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Correlation Between Malignant Phenotypes and Changes in Overall Proteolytic Capacity of Human Cervix and Liver Cancer

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Several proteolytic activities and the level of anti-trypsin in neoplastic tissues of human cervix and liver were compared to those in normal tissues to examine if any correlation exists between malignant behavior of the tumors and the changes in overall proteolytic capacity. Proteolysis against casein and insulin in cervix tumor was increased to 2-to 3-fold while that in liver tumor was reduced to one-tenth to one-half. By contrast, the level of anti-trypsin in cervix tumor was lowered to nearly one-tenth of that in normal tissues while the level rose to about 2-fold in malignant tissues of liver. On the other hand, the activities of plasmin-like protease and plasminogen activator were enhanced 10-20% over the activities in normals. These results suggest that the changes in proteolytic capacity are at least in part due to outbalance in either of proteolytic or its inhibitory activity over the other and occur distinctively to each tumor systems for their malignant behavior.

KEY WORDS: Proteolysis, Human cervix and liver cancer

Destruction of host tissues by proteolytic enzymes has been suggested to facilitate infiltration of neoplastic tissues (Liotta and Hart, 1982). For example, an increased production of cathepsin B in carcinomas of breast was demonstrated by comparing the enzyme's activity in normal to that in benign tissues (Poole *et al.*, 1978), suggesting a role for this protease in the expression of the aggressive malignant phenotype. Enhanced production and secretion of the serine protease, plasminogen activator, was also shown to associate with the neoplastic transformation of a variety of cell types (Robin, 1978), and therefore interest has concerned on the possible role of this enzyme in tumor invasion and metastasis.

In addition, a strong correlation was found to exist between the ability of tumor cells to produce spontaneous metastasis and possession of high levels of collagenase IV (Liotta *et al.*, 1980). The penetration of blood vessels, both during invasion and extravasation, is of pivotal importance in metastasis, and type IV collagen is one of the major structural proteins of basement membranes of blood vessels (Liotta *et al.*, 1980, 1982). However, the relative importance of a specific or a few proteolytic enzymes may vary from one tumor system to another or within the same type of tumor system, from one anatomic site to another. For example, many human cells are known to produce high levels of plasminogen activator, and examination of variant murine melanoma cell lines with different invasive capacities has failed to reveal a consistent correlation between malignant behavior *in vivo* and plasminogen activator production or secretion (Robin, 1978; Jones and DeClerck, 1982; Wang *et al.*, 1980). Therefore, it appears likely that a spectrum of proteases involve in metastasis within various types of tumor system.

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Recently, the activity of plasminogen activator in endothelial cells has been suggested to be regulated by changes in the level of a protein inhibitor, that is active against the enzyme, rather than by increase in the enzyme level itself (Hekman and Loskutoff, 1985; van Maruik *et al.*, 1984). Because there appears to exist endogenous inhibitors to nearly all known proteolytic enzymes (Horl and Heidland, 1982), it is possible that the changes in proteolytic activity in malignant tissues are due to outbalance of proteases to their inhibitors.

The present study was undertaken to determine the proteolytic capacity in neoplastic tissues in comparison with that in normal. However, we employed protein substrates, such as casein and insulin, that are susceptible to a variety of proteases (Goldberg *et al.*, 1981; Tanaka *et al.*, 1986) rather than to a specific enzyme. In addition, we measured the activity of a protein inhibitor active against trypsin in both normal and neoplastic tissues, because anti-trypsin is the most widely distributed protein in human tissues (Horl and Heidland, 1982; Travis and Salvesen, 1983) although its function other than that in blood plasma is still unclear.

Materials and Methods

Materials

[^{3}H]Casein was prepared by reductive methylation using [^{3}H]HCHO as described by Jentoft and Dearborn (1979). Insulin and fibrinogen were iodinated using chloramine T by the method of Greenwood *et al.* (1963). [^{3}H]HCHO and Na-^{125}I were obtained from New England Nuclear. All other chemicals were purchased from Sigma.

