Korean J. Zool. 31:185-190, 1988

Relation of the Activities of Plasminogen Activator and Plasmin-like Protease with Malignant Behavior of Skin Tumor of Rats

Yun Kee, Sang C. Park* Doo B. Ha, and Chin H. Chung

*Department of Zoology, College of Natural Sciences; *Department of Biochemistry, School of medicine, Seoul National University, Seoul 151-742, Korea*

To investigate whether malignant behavior of skin tumor correlates with changes in the level of proteolytic activities in skin tumors, rats were treated with 7,12-dimethylbenzanthracene followed by phorbor ester. Tumors induced upon the treatments exhibited more than 20-fold increase in the activity of plasminogen activator and about 3-fold of plasmin-like activity, as compared to those in treated controls. Furthermore, the former activity was raised to about 6-fold even in the preneoplastic tissues of the skin tissues. On the other hand, the proteolytic activity against casein and insulin decreased to several-fold in the tumor tissues while anti-trypsin activity remained similar in both tumor and controls. Thus, the increase in the activities of plasminogen activator and plasmin-like enzyme appears to occur as a characteristic to skin cancer and may involve in invasion and metastasis of the tumor.

KEY WORDS: Rat skin tumor, Proteolysis, Metastasis

Search for proteases secreted by tumor cells has been a corollary of the hypothesis that cancer invasion is an active process (Liotta and Hart, 1982). A number of different proteases including plasminogen activators and collagenases have been identified in malignant tissues (Strauli, 1980; Liotta *et al.*, 1980, 1981a, 1981b, 1982). These enzymes together with other unidentified proteases may function in a cascade fashion to facilitate infiltration of tumor cells through host barriers, one of their major components is collagen.

Fibrinolytic activity of tumor tissues was identified in 1935 (Strauli, 1980). A major component of fibrinolysis is produced by plasmin generated from serum plasminogen by the action of plasminogen activator (Reich, 1978). Although the latter enzyme is not an efficient protease for direct lysis of extracellular matrix, it can induce indirectly the cleavage of noncollagenous matrix components (Liotta *et al.*, 1981). In addition, plasmin can also activate proenzymes such as latent collagenases (Liotta *et al.*, 1981). Furthermore, the level of plasminogen activator has been reported to increase highly in malignant tumor cells such as mouse melanoma and human fibrocarcinoma (Jones *et al.*, 1975; Wang *et al.*, 1980). Such an elevation in the enzyme level reflects that the proteolytic activity involves in breakdown of fibrins accumulated along the blood vessels as well as of collagenous matrix, and therefore can facilitate infiltration or extravasation of tumor cells into circulatory systems.

In recent studies with human cervix tumors, the proteolytic activity against casein and insulin was found to increase several-fold while the level of anti-trypsin fell to nearly one-tenth of that in normal tissues (Kee *et al.*, 1988). Such a drastic change in proteolytic capacity in the tumor supports an idea of which a spectrum of proteases involve in malignant behavior of tumors rather

This work was supported by a grant from Korea Science and Engineering Foundation.

than a specific proteolytic enzyme does, particularly when considering that the protein substrates, casein and insulin, are known to be susceptible to a wide variety of proteases (Goldberg *et al.*, 1981; Tanaka *et al.*, 1986).

Therefore, the present study was performed to examine if the activities of plasminogen activator and plasmin-like protease increase as the tumor develops in rat skin because the skin cancer is metastatic and can easily be induced by the treatment of carcinogens. We also measured the proteolysis against casein and insulin as well as the activity of anti-trypsin to examine if their levels change upon the tumor development.

Materials and Methods

Materials

Normal and preneoplastic skin tissues of rat were dissected out from the abdominal region and minced to small pieces. The tissues were then homogenized after suspending them in 50 mM Tris-HCl (pH 8) containing 5 mM $MgCl_2$ and 10% (v/v) glycerol. The homogenates were centrifuged at 30,000 x g for 30 min to obtain the tissue extracts.

Insulin and fibrinogen were radioiodinated using chloramine T (Greenwood *et al.*, 1963). [^{3}H]Casein was prepared by reductive methylation as described elsewhere (Goldberg *et al.*, 1981; Chung and Goldberg, 1983). [^{3}H]HCHO and Na^{125}I were purchased from New England Nuclear. All other chemicals were obtained from Sigma.

