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Proenzyme to Urokinase-type Plasminogen Activator in Human Colon Cancer: *in vitro* Inhibition by Monocyte Minactivin after Proteolytic Activation

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Abstract—*Marked increases of plasminogen activator activity were observed in human colon cancer tissue, compared to corresponding normal tissues. This increase was attributable to urokinase-type activator (HPA52), with no increase evident in the level of the tissue-type plasminogen activator (HPA66).*

Human monocyte minactivin specifically inhibited HPA52 activity in cancer tissue homogenates and in colon cancer cell supernatants, an effect that was greatly enhanced by preincubation with plasminogen, indicating that the predominant form of HPA52 in tissue and the form that is secreted in vitro *is the proenzyme. Inactivation of HPA52 by minactivin was shown to be dependent on proteolytic activation of HPA52 proenzyme. Utilization of HPA52 activity by tumors* in vivo *could therefore be dependent upon a protease, such as plasmin, to generate the extracellular proteolytic activity necessary to digest the intercellular matrix and permit invasion of normal tissue structures by colon cancer cells.*

INTRODUCTION

PLASMINOGEN activating enzymes catalyse the formation of plasmin from the abundant plasma zymogen, plasminogen. Cells which secrete these enzymes are therefore able to recruit a proteolytic activity that is far in excess of that which the cells could produce directly [1, 2]. Many cell types can acquire the ability to secrete plasminogen activator under physiological stimulus [3, 4], pathological insult [5, 6] or malignant transformation [7, 8]. Most of these events appear to involve the urokinase type activator (HPA52)* (in humans, M_r 52,000 vs 66,000 for the tissue type) coded for by a different gene [9], and having no immunological cross-reactivity with the tissue-type activator (HPA66).

There is evidence that acquisition of the urokinase type activator may confer significant advantage on cancer cells, particularly in facilitating mobility through restraining tissue structures [10]. Indeed, several studies have reported increased levels of urokinase type activator (HPA52) in extracts of human tumor tissues [11–15]. This paper explores further the expression of plasminogen activators in human colon cancer, including some data on its level of activity in tumor vs normal tissue, the molecular species of the enzyme that is increased, the form in which it occurs (proenzyme or active enzyme), and the reactivity of tumor extract and tumor cell culture supernatant enzyme towards a specific inactivator, recently discovered in this laboratory, human monocyte minactivin [16].

METHODS

Tissue samples and homogenization

Tissue samples derived from 24 human colons were obtained immediately after surgical resection. The colonic mucosa was dissected free from the muscularis mucosa and then washed with Hank's solution. Where possible, macroscopically normal mucosa and frank carcinoma were sampled from each colon and stored frozen. Macroscopically nor-

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*Abbreviations used: CTA, Committee on Thrombolytic Agents; SDS, sodium dodecyl sulfate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS; Muramyl dipeptide,*N*-acetyl-muramoyl-L-alanyl-D-isoglutamine; HPA66, HPA52 and HPA36, human plasminogen activators of M_r 66,000, 52,000 and 36,000 respectively; Enzymes, thrombin (EC 3.4.21.5); plasmin (EC 3.4.21.7); kallikrein (EC 3.4.21.8); urokinase (EC 3.4.21.31).

mal mucosa was also obtained from 6 colons resected for benign conditions, including volvulus (2), diverticular disease (2), and adenomatous polyps (2). The cancer samples were obtained from 17 consecutive colons resected for colorectal carcinoma affecting 9 males and 8 females, age range 44–76 years. Using Dukes' classification to assess the degree of invasiveness, 4 cancers were classified Dukes' A, 6 as Dukes' B and 7 were Dukes' C and D. Their classification as normal or tumor tissue was confirmed by standard histological methods and the differentiation of colon cancer cells was graded. Five of the cancers were well differentiated, 7 moderately differentiated and 5 were classified as poorly differentiated. Portions of thawed material were later weighed and gently hand homogenised with 50 mM glycine buffer, pH 7.8, containing 0.5% Triton X100, using 10 μl of buffer per mg (wet weight) of tissue. The homogenates were clarified by centrifugation for 5 min at 9000 ***g*** in a Beckman microfuge.