Preparation of Tissue Extracts

Normal and neoplastic tissues of human cervix and liver were obtained from The Seoul National University Hospital. History of the patients corresponding to each tissue sample was obtained from Dr. Sang C. Park (School of Medicine, Seoul National University). The tissues (mostly 1-2 g) were either sonicated or homogenated after suspending them in 50 mM Tris-HCl (pH 8) containing 5 mM $MgCl_2$ and 10% (v/v) glycerol. The homogenates were then centrifuged for 30 min at 30,000 $\times$g to obtain the tissue extracts. The extracts were diluted with the same buffer to give final protein concentrations of 1 mg/ml for further use.

Assay of Proteolytic Actvity

The activities of plasmin-like protease and plasminogen activator were measured as described by Pitman (1985) with some modification. ^{125}I-labeled fibrinogen was diluted with unlabeled fibrinogen to give a final concentration of 0.1 mg/ml (5×10^5 cpm/ml). Aliquots (20 μg; 10^5 cpm) of this sample were put in 24-well culture dishes (0.2 ml/well) and dried under vacuum at 40°C for 48 hrs. Thrombin(0.25 U) was then added to each well for converting fibrinogen to fibrin. After incubating the dishes for 1 hr at 37°C, they were washed 5 times with phosphate-buffered saline (PBS). Reaction mixtures added to each well contained 50 μg of tissue extract, 0.1 M Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.1%(w/v) gelatin and 0.33 mIU of plasminogen. After incubating them at 37°C for 2 to 5 hrs, aliquots of 50 μl 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 and that in the presence of plasminogen but after subtracting the activity of plasmin-like enzyme accounts for the activity of plasminogen activator.

Proteolytic activity against casein or insulin was determined by following the hydrolysis of the substrate to product soluble in 10% (w/v) trichloroacetic acid (TCA)(Goldberg *et al.*, 1981). Reaction mixtures in 0.1 ml contained 50 μg of tissue extracts, 0.1 M Tris-HCl (pH 8), 10 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 (BSA) as a carrier and 50 μl of 40% TCA to precipitate proteins. The assay tubes were then kept on ice for 10 min and spun for 5 min. Aliquots (0.1 ml) of the supernatants were counted to measure the radioactivity of the acid-soluble products.

Assay of Protease Inhibitor

Assay of anti-trypsin was performed as described by Chung *et al.* (1983). Reaction mixtures contained 1 μg of trypsin, 0.1 M Tris-HCl (pH 8), 1 mM N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and 20 mM $CaCl_2$ in a final volume of 0.5 ml. Following incubation at 37°C for 30 min in the presence and absence of 50 μg of tissue extracts, 0.5 ml of 30% (v/v) acetic acid was added to stop the reaction. The absorption of p-nitroanilide released during the incubation period was measured at 410 nm. Protein was assayed as described by Bradford (1976).

Results

Activities of Proteases and Anti-trypsin in Normal and Neoplastic Tissues of Human Cervix.

To compare proteolytic capacity in neoplastic tissues to that in normal tissues of human cervix, casein and insulin were used as substrates. As shown in Figs. 1A and 1B, casein- and insulin-degrading activities in the tumor tissues are much higher than those in normal samples. The activity of specific serine proteases, plasmin-like enzyme and plasminogen activator, was also determined to be more or less higher in the neoplastic tissues than in normal ones (Fig. 2), but not as much as the proteolytic activity against casein or insulin. These results suggest that the increase in the overall capacity of proteolysis in the neoplastic cervix tissues occurs as a characteristic to the cancer, which may be related to tumor invasion.

To compare also protease inhibitory activity in the tumor tissues to that in normals, we have measured the activity of anti-trypsin as a model case. The activity of anti-trypsin was much lower in the neoplastic tissues than in normals (Fig. 1C), in contrast to the proteolytic activity against casein or insulin (Figs. 1A and 1B). Thus, the elevation of casein- and/or insulin hydrolyzing activity in the tumor tissues appears to be somehow related to the decrease in the level of anti-trypsin.

