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Original Paper

Modulation of the Proteolytic Balance Plasminogen Activator/plasminogen Activator Inhibitor by Enhanced N-*myc* Oncogene Expression or Application of Genistein

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The aim of this study was to determine whether enhanced expression of N-*myc* in a neuroblastoma cell line affects the balance of plasminogen activator/plasminogen activator inhibitor (PA/PAI), a shift towards proteolysis having been observed in other malignant tissues. Two transfected neuroblastoma cell lines with (WAC2 cells) or without (SH-EP007 cells) enhanced expression of the N-*myc* oncogene were examined by zymography and RNA extraction to determine UPA and PAI enzyme activity and uPA RNA and PAI RNA expression, respectively. The effect of genistein, an inhibitor of tyrosine protein kinase, on uPA/PAI was also investigated. Both the uPA/PAI-1 ratio at mRNA level and the PA/PAI ratio at protein activity level were higher in the more malignant, WAC2 cell line. Genistein attenuated uPA activity and stimulated PAI activity in both cell lines, leading to a decrease in the PA/PAI ratio. This effect was more pronounced in the more malignant, WAC2 cell line. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Urokinase- (uPA) and tissue-type (tPA) plasminogen activators are serine proteases of tryptic specificity. They are produced by many normal and transformed cells [1]. While tPA appears to play a major role in the prevention of intravascular clot formation, uPA may mainly be involved in tissue invasion and degradation and in tumour cell metastasis [2]. The precursor of uPA (pro-uPA) is secreted as a single chain protein and is essentially inactive. Cleavage of pro-uPA by plasmin, kallikrein, factor XIIa or cathepsin B yields the disulphide-linked two-chain active enzyme [3], which activates the zymogen plasminogen to plasmin and procollagenases to collagenases [1]. At least three serine protease inhibitors (serpins) are known to inhibit uPA: protease-nexin I and plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2, respectively). PAI-1 exhibits a much higher affinity towards uPA than PAI-2. PAI-1 is secreted as an active antiprotease but rapidly converts into a latent inactive form [3, 4].

uPA and PAI are secreted by many normal tissues or cells, where their activities are maintained at a delicate balance [5, 6]. A temporary shift towards proteolysis is noted during tissue remodelling and normal angiogenesis [7]. A lasting shift towards proteolysis is observed in malignant tissues and cells, enabling tumours to invade adjacent normal tissues, to induce tumour angiogenesis and finally to metastasise [5–7]. Such an imbalance of proteolysis and anti-proteolysis might predict the malignant potential of tumours and the derived cells and could possibly be used clinically as an indicator of the outcome of affected patients.

Neuroblastoma is the most common solid tumour of childhood [8]. Amplification of the N-*myc* oncogene resulting in high N-*myc* expression is thought to be causally involved in the progression of neuroblastoma to advanced stages of malignancy [9]. So far, the mechanism by which enhanced N-*myc* expression might contribute to neuroblastoma progression have remained unclear, but a modulation of proteolysis appeared possible. To test this hypothesis, we examined the ratio of expression of uPA and PAI-1 in two stably transfected neuroblastoma cell lines with normal or enhanced expression of the N-*myc* oncogene. We also investigated genistein, an inhibitor of tyrosine protein kinase, to determine whether it was able to reduce the PA/PAI ratio, with the possibility of future clinical application.

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MATERIALS AND METHODS

Materials

Culture media, serum and antibiotics were from Gibco (Paisley, U.K.) and trasylol from Bayer (Leverkusen, Germany). Nylon membranes and DIG-RNA labelling and chemiluminiscent detection kits were from Boehringer Mannheim (Mannheim, Germany). Megaprime DNA labelling system and radiolabelled dCTP were obtained from Amersham (Little Chalfont, U.K.). All other reagents were molecular biology grade and supplied by Sigma (St Louis, Missouri, U.S.A.), Bio-Rad (Hercules, California, U.S.A.), Merck (Darmstad, Germany) and Fluka (Buchs, Switzerland).

