Pergamon

European Journal of Cancer, Vol. 34, No. 4, pp. 566–569, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(97)10038-7

Original Paper

Urokinase-type Plasminogen Activator and Plasminogen Activator Inhibitors (PAI-1 and PAI-2) in Extracts of Invasive Cervical Carcinoma and Precursor Lesions

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In a previous study we reported a direct correlation between the degree of total proteolytic activity and the natural history of cervical carcinoma. The present work examined whether an increase in the urokinase-type plasminogen activator (uPA) and the plasminogen activator inhibitors (PAIs) is correlated with the natural history of cervical carcinoma. We measured uPA and PAI-1 activities and uPA, PAI-1 and PAI-2 antigen concentrations in cervical extracts from normal, squamous intraepithelial lesions (SIL) or invasive carcinoma patients. The uPA activity in invasive carcinoma extracts was 8.46 times that of normal extracts and 4.9 times that of SIL extracts. The PAI-1 activity in invasive carcinoma extracts was 1.3 times that of normal extracts and 1.24 times that of SIL extracts. uPA, PAI-1 and PAI-2 amounts were 25.7-, 12.1- and 7.9-fold higher, respectively, in invasive carcinoma than in SIL, and 39.1-, 21.38- and 27.3-fold higher, respectively, than in normal extracts. uPA and PAI-1 activities were 2.02- and 1.42-fold higher in extracts from patients with stages II–IV than those from stage I extracts, respectively. uPA, PAI-1 and PAI-2 amounts were 3.06-, 4.2- and 1.4-fold higher in extracts from patients with stages II–IV than those from stage I extracts, respectively. The increase in uPA activity and the antigen levels of uPA and PAIs (PAI-1 and PAI-2) in stages II–IV of invasive carcinoma of the cervix suggests that these components play an important role in invasion and metastasis in advanced stages of this tumour. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: cervical carcinoma, urokinase-type plasminogen activator, plasminogen activator inhibitors, PAI-1, PAI-2
Eur J Cancer, Vol. 34, No. 4, pp. 566–569, 1998

INTRODUCTION

IN RECENT years, our understanding of mechanisms that lead to invasion and metastasis has increased. These processes are controlled by successive events and involve movement of neoplastic cells across tissue boundaries and through host cellular and extracellular matrix barriers. Studies of tumour invasion and metastasis have focused on the role of tumour-associated proteases, such as metalloproteinases, cysteine-dependent proteinases and serine proteases of the plasmin/plasminogen activator system [1–4]. In this context, urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2) seem to be correlated with tumour cell invasion and metastasis in a number of human malignancies, including colon, breast and ovary [5–7]. We recently found a direct correlation between the natural history of cervical carcinoma and the total proteolytic activity present in cervical biopsies [8]. The

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Received 7 Jan. 1997; revised 27 Jun. 1997; accepted 10 Jul. 1997.

predominant catalytic class of proteinases in the tissue extracts was that of the metalloproteinases. The study also revealed that invasive carcinoma extracts had bands with proteolytic activity which were not present in normal tissue or biopsies with precursor lesions. This suggested the presence of uPA. Recently, Nuovo and associates found an increased ratio of mRNAs of matrix metalloproteinase (MMP)-9 and MMP-2 to those of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 in invasive cervical carcinoma and surrounding stromal cells in samples from patients with poor prognosis [9]. Concurrently, Kobayashi and associates showed that staining intensities of uPA and PAI-1 in tissue specimens are predictors of increased risk of lymph node metastasis in patients with stage II cervical cancer. They found that the overall survival rate was worst in patients with uPA staining confined to tumour stroma and with high antigen levels of uPA and PAI-1 [10]. However, they did not measure PAI-2 antigen values and PAI-1 activity nor did they include tissue specimens of patients with other stages of cervical cancer and precursor lesions. To investigate the role of uPA and PAIs in the natural history of cervical cancer, we measured antigen concentrations of uPA, PAI-1 and PAI-2 and uPA and PAI-1 activities in homogenates of normal tissues, squamous intra-epithelial lesions and invasive carcinoma of the human cervix.

MATERIALS AND METHODS

Patients and sample collections

Tissue samples were collected by biopsies or surgical resection. These were obtained from 35 patients with invasive carcinoma, 25 patients with high grade squamous intra-epithelial lesions (HGSIL) or low grade squamous-intra-epithelial lesions (LGSIL) and from 26 controls, who attended the Oncology or Gynecology Services at the Hospital Civil de Guadalajara, Mexico (age range 35–65 years). Diagnosis was made in accordance with The 1988 Bethesda System for Reporting Cytological Diagnosis of Squamous Cell Abnormalities [11]. Two different pathologists confirmed the diagnosis for all the specimens.