Colorimetric assays of plasminogen activators

The plasminogen activator content of tissue homogenates and cell culture supernatants were quantified by the assay method of Coleman and Green [17]. Supernatants from homogenates prepared as above were diluted 1 : 10 with homogenizing buffer prior to assay. Diluted samples of each homogenate (20 μl) were incubated with affinity-purified [18] human plasminogen (4 μg in 40 μl assay buffer) for 45 min at 37° C. The assay buffer contained 50 mM glycine, pH 7.8. 0.1% gelatin and 0.1% Triton X100.

SDS-PAGE and fibrin overlay zymogram

Since the colorimetric method measured plasminogen activation due almost entirely to HPA52 (melanoma HPA66 was virtually inactive in this assay [16]), the fibrin overlay method of Granelli-Piperno and Reich [19] was also used to compare normal and cancer tissue extracts. The tissue homogenates were applied to the non-reducing 11% acrylamide gel without further treatment, after incubation for 30 min at 37° C with affinity-purified human plasminogen [18], after incubation for 90 min at 23° C with monocyte minactivin [16] or after incubation first with plasminogen, and then with minactivin. Untreated samples of the above incubations (60 μl) were applied to the gel with SDS sample buffer (40 μl). Enzyme lysis bands were produced by incubation of the fibrin/agarose overlay for approx. 6–8 hr at 37° C.

Radial diffusion assays of plasminogen activators

Fibrinogen (Sigma type X), human plasminogen and thrombin (Sigma grade 1) were cast in a 1.25% agarose (Sea Plaque, FMC Corp., Rockland, ME, USA) gel matrix 1.2 mm thick. After clotting at 37° C, the gel was hardened at 4°C, and 3 mm wells were cut for the application of protease/minactivin incubation mixtures (5 μl). The proteases used for the incubations were porcine pancreatic kallikrein (1.5 μg; Sigma), porcine plasmin (150 ng; Sigma), mouse urokinase (3.6×10^{-3} CTA U), HPA66 (melanoma MM-170 culture supernatant), human urokinase (18×10^{-3} CTA U) and COLO 394 (see below) culture supernatant (containing 1.8×10^{-3} CTA U). These enzymes were incubated with minactivin (titration equivalent of 0.4 CTA U human urokinase standard) for 90 min at 23° C, then applied to the wells in the diffusion gel. The gel was incubated in a humidified box for 20 hr at 37° C, and the lysis produced enhanced by extensive washing in saline, followed by staining with amido black.

Culture of colon carcinoma cells

The human colon carcinoma cell lines COLO 394 and 397 [20] were obtained from Dr R. Whitehead, Ludwig Institute of Cancer Research, Royal Melbourne Hospital, Melbourne, Australia. Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) with 10% fetal calf-serum supplement and passaged twice a week. For studies of the effect of minactivin on tumor cell culture enzyme, approx. 3×10^6 cells/well were plated in 6-place multi-well plates (Linbro No. 76-058-05). After adherence overnight, the cells were washed with serum-free RPMI medium and cultured in this for 48 hr. Additions to the three experimental cultures were (1) human plasminogen (15 μg/ml), (2) plasminogen plus minactivin (equivalent of 1.4 CTA U of urokinase) or (3) plasminogen, minactivin and trasylol (18 μg/ml).

Minactivin preparation

Minactivin used in these experiments was prepared from human blood monocyte culture supernatants [16]. Fresh blood was spun to produce buffy coats of white cells, which were then purified by centrifugation on Ficoll Hypaque and the monocytes were then separated by centrifugal elutriation [21]. Adherent cultures on plastic dishes were cultured for 3 days with RPMI-1640 medium containing 0.05 μg/ml of muramyl dipeptide (adjuvant peptide, *N*-acetyl-muramoyl-L-alanyl/D-isoglutamine, Peninsula Laboratories, San Carlos, CA, U.S.A.). The minactivin in the supernatant was purified by two stages of affinity chromatography (R. Stephens, unpublished), giving a preparation which contained a single species of urokinase inhibitor as determined by gradient polyacrylamide gel electrophoresis and colorimetric assay of gel slices.