Cell culture

Human neuroblastoma cells of the SH-EP line were transfected with a vector containing a functional or a non-functional human N-*myc* oncogene and the resulting cell lines (WAC2 or SH-EP 007, respectively) were cloned as previously described [10]. N-*myc* gene content, as determined by Southern blotting, in WAC2 cells was 100-fold that of control SH-EP and SH-EP 007 cells. Neuroblastoma cells were grown in RPMI 1,640 medium supplemented with 10% calf serum and geneticin (in order to select neuroblastoma cells containing the transfected N-*myc* oncogene and a neomycin resistance gene which had also been integrated into the vector) at 37°C under 5% CO_2. All culture media contained streptomycin, penicillin and amphotericin [10]. Cell counts were carried out with a Coulter counter.

Conditioned media and cell extracts

To prepare conditioned media, cells were grown in 6-well plates. When subconfluent, medium was aspirated, cells were washed twice with phosphate-buffered saline (PBS) and each well received 1 ml of culture medium without serum and with 200 KIU of Trasylol/ml. Additionally, some wells received genistein (50 μM). After 24 h of incubation, conditioned media were collected. The cells were washed twice with PBS and harvested by scrapping into 0.5 ml of 0.2% Triton X-100 in 0.1 M Tris·HCl containing 200 KIU of Trasylol/ml and cells of duplicate wells were counted. Media and cell lysates were centrifuged at 1000×g for 20 min and the supernatants were collected and used in zymographic assays.

Zymographic assays

Aliquots of cell extracts and conditioned media normalised for equal cell numbers were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. Gels were washed twice in 2.5% Triton X-100 and twice in PBS (10 min each wash) and laid over a substrate gel prepared with agar (0.8%), plasminogen (40 μg/ml) and skimmed milk (1.5% in PBS). Gels were incubated under a moist atmosphere overnight at 4°C and subsequently incubated at 37°C. After 4–8 h, bands of proteolysis due to uPA activity were photographed under dark field. After an additional day of incubation at room temperature, the only white areas under dark field illumination were those corresponding to bands protected against proteolytic activity, that is, bands of PAI activity.

RNA extraction and hybridisation

The acid guanidinium-phenol-chloroform method [11] was used to isolate total RNA from cultured cells. 20 μg of RNA with an A_{260}/A_{280} ratio higher than 1.9 were applied into each lane of gels containing 0.8% agarose and 0.8 M formaldehyde and subjected to electrophoresis. Capillary transfer on to positively charged Nylon membrane was carried out overnight using 5 times concentrated SSC buffer. Baked and cross-linked membranes were hybridised. For uPA mRNA detection, a cDNA kindly supplied by D. Belin (University of Geneva Medical Center, Switzerland) was used to generate an antisense RNA labelled with digoxigenin according to manufacturer's instructions (Boehringer Mannheim); hybridisation and detection were also carried out according to manufacturer's instructions. Kodak X-OMAT AR films were exposed to the blots for 15–60 min at room temperature. For PAI-1 mRNA detection, a cDNA kindly supplied by P.A. Andreasen (Rigshospitalet, Copenhagen, Denmark) was used to prepare a ^{32}P-labelled probe with the Megaprime DNA labelling system. Probes with specific activity higher than 10^9 cpm/μg were used in hybridisation, carried at 42°C in 50% formamide, 5× SSPE, 5× Denhardt's reagent and 20 μg/ml of denaturated salmon sperm DNA for 16 h. Membranes were washed at high stringency: 20 min at 60°C with, successively, 2×, 0.5×, and 0.1× SSC containing 0.1% SDS. Films were exposed to the blots with intensifying screens at −80°C for 7–14 days. As an internal standard, mRNA for GAPDH was detected using a 1.3 kb GAPDH cDNA [12]. Additionally, homogeneity of quantity and quality of RNA loaded and transferred were checked by ethidium bromide staining. Autoradiograms were submitted to densitometric scanning and the expression levels, given as arbitrary units, were normalised taking GAPDH levels as an internal standard.

