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PAI-1 Promoter Polymorphism Modulates uPA-PAI Complex Accumulation by Breast Cancer Cells

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Key Words
Cancer · Breast · PAI-1 · Promoter · Polymorphism

Abstract

Objectives: The uPA-PAI system has been shown to play a role in the development of a more aggressive tumor phenotype. The PAI-1 promoter 4G/5G polymorphism, furthermore, regulates free plasma PAI-1 levels in patients with myocardial infarction. Our aim was to verify if the different polymorphisms in the PAI-1 promoter are also associated with alterations in the intracellular accumulation of uPA-PAI complexes in human breast cancer. ***Methods:*** Accumulation of uPA-PAI complexes inside the tumor cells was determined by means of immunohistochemistry, as previously described by our own group, and two extremely different sets of tumors were chosen, one of them with strong uPA-PAI complex reactivity inside more than 50% of tumor cells, the other with no demonstrable reactivity at all. Finally, the 4G/5G polymorphism of the PAI-1 promoter was studied in all of them by means of DNA extraction, PCR amplification of the PAI promoter sequence, and restriction polymorphism typing. ***Results:*** Absence of intracellular uPA-PAI complex accumulation was significantly associated with the prevalence of the 4G allele and, conversely, the presence of uPA-PAI complexes inside the tumor cells was significantly associated with 5G/5G homozygosity (logistic regression, p = 0.0128). Furthermore, none of the 7 5G/5G homozygous tumors showed histological grade 3, as did 6/21 tumors in the group where the 4G allele was present. In spite of the low case number, this association of the 5G/5G polymorphism with a less aggressive phenotype almost reached statistical significance (Spearman's correlation test, p = 0.118). ***Conclusions:*** The 4G/5G polymorphism of the PAI-1 promoter seems indeed to be associated with different rates of uPA-PAI complex internalization by breast cancer cells. Complex accumulation inside the tumor cells is significantly related to 5G/5G homozygosity, and this shows a trend towards an association with a less aggressive, better-differentiated tumor phenotype.

Introduction

The plasminogen activation system plays an important role in many physiologic processes, such as wound healing or inflammation, and seems also to be involved in tumor

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development [1]. Of the two known natural plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), it is the latter which plays the most important role in the degradation of the matrix surrounding the tumor cells, one of the first steps in the metastatic process [2]. The inactive form of uPA (pro-uPA) is secreted mainly by stromal cells (and to a much lesser extent also by tumor cells), and is activated by binding to its cell surface receptor (uPA-r). Active, receptor-bound uPA in its turn activates the passage of plasminogen to plasmin, a strong proteolytic enzyme, thus creating a localized microenvironment of matrix degradation, through which migration of the tumor cell is facilitated. In the long run, however, such an unspecific proteolytic effect in its immediate surrounding would be dangerous for the tumor cell itself, so that the whole process is held in control by a negative feedback loop, mediated by specific inhibitors of uPA, called plasminogen activator inhibitors (PAI). Two of them are known (PAI-1 and PAI-2), the most important being PAI-1, which was first identified as a powerful prognostic factor in breast cancer by Schmitt et al. [3], something corroborated in a recent report by the same group, involving a large series of patients and long-term follow-up [4]. Only the active forms of uPA and PAI-1 bind with each other, forming stable uPA-PAI complexes. We have recently been able to show that these are internalized by tumor cells, in the presumably last step of the chain, thus removing free PAI-1 that eventually might enter the blood stream from the extracellular environment [5]. In that same study, we also found that this phenomenon was associated with significantly higher levels of hormone receptor expression (i.e. it seemed to be estrogen dependent), as well as with lower nuclear grade and lower proliferation, all of which define a less aggressive tumor phenotype.

The balance between uPA and PAI activity may not be the only control mechanism of the whole uPA system. There seems to exist a more sophisticated fine-tuning of the just described negative feedback loop, in which the PAI-1 promoter plays a key role. In fact, a particular allele (4G) of a recently described polymorphism of the PAI-1 promoter sequence (4G/5G) is associated with higher plasma PAI activity in patients having suffered myocardial infarction before the age of 45 [6]. It may be speculated that, by inhibiting physiological clot degradation in these patients, free PAI increases the possibilities for coronary artery thrombosis and, hence, myocardial infarction. To verify if PAI-1 promoter polymorphism acts similarly on PAI-1 activity in tumors, we have studied the 4G/5G polymorphism in a series of breast cancers and have correlated the relative frequency of each allele with the accumulation of uPA-PAI complexes inside the tumor cells.