Induction of Skin Cancer

Abdominal skin of rats was treated with 0.5 ml of 7,12-dimethylbenzanthracene (DMBA; 40 mg/ml in acetone). Twenty-four hours after the treatment, one group of the rats was applied only once with 2 μg of croton oil (i.e., phorbor ester) and the other group was with the same amount of the reagent but twice in a week-period for 5 times. Skin tissues from the rats were obtained at 10-hr or 100- to 200-day after the last treatment. During 100-200 days, more than 70% of the rats applied repeatedly with phorbor ester after treating DMBA developed tumors while less than 5% did for the rats applied once with DMBA, with the reagent followed by phorbor ester, or repeatedly but only with phorbor ester.

Assays

The avtivities of plasmin-like protease and plasminogen activator were assayed as described by Pitman (1985) with some modifications. Radioiodinated fibrinogen was diluted with unlabeled fibrinogen to a final concentration of 0.1 mg/ml (5 x 10^5 cpm/ml). Aliquots (20 μg) of this sample were put in 24-well cluster dishes (0.2 ml/well) and dried under vacuum at 40°C for 2 days. Thrombin (0.25U) was then added to each well for converting fibrinogen to fibrin. After incubating the dishes for 1 hr at 37°C, they were washed five-times with phosphate-buffered saline. Reaction mixtures added to each well contained 50 μg of tissue extract, 0.1M Tris-HCl (pH 8), 0.1% (w/v) gelatin and 0.33 mIU of plasminogen. After incubating them at 37°C for 2 to 5 hrs, 50 μl aliquots were removed and the radioactivity released as soluble form was measured using a liquid scintillation spectrometer. Thus, the radioactivity appeared without the addition of plasminogen represents the activity of plasmin-like protease (PLP) and that in its presence but after subtrating the activity of PLP corresponds to the activity of plasminogen activator (PA).

Casein- or insulin-degrading activity was determined by following the hydrolysis of the substrate to acid-soluble products (Goldberg *et al.*, 1981; Chung and Goldberg, 1983). Assay of anti-trypsin was carried out as described by Chung *et al.* (1983). Protein was assayed by the method of Bradford (1976).

Results

Activities of Plasmin-like Protease and Plasminogen Activator

To examine if the level of proteolytic activity changes upon tumor development, rats were treated on their skin with DMBA followed by application of phorbor ester for various periods. After the treatments, skin tissues were dissected out at a number of sites (n=6) from abdominal region and their extracts were prepared. Because no

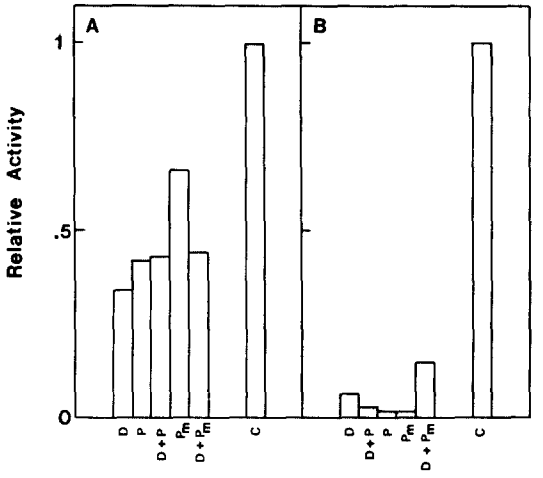

Fig. 1. The activities of (A) PLP and (B) PA in treated controls, preneoplastic and neoplastic tissues of rat skin. Assays were performed as described in the Materials and Methods by incubating 100 μg of the tissue extracts for 5 hr at 37°C in the fibrinogen-coated wells. The highest fibrinolytic activity exhibited by one of the extracts is expressed as 1.0, and the activities in other samples were as the values relative to it. Treated controls: D, treated once with DMBA; P, with phorbor ester (croton oil); D+P, once with DMBA followed by once with phorbor ester; Pr, repeatedly with phorbor ester. Preneoplastic tssues: D+Pr, treated once with DMBA followed by repeatedly with phorbor ester. Neoplastic tissues: C, tissues which developed tumors after the treatment as of D+Pr.