Tissue extraction

After removing necrotic and bloody areas, the samples were frozen at −70°C until extraction. Extracts were prepared from 100–150 mg (wet weight) tissue samples as described previously [8]. Briefly, the specimens were homogenised in a high-speed mixer-homogeniser (Polytron PT 3000, Kinematica AG, Brinkmann, Switzerland) for 5 min at 15 000 rpm, in 4 ml of 0.15 M NaCl at 4°C. After three freeze–thaw cycles, each homogenate was sonicated (Sonic Dismembrator Brand, U.S.A.) twice at 21 kilocycles/s for 1 min at 4°C. The homogenate was centrifuged at 8 000 *g* for 10 min at 4°C and the supernatant divided into aliquots and stored at −70°C until analysed. The protein concentration of the extracts was determined according to Bradford [12].

Activity assay for the quantitative determination of functional uPA

The determination of uPA activities was performed using a specific immunosorbent activity assay (Chromolize™ uPA, Biopool, Umeå, Sweden). In brief, 20 µl of tissue extracts were added to a micro-test plate well coated with monoclonal antibodies (MAb) directed against uPA, which also contained 10 µl of uPA depleted plasma. The uPA was allowed to adsorb to the micro-test plate and the non-adsorbed material was then washed out. The bound enzyme was activated by addition of a plasmin solution. After thorough washing with buffer, the activity of uPA was determined by adding a reagent containing plasminogen, poly-D-lysine and a plasmin sensitive chromogenic substrate. The amount of yellow colour was determined by measuring the absorbance at 405 nm and was proportional to the amount of uPA present in the tissue extracts or standard.

Activity assay for the quantitative determination of PAI-1

The determination of PAI-1 activity was performed using a chromogenic, indirect enzymatic assay involving two steps (Spectrolyse® (pL) PAI, Biopool, Umeå, Sweden). In step one, 25 µl of tPA (40 IU/ml) were added to the tissue extract sample (25 µl) and allowed to react with the PAI-1 in the extract. The sample was then acidified to eliminate alpha-2-antiplasmin and other plasmin inhibitors, and diluted. In step two, the residual tPA activity was measured by addition of a solution of Glu-plasminogen, poly-D-lysine and chromogenic substrate at neutral pH. The PAI content of the sample was expressed as the difference between the amount of tPA added and the amount of tPA found. The absorbance was measured at 405 nm and 492 nm and the difference was used in the calculation of tissue extract PAI activities.

Enzyme immunoassay (ELISA) for quantitative determination of human uPA

This antigen was measured using an enzyme immunoassay (Biopool TintElize® uPA, Biopool, Umeå, Sweden) that uses the double antibody principle and measures single-chain urokinase and a high molecular form of uPA with approximately the same efficiency. Mouse antihuman uPA MAb was incubated with 50 µl of the tissue extracts for 2 h, a horseradish peroxidase labelled goat antihuman uPA-IgG was then added to form a 'sandwich' enzyme-linked immunosorbent assay and *ortho*-phenylendiamine was added as the substrate. The assay included the use of quenching and non-specific antibodies to exclude false positive results (immunological specificity and accuracy controls).

Enzyme immunoassays for quantitative determination of human activator inhibitor, type 1 (PAI-1) and human activator inhibitor, type 2 (PAI-2)

Total (latent, active and complexed) PAI-1 or PAI-2 were determined using the TintElize® PAI-1 ELISA (Biopool, Umeå, Sweden) or the TintElize® PAI-2 ELISA (Biopool, Umeå, Sweden), respectively. In brief, the PAI-1 or PAI-2 present in sample extracts (20 µl) was reacted with mouse monoclonal antihuman PAI-1 or PAI-2 for 2 h. Horseradish peroxidase labelled goat anti-human PAI-1-IgG or PAI-2-IgG was used to form a 'sandwich' ELISA assay and *ortho*-phenylendiamine was added as the substrate. The assay included the use of quenching and non-specific antibodies to exclude false positive results (immunological specificity and accuracy controls).