Materials and Methods

We studied archival, paraffin-embedded tumor samples from breast cancer patients diagnosed and operated upon at the 'Centro de Patología de la Mama, Fundación Tejerina', Madrid, Spain.

Immunohistochemistry

For the detection of uPA-PAI complexes, we have followed the immunohistochemical method described by us previously [5]. Briefly, 5-μm sections from paraffin-embedded invasive breast carcinomas were mounted on poly-*L*-lysine-coated slides, deparaffinized, rehydrated and submitted to heat-induced epitope unmasking in citrate buffer. Subsequently, they were quenched in H_2O_2-methanol, to eliminate endogenous peroxidase activity, incubated with blocking serum, and finally with the AB775 polyclonal anti uPA-PAI antibody (Chemicon International, Temecula, Calif., USA; also to be purchased as Bender MedSystems BMS4139 antibody from Boehringer Ingelheim, Heidelberg, Germany), diluted 1:1,000. This antibody was characterized by the group having produced it and shown by means of reverse zymography to be specific for uPA-PAI complexes [7]. All subsequent steps were the standard ones corresponding to the streptavidin-biotin-peroxidase technique usually employed by us [5], and were carried out using a commercially available kit (Histostain-SP, Zymed, San Francisco, Calif., USA), to ensure uniformity of results. After performing the procedure, two extreme sets of tumors were chosen, the first corresponding to 13 samples with strong staining for uPA-PAI complexes in more than 50% tumor cells (fig. 1a), the other one to 15 showing the opposite feature, i.e. no staining at all (fig. 1b). The study of the 4G/5G polymorphism in the PAI-1 promoter sequence was carried out in these 28 tumors.

DNA Preparation and Polymorphism Analysis

Genomic DNA was isolated from paraffin-embedded tissue using a kit for DNA purification (Quiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was determined by absorbance measurement at 260 nm. Amplification of the PAI-1 sequence from genomic DNA, restriction polymorphism (4G/5G) typing and gel analysis were carried out following protocols reported elsewhere [8], with slight modifications. Briefly, 0.5 μg of tumor DNA was amplified by PCR in a thermal cycler (BioRad, USA) using a 22-mer forward oligonucleotide (5′-CACAGAGAGAGTCTGGCCACGT-3′) and a 21-mer reverse oligonucleotide (5′-CCAACAGAGGACTCTTGGTCT-3′) in a final volume of 50 μl. The number of cycles was increased to 40, being higher than recommended [8], in order to improve the yield of amplified PAI-1 sequence, since amplification from archival, paraffin-embedded tissue is known to be less efficient [9]. Then, 20 μl of the amplification products were digested for 3 h with 1 U of *Bsi YI* (Roche Diagnostics, Germany) restriction enzyme, and the fragments were separated by 10% polyacrylamide nondenaturing gel electrophoresis. DNA fragments were visualized by silver staining (fig. 2). The sizes of *Bsi YI* fragments were estimated by comparison with known size markers *(Msp-I*-digested pUC19 DNA, Fermentas, Lithuania). The digestion step was repeated using another 20 μl of PCR products of each sample to confirm the results.

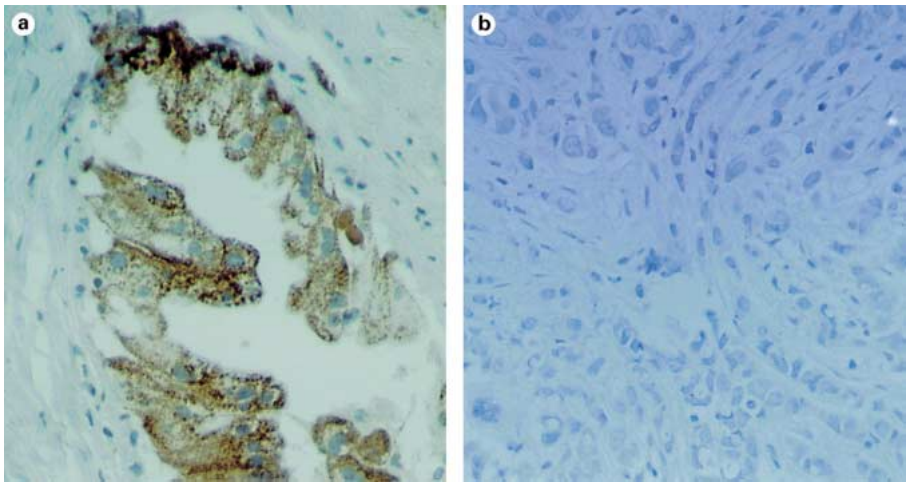

Fig. 1. a Accumulation of uPA-PAI complexes inside breast cancer cells. AB775 (BMS4139) polyclonal antibody. Streptavidin-biotin-peroxidase. × 250. b Absence of uPA-PAI complexes inside breast cancer cells. AB775 (BMS4139) polyclonal antibody. Streptavidin-biotin-peroxidase. × 250.