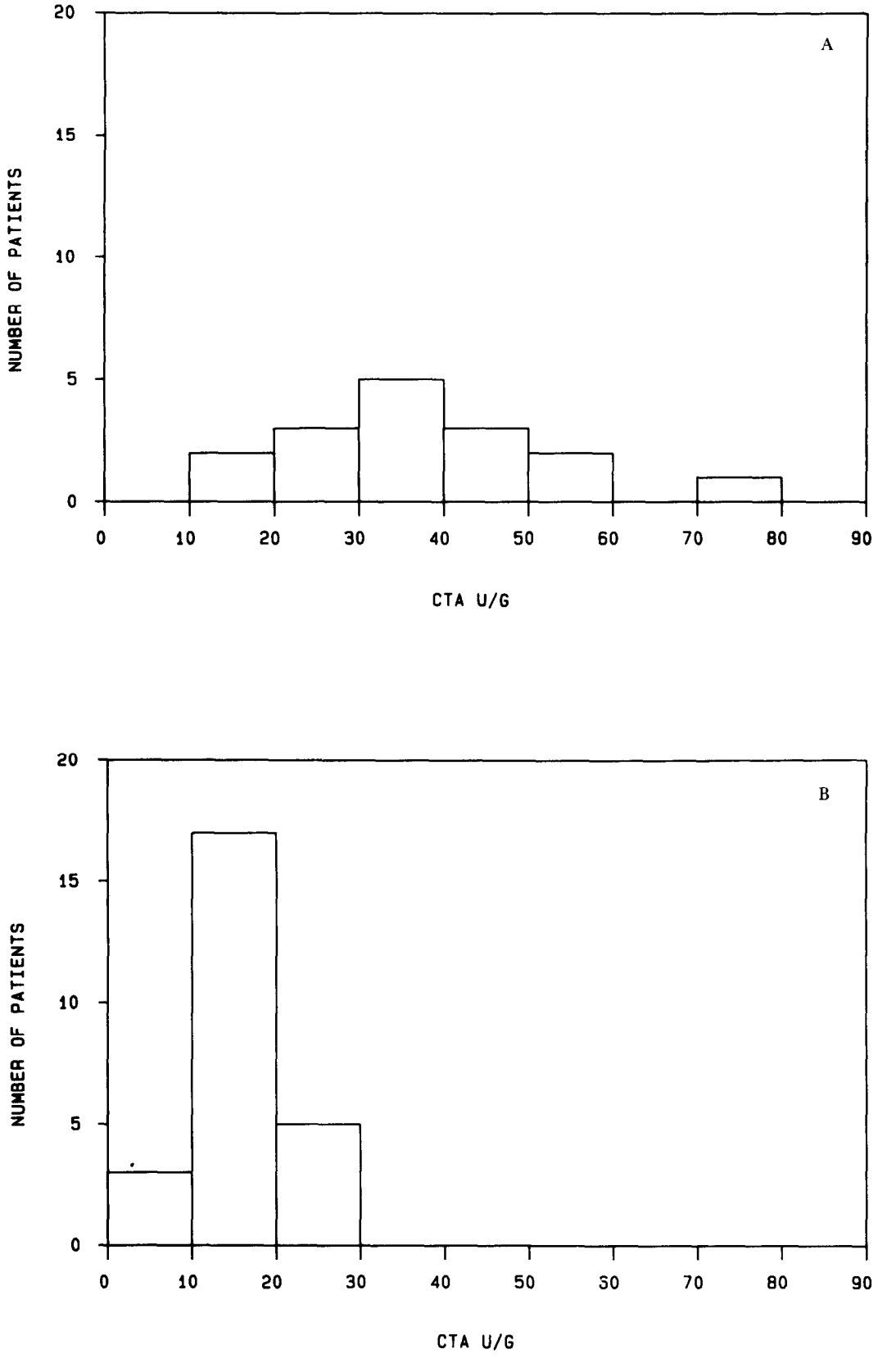

Fig. 1. Frequency distribution histograms showing the results from colorimetric assays of plasminogen activator in homogenates of colon cancer tissue (A) and from histologically normal areas of cancer-bearing colons (normal colon) (B). The plasminogen substrate contained traces of plasmin, such that results express the sum of proenzyme and active enzyme. The activity is expressed as CTA U/g tissue, based on measurements of urokinase standard made under the same conditions. 1×10^{-3} CTA U *of this urokinase produced an absorbance of* 0.16 *at* 412 nm.