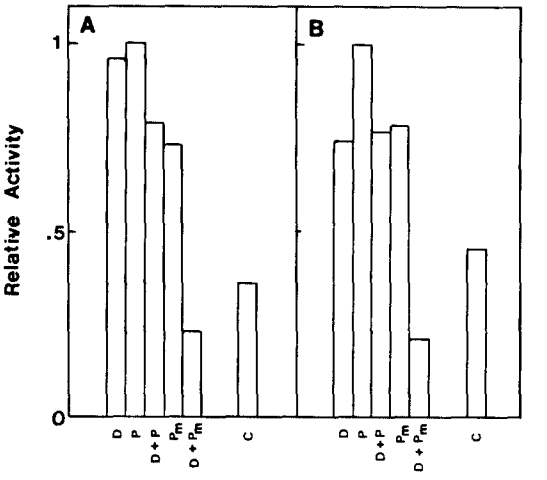

Fig. 2. Proteolytic activity against (A) casein and (B) insulin in treated controls, preneoplastic and neoplastic tissues of rat skin. Reaction mixtures in 0.1 ml contained 50 μg of the extracts, 0.1M Tris-HCl (pH 8), 5 mM $MgCl_2$, and 10 μg of [^{3}H]casein or 5 μg of ^{125}I-insulin. After incubating them at 37°C for 1 hr, 50 μl of 1%(w/v) bovine serum albumin as a carrier and 50 μl of 40% TCA to precipitate proteins. The mixtures were centrifuged for 5 min and the radioactivity in the acid-soluble fraction was determined in a liquid scintillation spectrometer. Relative activity in each sample was expressed as in Fig. 1. Abbreviations represent samely as described in the legend to Fig. 1.

tumor was developed in the rats treated once with DMBA, phorbor ester, or one after the other and repeatedly but with phorbor ester only, we refer the tissues obtained from these groups as treated controls. On the other hand, the rats applied with DMBA once followed by multiple treatments of phorbor ester eventually developed tumors, and therefore their skins were referred to as pre-neoplastic tissues. Finally, the tissues that developed tumors were as neoplastic tissues.

As shown in Fig. 1A, the activity of PLP in treated controls was more or less the same to that in preneoplastic tissues while the activity in neoplastic tissues increased to about 2-fold. On the other hand, the activity of PA in the preneoplastic tissues increased significantly as compared to that in treated controls (Fig. 1B). Furthermore, the level of PA was dramatically elevated in the neoplastic tissues. These results clearly show that the rise in PLP and PA activities is one of the malignant phenotypes of skin cancer and that the increase particularly of PA is implicated with the tumor development.

Proteolysis against Casein and Insulin and Antitrypsin Activity

Casein and insulin are known to be susceptible to a wide variety of proteases (Goldberg *et al.*, 1981; Tanaka *et al.*, 1986). Therefore, using the protein substrates, we have examined if overall capacity of proteolysis changes upon the development of tumors in skin tissues. Fig. 2 shows that both the casein- and insulin-degrading activities in preneoplastic tissues decline markedly when compared to those in treated controls. In addition, the activities in neoplastic tissues remained as low levels as in the preneoplastic tissues. Thus, the decrease in proteolytic capacity appears to occur as if it is necessary for the development of skin cancer.

Table 1. Summary of the activities of proteases and AT in treated controls, preneoplastic and neoplastic tissues of rat skin.

Activity	% Proteolysis or % inhibition*			Ratio(C/N)
	Treated control(N)	Preneoplastic tissues	Neoplastic tissues(C)	
PA	1.9±0.6	6.5±0.4	46.4±8.6	24.42
PLP	12.8±2.2	11.8±1.9	26.7±1.8	2.09
Caseinases	52.1±1.9	12.9±1.1	13.6±7.2	0.26
Insulinases	24.2±2.1	6.1±0.7	8.7±4.4	0.36
AT	15.2±3.6	6.9±0.6	15.1±5.9	0.99

These data were obtained by taking the mean values of the data in Figs. 1-3. Proteolytic activity was expressed as percentages of each respective protein substrate released into medium or converted to acid-soluble products by the tissue extracts. Percent inhibition corresponds to the extent of inhibiting the BAPNA-hydrolyzing activity of trypsin (1 μg) by the extracts.

*X + S.E. (S.E. =S.D./n: X, mean; S.E., standard error; S.D., standard deviation; n=6, number of cases).