Calculations and statistical analysis

The activities of the activators in tissue extracts were expressed as ng uPA/mg protein. PAI activities were expressed as U/mg protein. One unit of PAI activity is defined as the amount of PAI that inhibits one international unit of human single chain tPA, as calibrated against the international standard for tPA lot 86/670 (distributed by NIBSAC, Blanche

Table 1. *Biological activities of uPA and PAI-*1 *in samples of invasive cervical carcinoma and precursor lesions*

Groups	uPA (ng/mg protein)*	PAI-1 (U/mg protein)*
Normal ($n=26$)	0.15 ± 0.20†	27.13 ± 11.04‡
SIL ($n=25$)	0.26 ± 0.24†	28.64 ± 13.39‡
Invasive carcinoma ($n=35$)	1.27 ± 0.46	35.59 ± 13.82

*Mean (S.D.). †Versus invasive carcinoma, $P<0.0001$. ‡Versus invasive carcinoma, $P<0.05$.

Lane, South Mimms, Potters Bar, Hertfordshire, U.K.). Antigen concentrations were expressed as ng/mg protein. Results are given as mean (± S.D.) of four experiments. Differences between group means were tested for significance using the Kruskal–Wallis and Mann–Whitney *U* tests.

RESULTS

Thirty two of the 35 epidermoid invasive carcinoma of the uterine cervix (91%) were large cell non-keratinising type, and three (9%) were large cell keratinising type. Fifty-two per cent (13/25) of SIL were of high grade and 48% (12/25) of low grade.

The within-assay variation was determined and in all assays the coefficient of variation (CV) was below 6.2%. In the between-assay variation the CV ranged between 9.1 and 12.3%.

There was significantly higher uPA activity in invasive carcinoma extracts in comparison with normal and SIL extracts (Table 1). Invasive carcinoma uPA extract activity was 8.46 times that of normal extracts and 4.9 times that of SIL extracts. There was a slight, but not statistically significant, increase in SIL extracts over the controls ($P=0.06$). The PAI-1 activity in invasive carcinoma extracts was 1.3 times that of normal extracts and 1.24 times that of SIL extracts.

The mean antigen concentration of uPA in invasive carcinoma extracts was 39.1 times that of normal extracts and 25.7 times that of SIL extracts (Table 2; $P<0.0001$). The mean antigen concentration of PAI-1 in invasive carcinoma extracts was 21.28 times that of normal extracts. The mean antigen concentration of PAI-1 in SIL extracts was 1.76 times that of normal extracts, but 12.1 times lower than that in invasive carcinoma ($P<0.0001$). The mean PAI-2 antigen concentration of invasive carcinoma extracts was 27.3 times that of normal extracts and 7.9 greater than that of SIL extracts ($P<0.0001$).

Significant correlation was seen between the value of uPA activity and the uPA antigen level (correlation coefficient 0.8036; $P<0.001$). However, the correlation was not significant between the value of PAI-1 activity and PAI-1 antigen level (correlation coefficient 0.3560).

To test whether the increase of activity or antigen content of uPA, PAI-1 and PAI-2 in tissue samples of patients with invasive cervical carcinoma was correlated with the clinical stage, we analysed the results of invasive carcinoma extracts according to FIGO classification: stage I (11 patients), and stages II–IV (24 patients). The mean uPA and PAI-1 activities were significantly higher in extracts of patients with stages II–IV than in extracts of patients with stage I (Table 3). The mean uPA, PAI-1 and PAI-2 levels in stages II–IV were 3.06-, 4.2- and 1.4-fold, respectively, that of stage I extracts (Table 4). The differences for uPA and PAI-1, but not PAI-2, were significant.

Table 3. *Activities of uPA and PAI-*1 *in samples of invasive cervical carcinoma according to FIGO classification*

Stage(s)	uPA (ng/mg protein)*	PAI-1 (U/mg protein)*
I ($n=11$)	0.80 ± 0.41†	29.59 ± 12.58‡
II–IV ($n=24$)	1.62 ± 0.40	42.22 ± 18.90

*Mean (S.D.). †Versus stages II–IV, $P<0.01$.‡Versus stages II–IV, $P<0.05$.