To quantitate more precisely the rise and fall of the proteolytic and the anti-trypsin activities, the mean values of the activities were calculated using the data shown in Figs. 1 and 2. Table 1 summarizes the extent of increase or decrease in the acti-

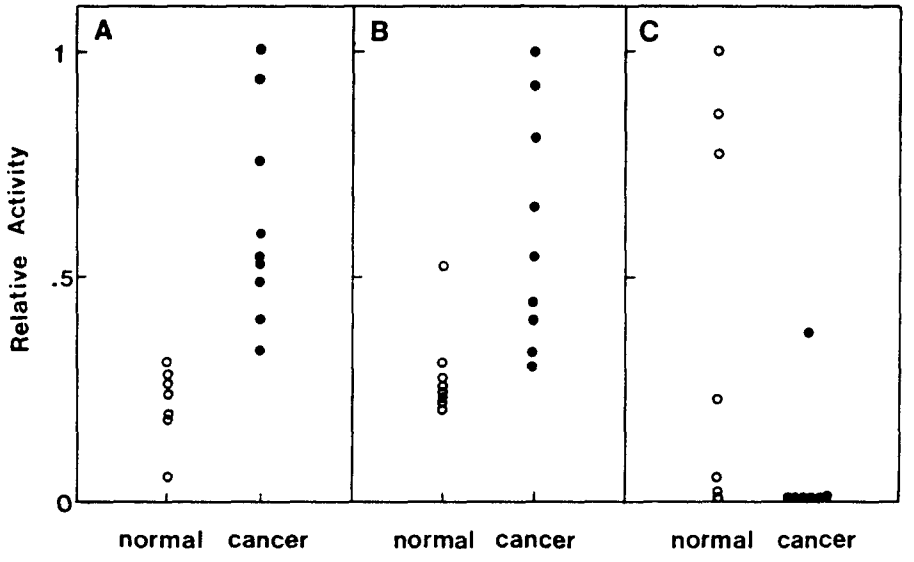

Fig. 1. Levels of proteolytic activity against casein (A) and insulin (B) and of anti-trypsin (C) in normal and neoplastic tissues of human cervix. Assays were performed as described in Materials and Methods by incubating 50 μg of the tissue extracts for 1 hr at 37°C. The highest activities against the protein substrates in a neoplastic tissue were expressed as 1.0, and the others as the values relative to it. For anti-trypsin, the highest activity of a normal sample was expressed as 1.0.

Table 1. Summary of the activities of proteases and anti-trypsin in normal and neoplastic tissues of human cervix.

Proteases	% Proteolysis or % Inhibition		Ratio (C/N)
	Normal(N)	Cancer(C)	
Caseinases	2.6±0.2	7.0±0.9	2.69
Insulinases	8.0±1.0	17.3±2.5	2.16
PLP	17.6±1.6	21.3±0.9	1.19
PA	20.0±5.7	22.6±4.2	1.13
Anti-trypsin	14.1±6.6	1.9±1.9	0.15

These data were obtained by taking the mean values of the data in Figs. 1 and 2. Proteolytic activity was expressed as the percent of respective protein substrates 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. PLP, plamin-like protease. PA, plasminogen activator.

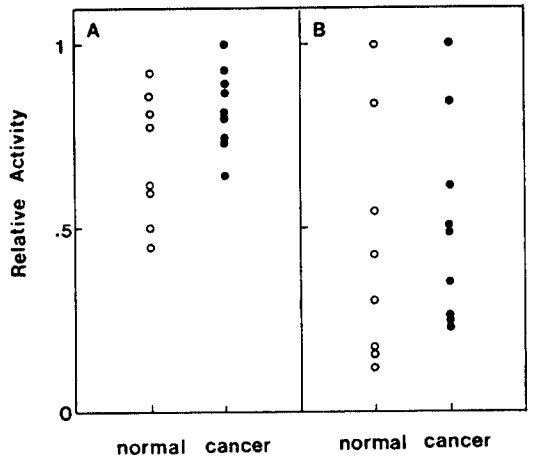

Fig. 2. Levels of the activities of plasmin-like protease (A) and plasminogen activator (B) in normal and neoplastic tissues of human cervix. Assays were carried out as decribed in Fig. 1, but by incubating the tissue extracts for 5 hrs at 37°C using the fibrinogen-coated wells. Relative activities were expressed as in Fig. 1.

vities in the neoplastic tissues of human cervix.