RESULTS

Plasminogen activator content of normal and tumor colon tissue samples

Diluted homogenates of colon mucosa were assayed for plasminogen activator by the method of Coleman and Green [17] which measured the total content of plasminogen activator enzyme (i.e. proenzyme and active enzyme) because the plasminogen substrate used contained trace amounts of plasmin sufficient to activate proenzyme [22]. Furthermore, since this assay had very low sensitivity to tissue-type enzyme [16], it measured predominantly urokinase-type activator. Direct hydrolysis of the lysine thioester plasmin substrate by plasminogen-independent neutral proteases in the diluted homogenates did not contribute significantly to color development. Both histologically normal areas of cancer-bearing colons, and frank colon cancer tissue were studied. The results of these quantitative assays are shown in Fig. 1.

The activity of normal tissue homogenates was tightly centered, with a mean value of 16 ± 5 CTA U/g tissue. While very few samples showed active urokinase bands on zymograms after SDS-PAGE (see below), the more sensitive colorimetric assay showed that *all* homogenates of normal tissue contained some plasminogen activator contributing to this assay. In the absence of added fibrin, it was most likely this contribution was from low levels of HPA52 and not from the ubiquitous HPA66 [11, 23]. We have previously shown that melanoma culture supernatant HPA66 does not produce significant color in this assay [16].

Cancer homogenates, however, showed a wide spectrum of plasminogen activator activity, covering a 10-fold range of color yield (Fig. 1). These results no doubt reflect, in part, the variation in areas of tumor sampled as well as tumor necrosis. The mean activity was 36 ± 17 CTA U/g tissue, more than double that of the mean for normal tissue homogenates. (An unpaired Student's t-test for a difference in the mean gave $P < 0.005$). This ratio, however, included all material sampled. When the tumor samples were paired with the corresponding samples of normal tissue from each colon (Fig. 2), the mean ratio increased to over 3, with some pairs having a ratio of more than 5.

Zymograms of plasminogen activators in homogenates of colon mucosa

Homogenates of colon mucosa from histologically normal areas of cancer-bearing colons typically produced only one major band of lysis on fibrin-agarose zymograms after SDS-PAGE (Fig. 3, lane 1). This band was produced by a plasminogen-dependent enzyme migrating with the same mobility as the HPA66 of human melanoma culture supernatant. Minor bands were present at M_rs of approximately 96,000 (HPA96) and 110,000 (HPA110). Occasionally, an homogenate of normal tissue also produced a band migrating as the high molecular mass form of urokinase, i.e. HPA52.

Homogenates of cancer tissue typically produced the enzyme pattern seen in Fig. 3, lane 2. A major band of HPA52 was always present, together with