Results

In tumors without demonstrable intracellular accumulation of uPA-PAI complexes, the 4G allele of the PAI-1 promoter polymorphism was highly prevalent (14/15 cases), whereas in the group with immunohistochemically detectable uPA-PAI complex accumulation inside the tumor cells, alleles 4G and 5G were evenly distributed (7:6). When the PAI-1 promoter showed 5G homozygosity (5/5), finally, uPA-PAI complexes were detectable by immunohistochemistry in 6 of 7 instances. This difference in the relative prevalence of the 4G and 5G alleles in relation with the uptake of uPA-PAI complexes by tumor cells was statistically significant (Fisher's exact test, p = 0.028), despite the low number of cases involved. In a logistic regression model, the odds ratio for the occurrence of the 4G allele if no uPA-PAI complexes were detectable inside the tumor cells was 12.0 (1.07–134.34), and the corresponding p value (0.0128) showed again that this distribution was not attributable to pure chance.

When considering other features defining the tumor phenotype, it is remarkable that none of the 7 5G/5G homozygous tumors showed histological grade 3, as did 6/21 tumors in the group where the 4G allele was present. In spite of the low case number, this association of the 5G/5G polymorphism with a less aggressive phenotype almost reached statistical significance (Spearman's correlation test, p = 0.118). However, other features of a less aggressive tumor phenotype which we found significantly associated with the immunoreactivity for uPA-PAI complexes in our previous study [5], such as low nuclear grade and hormone receptor expression, were not significantly related to 5G/5G homozygosity in the present one. This, however, may again be attributable to the low case number of this pilot study, which had completely different aims.

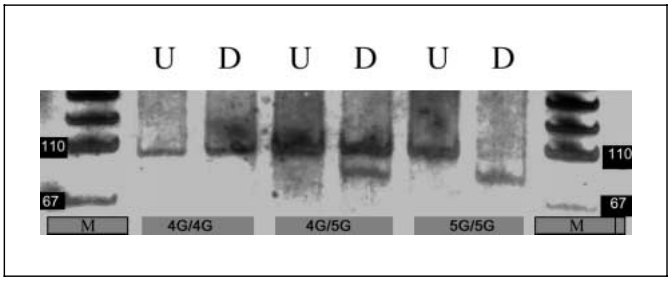

Fig. 2. Electrophoretic determination of the single base pair insertion/deletion polymorphisms of the PAI-1 gene promoter. PCR amplification of human genomic DNA and digestion by restriction enzyme *Bsl*-I. M = Molecular weight standard; 4G/4G = homozygote for the deletion allele; 4G/5G = heterozygote; 5G/5G = homozygote for the insertion allele; U = undigested amplified product; D = fragments obtained after endonuclease restriction.

Together with all studied clinical and pathological features of the tumors, the results are summarized in table 1.

Discussion

Eriksson et al. [6] have previously reported that high total PAI-1 levels in plasma are associated with a higher prevalence of the 4G allele of the PAI-1 promoter polymorphism. In that same study, they found that this was also associated with a higher incidence of myocardial