RESULTS

Expression of uPA and PAI-1 mRNA in neuroblastoma cells

A model system for malignancy in neuroblastoma has been recently described [10]. This model consists of two neuroblastoma cell lines (named SH-EP 007 and WAC2) generated from the homogeneous SH-EP neuroblastoma line: SH-EP 007 by transfection with a vector containing a non-functional N-*myc* oncogene; WAC2 by transfection with a functional N-*myc* oncogene. SH-EP 007 cells express N-*myc* at normal levels, they have little proliferative potential and do not generate tumours in nude mice. In contrast, WAC2 cells express N-*myc* gene product at 100-fold enhanced levels, they bear an extensive proliferative potential and generate tumours in nude mice [10]. Thus, SH-EP 007 and WAC2 exhibit characteristics of cells derived from human neuroblastomas at initial and advanced, metastatic stages of the disease, respectively [9].

Figure 1 shows the expression of uPA and PAI-1 mRNA. The expression of uPA was similar in both neuroblastoma cell lines (lanes 1 and 2). In contrast, expression of PAI-1 differed considerably: it was high in SH-EP 007 cells and very weak in WAC2 cells. Quantification of the expression levels of the mRNAs by densitometry revealed that the uPA/PAI-1 ratio was 5-fold higher in the more malignant WAC2 cells (results not shown).

Activities of PA and PAI

In order to determine whether uPA and PAI-1 steady state mRNA levels correlated with bioactivity, we prepared conditioned media and cell extracts and examined PA and PAI activities using zymographic assays (Figure 2).

PA activity could be detected in extracts but not in conditioned media of SH-EP 007. Two bands of lower intensity

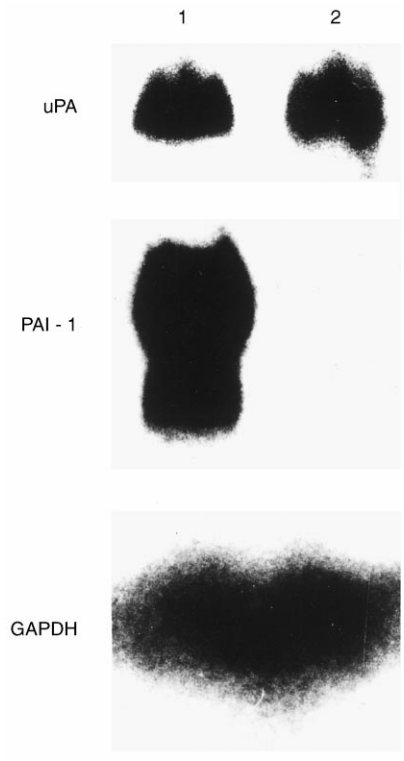

Figure 1. Expression of uPA and PAI-1 mRNAs in neuroblastoma cells with or without enhanced expression of the N-*myc* oncogene. Northern blots were carried out as described in 'Materials and Methods'. GADPH was used as an internal control. Lane 1, SH-EP 007 cells; lane 2, WAC2 cells.