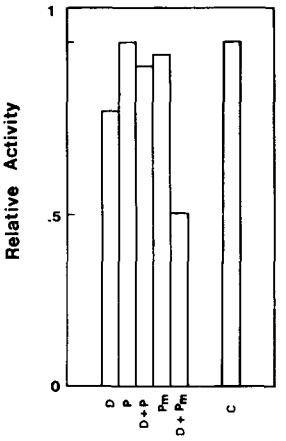

Fig. 3. Level of AT in treated controls, preneoplastic and neoplastic tissues. The activity of AT was measured by incubating reaction mixtures (0.5 ml) containing 1 μg of trypsin, 0.1M Tris-HCl (pH 8), 1 mM N-benzoyl-DL-arginine-p-nitroanilide (BAPNA), and 20 mM $CaCl_2$ in the presence and absence of 50 μg of the tissue extracts. The reaction was terminated by adding 0.5 ml of 30% (v/v) acetic acid, and the absorption of p-nitroanilide released during the incubation period was measured at 410 nm. Relative inhibitory activity and abbreviations are shown as in Fig. 1.

Anti-trypsin (AT) is one of the most widely distributed proteins in animal tissues (Horl and Heidland, 1982; Travis and Salvessen, 1983), and has been suggested to play a role in control of intracellular proteolysis through its inhibitory activity against proteases (Kee *et al.*, 1988). Therefore, the level of AT was measured to see if it changes upon the development of skin cancer. As shown in Fig. 3, the activity of AT in treated controls is nearly identical to that in neoplastic tissues. In contrast, the AT activity in preneoplastic tissues was about one-half that in the others. These results indicate that the activity unconcerns with the changes in overall proteolytic capacity in pre-neoplastic and neoplastic tissues. However, it is still unclear why the AT level decreases upon the treatment of DMBA followed by multiple application of phorbor ester.

Discussion

The present study demonstrates that the activities of PA and PLP and the proteolysis against casein and insulin change during the development of tumors on rat skin while the level of AT activity in the tumor stays at a similar level to that in normal tissues. These results are summarized in Table 1.

We have recently reported that overall proteolytic capacity in neoplastic tissues of human cervix increases several-fold with a concomitant decrease in the inhibitory activity against trypsin but without any significant alternations in the activites

of specific proteases such as PA and PLP (Kee *et al.*, 1988). It has therefore been suggested that a spectrum of proteases concertedly participate in malignant behavior of tumors rather than a single or a few specific proteolytic enzyme(s) involves. However, in contrast to the previous findings, skin tumors of rat exhibited a marked increase in PA activity with several-fold reduction in the proteolytic activity against casein and insulin. Thus, it seems quite clear that the relative importance of individual proteases for malignant phenotype varies from one tumor system to another or, within the same type of tumor system, from one anatomic site to another.

Of particular interest in the case of skin tumor was that the level of PA activity is higher in preneoplastic tissues and gets much greater in neoplastic tissues than that in treated contols. Such a tumorigenic time-dependent increase in the activity implicates a possible role of PA in the development of tumor itself. Alternatively, the rise of PA activity in preneoplastic stage of skin tissues could be a prerequisite for the invasive and metastatic character of fully-developed skin cancer. Despite that the function of PA has not yet been cleared other than its role in the process of blood clotting, the latter possibility seems likely when considering that PA can induce the hydrolysis of noncollagenous matrix components and can generate plasmin which activates latent collagenases (Liotta *et al.*, 1981). It is noteworthy that the activity of PLP in skin tumors also increases several-fold.

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(Accepted June 30, 1988)

Plasminogen Activator 및 Plasmin-like Protease 활성도의 변화와 쥐 피부암의 악성 종양형질과의 연관관계

기윤 · 박상철* · 하두봉 · 정진하 (서울대 자연대 동물학과, *의과대 생화학교실)

피부암의 형성과 피부조직에서 나타나는 단백질 분해활성의 변화와의 상관관계를 조사하기 위하여, 쥐의 피부에 7,12-dimethylbenzanthracene과 phorbor ester를 차례로 처리하였다. 이 결과, 암이 유발된 피부조직에서는 대조군에 비하여 plasminogen Activator (PA)의 활성도는 약3배가 높게 나타났다. 더우기, PA의 활성도는 암 유발 전(preneoplastic stage)의 조직에서도 약6배 정도 증가하였다. 반면, casein과 insulin의 분해활성도는 암조직에서 현저히 감소되었고 anti-trypsin의 활성도는 대조군의 것과 거의 비슷하게 나타났다. 따라서, PA 및 PLP 활성도의 증가현상은 피부암에서 특징적으로 나타나는 것으로 보이며, 이 암의 전이 및 침투에 밀접한 관계가 있는 것으로 사료된다.