2+} concentration.¹ Rosty and colleagues⁴ show a statistically significant association between the expression of protein S100A4 and hypomethylation in the first intron of the S100A4 gene. They also suggest that rather than an epigenetic phenomenon, the hypomethylation of S100A4 may reflect clonal selection during cancer progression. These findings are in agreement with previous studies showing that hypermethylation of the S100A4 gene is involved in transcription silencing.^{46,77-79} In this context, it is noteworthy

that the first intron of the S100A4 gene contains several negative and positive regulatory elements^{80,81} that in the mouse interact with numerous factors, such as nuclear factor-kB, or recently characterized regulatory proteins.^{82,83} These enhancer and silencer elements may be strongly affected by the methylation status of the gene.

Conclusions and Outlooks

The information gathered throughout the past few years demonstrate that protein S100A4 is involved in the regulation of cancer invasiveness and metastasis. Clinical studies are beginning to elucidate the prognostic significance of this protein in human tumors. It is likely that our knowledge on S100A4 as a prognostic, predictive, or even diagnostic factor will dramatically increase with the development and commercial availability of antibodies. However, pathologists involved in the quest for prognostic markers in human neoplasia are, at the same time, also bound to focus on one of the most important objectives of pathology, ie, to foster a better comprehension of the pathogenesis of neoplasms and other diseases. To achieve these goals, the functions of S100A4 need to be further investigated. In the future, novel intra- and extracellular targets of S100A4 protein will probably be identified. We still need to understand how the conformational status of this protein, and in particular the formation of protein heterodimers with other members of the S100 protein family may influence cellular functions. The study of interactions of S100A4 with proteins involved in the control of the cell cycle, either in the wild-type or in a mutated form, and the interactions with adhesion molecules seems to be a promising research area. Hopefully, a more extensive knowledge on S100A4 will eventually allow the development of novel therapeutic strategies.

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