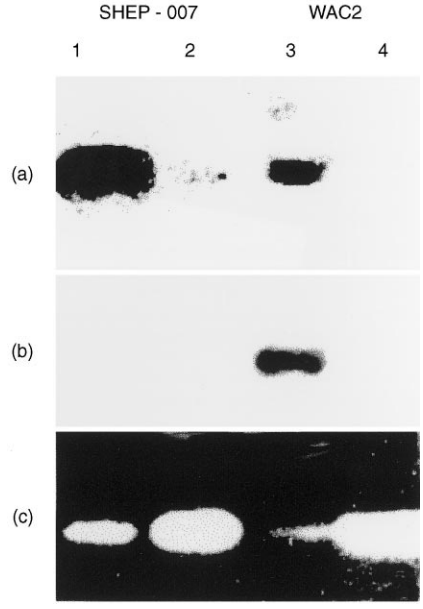

Figure 2. Zymographic assay of uPA and PAI activities in neuroblastoma cells with or without enhanced expression of the N-*myc* oncogene. Zymography was carried out with both cell extracts and conditioned media as described in 'Materials and Methods'. uPA activity was detected in both cell extracts (a) and conditioned media (b), whereas PAI activity was only detected in conditioned media (c). Control (lanes 1, 3) and genistein-treated (lanes 2, 4). SH-EP 007 (lanes 1, 2) and WAC2 (lanes 3, 4) cells were examined.

were detected in WAC2 cell extracts, though the intensity of the upper band was so low that it cannot be observed in Figure 2 (see position A3); this upper band could be a PA: PAI complex, as previously detected in other cells [13, 14]. In contrast to SH-EP 007 cells, an intense band of PA activity could be detected in WAC2 cell conditioned media (lane 3, b).

We next examined extracts or medium conditioned by the cells for PAI activity. No activity could be detected in cell extracts (data not shown). However, a signal of PAI activity was found in medium conditioned by SH-EP 007 cells. PAI activity was lower in medium conditioned by WAC2 cells (see Figure 2). These activity levels correlated with the PAI-1 mRNA expression levels above mentioned. Thus, PAI-1 expression and activities contrast with those of uPA in the respective cell lines. The PA/PAI activity ratio was higher in the more malignant cell line, as it was with the PA/PAI ratio at mRNA expression level.

Modulatory effect of genistein

The isoflavonoid genistein can inhibit tyrosine kinase [15], arrest cell cycle [16, 17], and inhibit the proliferation of cells derived from tumour cells, including the cell lines used in the present study. In fact, 24 h incubations in media without serum in the presence of 50 μM genistein inhibited proliferation of both cell lines (Table 1). This effect of genistein on neuroblastoma cell proliferation was more pronounced in the cell line with enhanced N-*myc* oncogene expression (WAC2). Genistein also produced a remarkable effect on neuroblastoma cell morphology: in both cell lines, genistein-treated

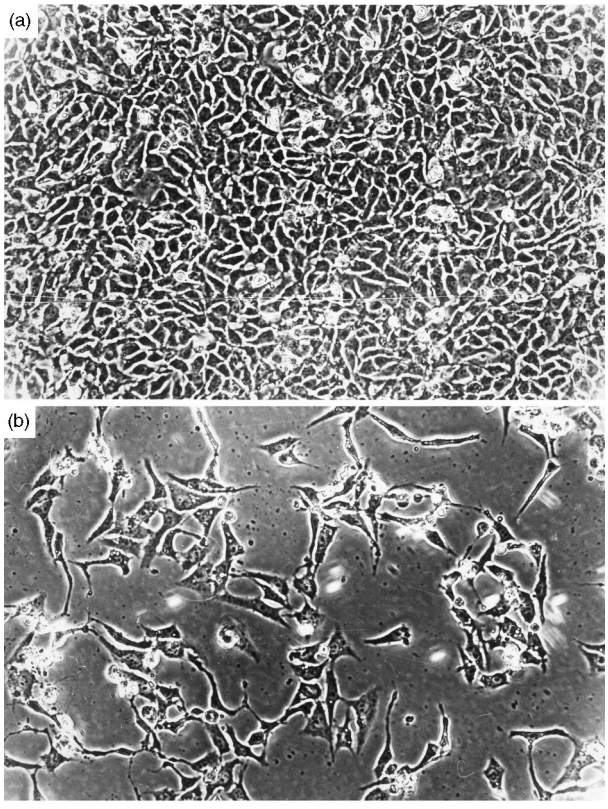

Figure 3. Morphology of WAC2 cells. Cells were cultured as described in 'Materials and Methods'. When subconfluent, medium were removed, cells were washed twice and new medium without serum was added. Cells were incubated for another 24-h period in the absence (a) or presence (b) of 50 μM genistein.