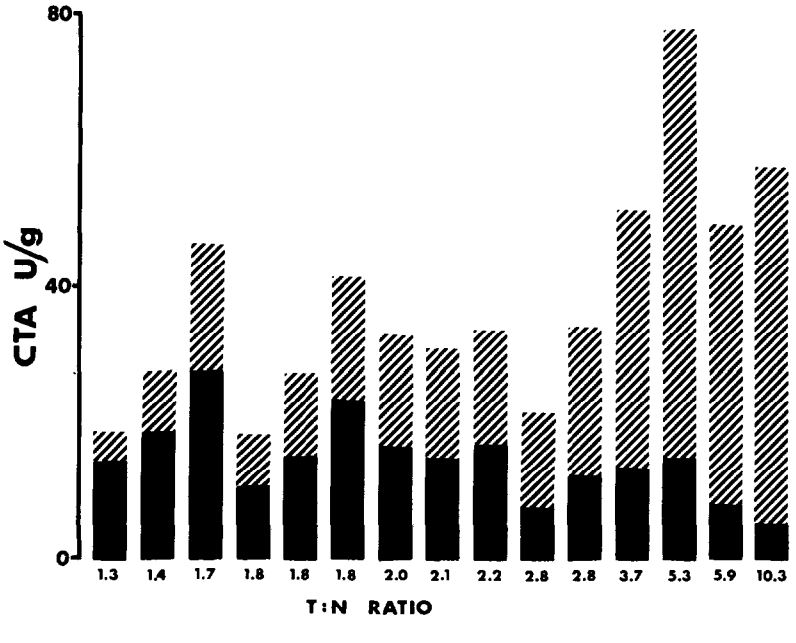

Fig. 2. Plasminogen activator activity of 15 colon tumor specimens (striped rectangles) compared to the activity in histologically normal tissue from the same colons (solid rectangles). The samples are ranked left to right according to the increasing value of the tumor : normal ratio (T/N).

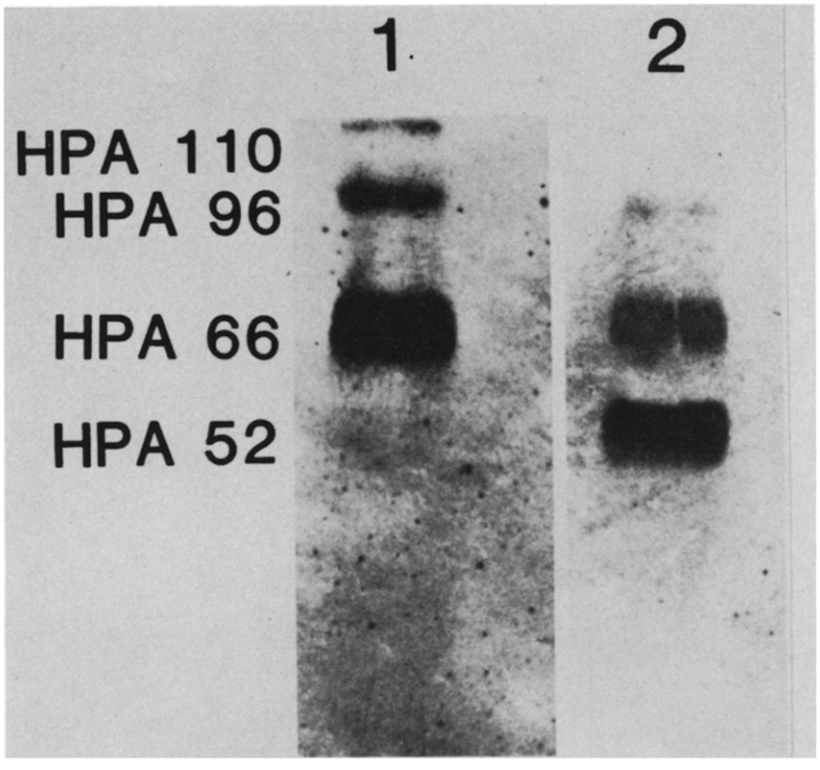

Fig. 3. *Types of plasminogen activator found in typical homogenates of normal mucosa (lane* 1*) and colon tumor (lane* 2*) as shown by fibrin agarose overlay after SDS-PAGE. Zones of lysis are: plasminogen activator of* M, 110,000 (*HPA*110); *plasminogen activator of* M, *96,000 (HPA96); plasmin* M, *85,000 and plasminogen activator of* M, 66,000 (*HPA*66).