Activities of Proteases and Anti-trypsin in Normal and neoplastic Tissues of Human liver.

Proteolytic activity against casein or insulin was also measured to compare the activity in tumor tissues to that in normal ones of human liver. By contrast to the activities in human cervix, both activities were much lower in the neoplastic liver tissues than in normals (Figs. 3A and 3B). Furthermore, the activity of anti-typsin in the tumor tissues was greater than that in normal liver (Fig. 3C), again conversely to the activity in the neoplastic cervix tissues (Fig. 1C). Thus, it appears likely that the decrease in the proteolytic activity is due to outbalance of the inhibitory activity in liver cancer although it is still unknown if anti-trypsin is able to inhibit the casein- and/or insulin-degrading activity.

Table 2. Summary of the activities of proteases and anti-trypsin in normal and neoplastic tissues of human liver.

Proteases	% Proteolysis or % Inhibition		Ratio (C/N)
	Normal(N)	Cancer(C)	
Caseinases	2.0±0.4	0.3±0.2	2.11
Insulinases	7.4±0.3	3.7±0.8	0.50
PLP	18.5±1.7	20.4±1.5	1.10
PA	14.0±5.7	15.6±5.5	1.11
Anti-trypsin	14.8±1.9	30.6±5.1	2.07

These data were obtained as in Table 1, but from the data of Figs. 3 and 4. *X±S.E. PLP, plasmin-like protease. PA, plasminogen activator.

The activities of plasmin-like protease and plasminogen activator increased slightly in the tumor tissues of liver (Fig. 4), similarly to those in the neoplastic cervix tissues (Fig. 2). Such an increase to a small extent is presently unclear if it is significant in malignant behavior of the tumors. Table 2 summarizes the increase or decrease in the proteolytic activities and the inhibitory activity against trypsin in the neoplastic liver tissues.

Discussion

The present study demonstrates that the proteolytic capacity has changed in the neoplastic tissues of human cervix as well as liver tissues. Although it is beyond discussion why the casein- and insulin-degrading activity increased several--

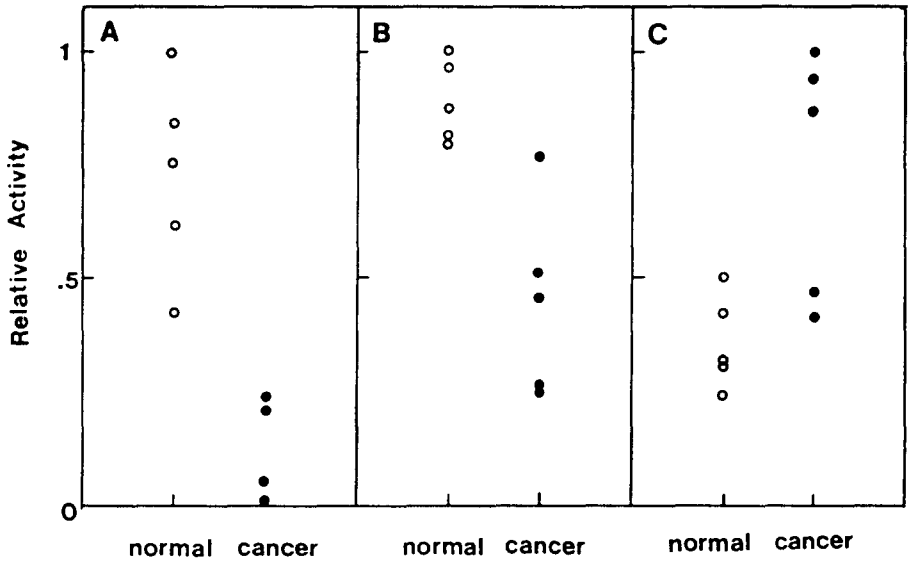

Fig. 3. Levels of proteolytic activity against casein (A) and insulin (B) and of anti-trypsin (C) in normal and neoplastic tissues of human liver. All parameters were determined as described in Fig. 1.