Table 1. Clinical and histological features of the tumors studied

Sample	PAI-1 promoter polymorphism	Intracellular uPA-PAI-1 complex accumulation	Histology	Tumor size	Nodal invasion	Nuclear grade	Histologic grade	ER	PR
1	4/4	–	ductal	T1	–	3	2	+	+
2	4/4	–	ductal	T2	–	3	3	–	–
3	4/4	–	ductal	T2	–	3	2	–	–
4	4/4	+	ductal	multifocal	–	3	3	+	–
5	4/4	–	lobular	T2	+	3	2	+	–
6	4/4	–	ductal	multifocal	+	3	2	+	+
7	4/4	+	ductal	multifocal	+	3	2	+	+
8	4/4	+	ductal	multifocal	+	3	2	+	+
9	4/4	+	ductal	multifocal	–	2	2	+	+
10	4/4	+	ductal	T2	+	3	3	–	–
11	4/4	–	lobular	T1	+	2	1	–	+
12	4/4	–	ductal	T1	–	1	1	+	+
13	4/5	–	ductal	T1	–	2	2	+	+
14	4/5	–	lobular	T2	+	2	2	+	+
15	4/5	+	ductal	T1	+	3	3	+	+
16	4/5	+	ductal	T1	+	3	3	–	–
17	4/5	–	ductal	T1	–	2	2	–	–
18	4/5	–	ductal	T1	–	3	3	+	+
19	4/5	–	ductal	T1	–	3	2	–	–
20	4/5	–	ductal	T1	–	2	2	+	+
21	4/5	–	lobular	T1	+	2	2	–	+
22	5/5	–	lobular	T1	+	2	2	–	–
23	5/5	+	ductal	T1	+	2	2	–	–
24	5/5	+	ductal	T1	+	2	2	+	+
25	5/5	+	ductal	multifocal	+	3	2	+	–
26	5/5	+	ductal	T1	–	2	2	+	+
27	5/5	+	ductal	T1	–	3	2	+	+
28	5/5	+	lobular	T1	–	3	2	+	+

ER = Estrogen receptors; PR = progesterone receptors.

infarction. It seems logical that a functioning uPA-plasminogen system may have a protective effect against intravascular thrombosis, and hence the deleterious effect of high levels of free plasma PAI-1, which, by inhibiting uPA, could counteract this protective mechanism.

The present study demonstrates that in breast cancer the 4G allele is associated with significantly lower accumulation of uPA-PAI complexes inside the tumor cells, which is in contrast to the previously published results of Eriksson et al. They reported that total circulating PAI-1 levels are increased in the presence of the 4G allele, and, consequently, the uPA-PAI complex formation should also be increased. However, complex formation may indeed be increased, but their degradation too, before they are taken up by tumor cells. Since our immunohistochemical technique is aimed at the last step of the chain, i.e. the presence or absence of uPA-PAI complexes inside tumor cells, an increased extracellular degradation of the former before detection cannot be excluded. Furthermore, in human breast cancer, Pedersen et al. [10] have shown that total uPA or PAI are not single entities, but rather a compendium, including pro-uPA, uPA bound to its receptor (uPA-r), latent PAI-1, active PAI-1, and finally uPA-PAI complexes. They also state that the latter can only be formed from their active uPA and PAI-1 precursors. Thus, the complexes detected by us by means of immunohistochemistry do not indirectly reflect total PAI present in the tissues, but rather, at best, a (variable) fraction of it, i.e. active PAI (and active uPA). Finally, and most importantly, recently Pedersen et al. [11] have again studied uPA, PAI-1 and uPA-PAI complex concentration separately in a large series of 342 breast tumors and have

determined that, indeed, uPA and PAI-1 concentrations predicted a significantly shorter survival, whereas, contrarily, increased uPA-PAI complex concentrations predicted a significantly longer survival, and were furthermore significantly associated with less aggressive features of the tumors (smaller size, lower grade and less axillary nodal invasion). The almost significant association we have found in the present study between 5G homozygosity of the PAI-1 promoter and lower tumor grade points in the same direction, since we also found that 5G homozygosity was significantly associated with an increased presence of uPA-PAI complexes. Coincidentally, in our previous pilot study on the localization of uPA-PAI complexes inside breast tumor cells [5], we also found this feature to be associated with a less aggressive tumor phenotype (lower proliferation, lower nuclear grade and higher expression of hormone receptors). In the present one, 5G homozygosity of the PAI-1 promoter was associated with estrogen receptor expression in 72% of the tumors, compared to 62% in the rest, but this difference was not statistically significant, given the small sample size. However, if confirmed (or increased) in a larger series, such as our previous one, this 10% difference might attain statistical significance.

Finally, in agreement with the findings of Pedersen et al. [11], uPA and PAI-1 levels were directly (and significantly) correlated with each other, the uPA-PAI-1 complex formation was variable and not related to either of them (but, intriguingly, associated with a better prognosis, in contrast with total PAI-1 levels, which indicated a worse prognosis). Therefore, it seems that complex formation is not merely a gross reflection of previous PAI activity, but rather a distinct phenomenon of variable intensity, which is associated with benign features of the tumors.

In summary, our present study seems to indicate that, in contrast with total PAI-1 levels in breast cancers, which reflect a bad prognosis and are associated with a higher prevalence of the 4G allele in the PAI-1 promoter, complex accumulation in the tumor cells, reflecting a better prognosis and a less aggressive phenotype, is associated with the opposite feature, i.e. 5G homozygosity.

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