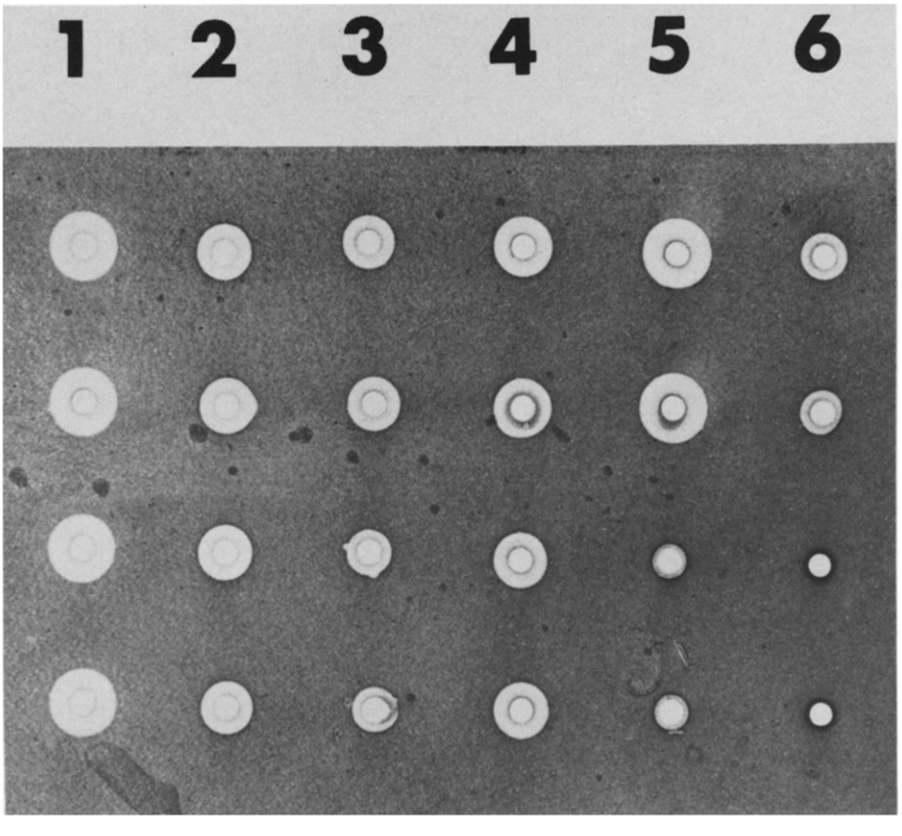

Fig. 4. *Specificity of minactivin shown by inhibition of fibrinolysis in an agarose diffusion gel. The top two rows contain proteases with control buffer, the bottom two rows contain minactivin (titration equivalent of* 0.4 *CTA U urokinase) treated proteases (see Methods). The proteases were: Lane* 1, *procine pancreatic kallikrein; lane* 2, *porcine plasmin; lane* 3, *mouse urokinase; lane* 4, *human melanoma cell culture supernatant containing HPA*66; *lane* 5, *human urokinase; lane* 6, *colon tumor cell culture supernatant containing HPA*52.