Table 1. *Effect of genistein on neuroblastoma cell proliferation*

Cell line	Control cells$\times 10^{-5}$	Genistein treated cells$\times 10^{-5}$
SH-EP 007	7.56 ± 0.04	4.03 ± 0.09
WAC2	12.25 ± 0.21	3.57 ± 0.00

cells emitted dendrite-like projections, suggesting that they had acquired a differentiated phenotype (see Figure 3). To test whether genistein could induce some additional modulatory effect on the PA/PAI balance, we exposed cells to genistein and examined conditioned media or cell extracts for uPA or PAI activity using zymographic assays. As seen in Figure 2, genistein attenuated uPA activity and stimulated PAI activity in both cell lines. This stimulatory effect was more pronounced in the WAC2 neuroblastoma cell line.

DISCUSSION

The present study was undertaken in order to study the balance of plasminogen activator (uPA) and its inhibitor (PAI-1) as an indicator of the invasive potential of cells derived from neuroblastoma, the most common solid paediatric tumour. Previous studies have shown that a delicate balance of both enzymes exists in normal tissues and that this balance can shift towards proteolysis in solid tumours [4–7]. This allows a tumour to digest the underlying basement membrane, to invade adjacent tissues and to induce angiogenesis necessary for further tumour spread and metastasis. If such a shift in favour of proteolysis would prevail in general, it could be used to predict the clinical behaviour of tumours in terms of their invasive and malignant potential and the prognosis of the affected patients [18–20]. Such information might also be used clinically to select certain therapeutic strategies.

Here, we examined uPA and PAI-1 expression and bioactivity in cells derived from neuroblastoma. In the neuroblastoma model system used in this study, the uPA/PAI-1 ratio of the expression levels of mRNA and the ratio PA/PAI of activities was higher in WAC2, a human neuroblastoma cell line rendered particularly malignant through establishment of a high N-*myc* oncogene expression [10]. These findings suggest that high N-*myc* oncogene expression could contribute to the aggravated malignant behaviour of neuroblastomas by modulating their proteolytic balance and allowing them to invade and penetrate adjacent tissues. Interestingly, it has been previously shown that the induction of urokinase brought about by serum or EGF parallels that of c-*myc* mRNA levels during the transition from quiescent to proliferative state in normal mouse fibroblasts and keratinocytes [21]. It is noteworthy that the malignant WAC2 neuroblastoma cell line had high levels of soluble PA activity in its conditioned media; a possible explanation could be that the amount of uPA produced by these cells is so high that it exceeds the capacity of the high affinity uPA receptors (uPAR) and, eventually could produce a down-regulation of uPAR expression, as previously reported for a colon cancer cell line [22]. Much more experimental effort should be devoted to clarify these speculations.

Despite all these promising data, a shift towards u-PA activity should not be considered a unique indicator of malignant potential. Tumour promotion, invasion and metastasis are very complex phenomena in which many other gene products should be involved, including uPA receptor and other matrix proteases and their inhibitors [23–25]. Nevertheless, it seems that in a clinical setting a shift of a given tumour towards proteolysis could signal an extensive malignant potential of that tumour and warrant a more intensive therapy. Such a therapy could make use of established chemotherapeutic drugs. Alternatively, the present study suggests that genistein could be used as a therapeutic agent, since it inhibits the activity of uPA while stimulating that of PAI-1. Through this behaviour, it might prevent localised tumours from metastasising. It has been previously demonstrated that genistein inhibits the proliferation of paediatric tumour cells [8] and angiogenesis *in vitro* [26]. Together with the activities described by others [8, 15, 17], these properties suggest that genistein should be investigated in further studies to determine its usefulness as therapy in solid malignant tumours.

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