DISCUSSION

We have previously demonstrated that there is a direct correlation between the natural history of cervical carcinoma and the degree of total proteolytic activity present in samples of normal cervix, SIL and invasive carcinoma [8]. In this work we used a tissue extraction procedure that preserves the activity of proteolytic enzymes [8]. The suitability of the activity assays for uPA and PAI-1 and the ELISA for uPA, PAI-1 and PAI-2 for the measurement of activities and antigen levels in tissue extracts, respectively, has also been well documented [13, 14]. We found that activity and antigen concentrations of uPA were significantly increased in invasive carcinoma, especially in stages II–IV, when compared with normal and SIL. The results in these last two groups were not significantly different. Moreover, our results show that both PAI-1 and PAI-2 antigen concentrations were significantly greater in invasive carcinoma than in normal and SIL extracts, while PAI-1 activity was slightly greater in invasive carcinoma than in normal and SIL extracts. It has been reported that active, latent and complexed forms of PAI-1 and PAI-2 give approximately the same response with the ELISA assays used in this work [15]. Kobayashi and associates found that most PAI-1 obtained from cervical carcinoma extracts is in the latent form, and demonstrated, by Western blot analysis, the presence of uPA/PAI-1 complexes in homogenates from cervical cancer tissue [10]. These data support the finding in our study that tissue PAI-1 is predominantly in the latent form.

Our results do not include information about the outcome and relapse of the patients studied because the follow-up time was not long enough. Our results do provide new information with regard to the increase of PAI-2 antigen

Table 2. *Total uPA and PAIs in samples of invasive cervical carcinoma and precursor lesions as measured by ELISA technique*

Groups	uPA* (ng/mg protein)	PAI-1* (ng/mg protein)	PAI-2* (ng/mg protein)
Normal ($n=26$)	0.23 ± 0.32†	1.38 ± 1.96†	1.28 ± 3.04
SIL ($n=25$)	0.35 ± 0.34†	2.43 ± 3.20†	4.41 ± 6.29
Invasive carcinoma ($n=35$)	9.00 ± 5.40	29.37 ± 32.92	34.97 ± 40.95‡

*Mean (S.D.). †Versus invasive carcinoma, $P<0.0001$. ‡Versus normal and SIL, $P<0.0001$.

Table 4. Total uPA and PAIs in samples of invasive cervical carcinoma according to FIGO classification as measured by ELISA technique

Stage(s)	uPA* (ng/mg protein)	PAI-1* (ng/mg protein)	PAI-2* (ng/mg protein)
I ($n = 11$)	3.62 ± 2.43†	12.36 ± 12.25‡	32.56 ± 26.08
II–IV ($n = 24$)	11.07 ± 3.85	51.50 ± 56.01	45.45 ± 41.74

*Mean (S.D.). †Versus stages II–IV, $P < 0.001$. ‡Versus stages II–IV, $P < 0.01$.

levels in invasive carcinoma in comparison with levels in precursor lesions and normal extracts. Although the functional role and clinical significance of the PAI-2 increase in cervical carcinoma is unknown, Foekens and colleagues reported that breast tumour patients with high levels of uPA, and low levels of PAI-2, have a very poor prognosis [16]. The aim of this work was to investigate the role of uPA and PAIs in the natural history of cervical cancer, but we did not find any important differences between normal and SIL extracts, and between low and high grade SILs in terms of uPA and PAI activity and antigen levels. The increase in activity and antigen levels of uPA and PAI (1 and 2) in stages II–IV of invasive carcinoma of the cervix suggests that these components play an important role in invasion and metastasis. These findings are somewhat different to those in our previous work, where we demonstrated an early increase of total proteolytic activity from SIL with a predominance of metalloproteinases [8]. Although the interactions between the plasminogen activator system and metalloproteinases and their inhibitors that occur *in vivo* in the natural history of cervical carcinoma remain unclear, our results are in agreement with data reported for other tumours, such as melanoma, where plasminogen activators, their inhibitors and urokinase receptor, increase in the late stages of melanoma progression [17].

Schmalfeldt and associates reported that receptors for uPA, PAI-1, PAI-2 and uPA increase in metastasis of the omentum from ovarian cancer over primary tumours [7]. In addition, in human breast tumours, the presence of uPA-positive cells is an important independent variable for the identification of patients with high risk of recurrence, just as high concentrations of uPA and PAI-1 in cytosolic extracts are associated with poor prognosis [18].

Our findings raise many questions about the biological role and clinical implications of uPA and PAIs in cervical carcinoma, in particular the determination of whether the uPA and PAI activities or antigen levels are helpful as predictors of relapse or survival time. It will be necessary to analyse a large number of samples and to have an extended follow-up of the patients to answer these questions. Our results suggest that it would be profitable to investigate uPA and PAI regulation and search for compounds that reduce the activities or synthesis of uPA for clinical applications.

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Acknowledgements—The authors wish to thank Dr Mark West for careful revision of the manuscript, Dr Alfredo de Jesús Celis de la Rosa for helpful suggestions used in the statistical analysis. This work was supported by a grant from CONACYT (3975P-M9608) to A. Daneri-Navarro.