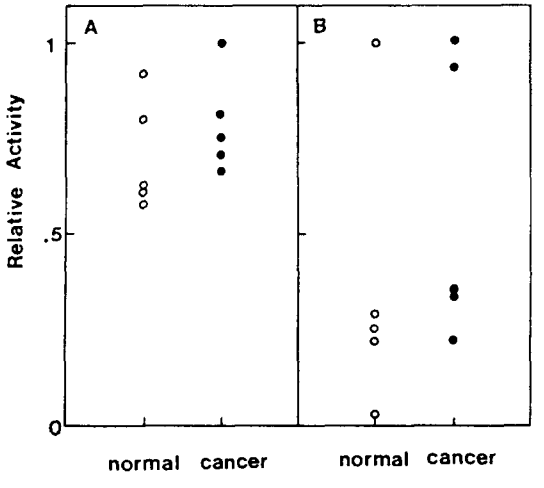

Fig. 4. Levels of the activities of plasmin-like protease (A) and plasminogen activator (B) in normal and neoplastic tissues of human liver. The activities were assayed and expressed as described in Fig. 3.

fold in the former case of tumor, but rather decreased a great deal in the latter case, it seems clear that one of the major changes in biochemical phenotype in the two types of the cancer is the alteration of protein catabolism.

Of particular interest is that, in both tissues, the level of anti-trypsin is inversely related with the level of proteolytic activity against casein and insulin. This kind of relationship clearly indicates that the protein breakdown in these tissues is tightly regulated by the changes in the levels of these activities. In addition, such an unbalance should provoke a gross change in the mobilization of cell proteins in the malignant tissues or, if the inhibitor or the protease(s) is secreted to extracellular matrix, of the proteins nearby the cells. Thus, if the proteases with elevated levels get secreted or if the secreted inhibitors get reduced to low levels and therefore unable to prevent the proteolysis in extracellular matrix, tumors (e.g., cervix cancer) would become metastatic through the increased proteolytic reaction by which the infiltration and extravasation of tumor cells are facilitated.

Recently, two distinct proteins having anti-trypsin activity have been isolated from cultured human hepatoma cells and shown to be associated with tumor (McKeehan *et al.*, 1986). These proteins stimulate specifically the proliferation of human endothelial cells to over 5-fold when compared to the cells cultured in their absence. In addition, it has been found that the anti-trypsin obtained from human pancreas contains strong

sequences homologies with tumor-associated growth factors, such as epidermal growth factor-like proteins and platelet-derived growth factor (Bowen-Pope *et al.*, 1984; Sheving, 1983; Todaro *et al.*, 1980). Similarly to these proteins, the increased activity of anti-trypsin in neoplastic tissues of human liver (Fig. 2C) may also be associated with the tumor, for example, by promoting the proliferation of the neoplastic cells.

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인체의 자궁암과 간암조직에서의 단백질 분해활성의 변화

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인체의 자궁암과 간암조직들이 나타내는 몇 종류의 단백질 분해효소들과 Anti-trypsin의 활성도를 정상조직의 것들과 비교하여서, 암의 종양성 형질과 단백질 분해활성의 변화사이에 어떤 상관관계가 있는지를 조사하였다. Casein과 Insulin의 분해 활성도는, 자궁암에서 2~3배정도 증가하는 반면, 간암에서는 1/2에서 1/5정도로 감소하였다. 이와는 대조적으로, Anti-trypsin의 활성도는 자궁암에서 약 1/10정도로 감소하였고 간암에서는 2배 가량 증가하였다. 한편, Plasmin-like enzyme과 Plasminogen activator의 활성도는 자궁암과 간암조직 모두에서 정상조직에서보다 10~20% 정도 높게 나타났다. 이러한 결과는, 정상조직 내의 단백질 분해활성도가 단백질 분해효소들과 이들의 활성을 저해하는 단백질들의 균형에 의하여 조절됨을 시사하며, 암조직들에서는 각 암조직들의 종양특이성에 따라 단백질 분해효소와 저해단백질들 사이의 균형이 깨어짐에 따라 단백질 분해활성도가 다르게 나타남을 보여준다.