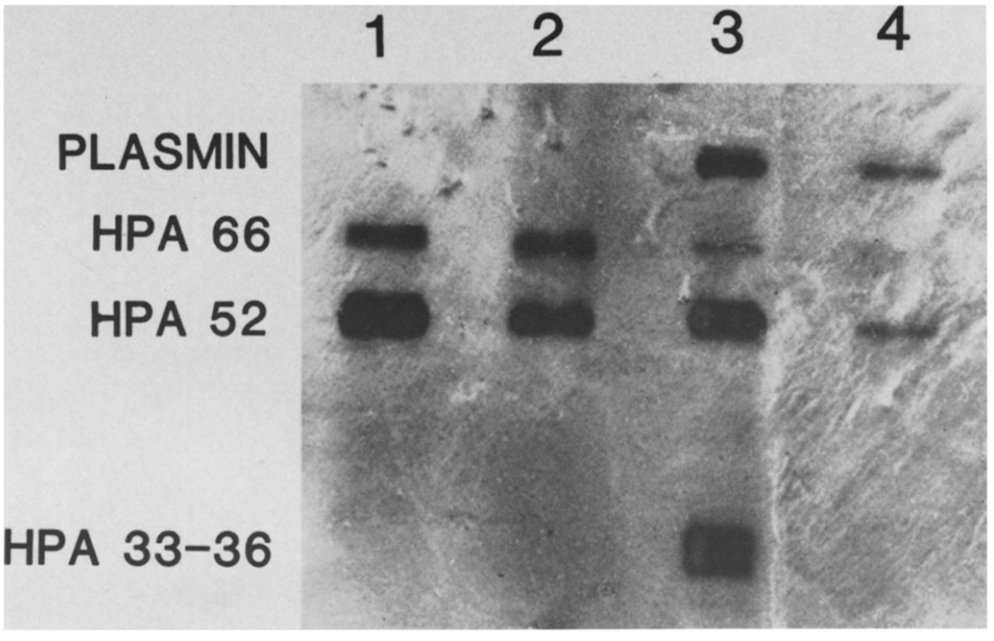

Fig. 5. The effect of minactivin on plasminogen activators in a sample of colon cancer homogenate. Lane 1 shows the untreated sample. Lane 2 shows the effect of preincubation for 60 min at 23° C (titration equivalent of 1.4 CTA U urokinase) with minactivin. Lane 3 represents the effect of preincubation for 30 min at 37° with human plasminogen and then for 60 min at 23° with control buffer. In lane 4, the homogenate was preincubated for 30 min at 37° C with plasminogen, and then exposed to minactivin for 60 min at 23° C. Zones of lysis are: plasmin M_r 85,000; *HPA66; HPA52 and HPA* 33-36.

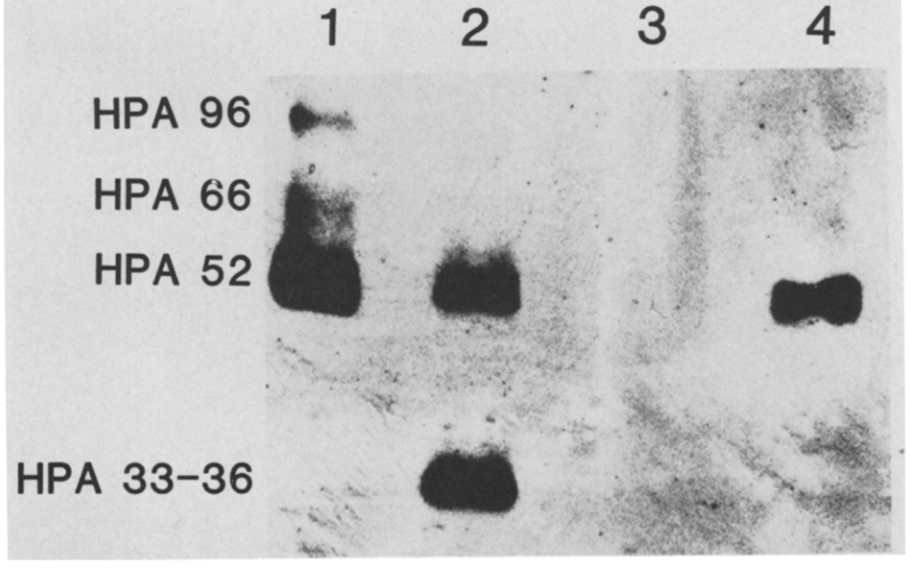

Fig. 6. Effect of minactivin on plasminogen activator in the culture supernatant of COLO 394 cells. Lane 1, culture with no additions; lane 2, with plasminogen; lane 3, with plasminogen and minactivin (titration equivalent of 1.4 CTA U urokinase); lane 4, with plasminogen, trasylol and minactivin. Zones of lysis are: plasminogen activator of M, 96,000 (*HPA* 96); *HPA66; HPA52 and HPA* 33–36.

a second band of varying relative intensity (sometimes almost absent), representing the HPA66 also seen in normal mucosa homogenates. The other minor bands noted above were usually very weak or absent.

Specific inactivation of urokinase-type activators in colon mucosa homogenates

We have recently described a urokinase inactivator which is produced by human blood monocytes after activation *in vitro* [16, 24, 25]. This inactivator, which we have called minactivin, has a specificity for urokinase-type plasminogen activators, and does not inactivate kallikrein, plasmin or HPA66 (Fig.4). It strongly inhibits human urokinase containing HPA52 and HPA33-36, as well as a plasminogen activator of M_r 52,000 produced in culture supernatants of a human colon tumor cell line (COLO 394,[20]). By contrast, mouse urokinase activity is only slightly affected by minactivin (Fig. 4). Minactivin was therefore tested with the enzymes present in colon mucosa homogenates. We have previously shown that minactivin forms inactive products with the HPA52 and HPA36 forms of urokinase, and these only regain very low activity after SDS-PAGE [16].

Tumor homogenates produced prominent HPA52 lysis bands (Fig. 5, lane 1), which could be reduced in size by pretreatment with minactivin (Fig. 5, lane 2). Pretreatment with plasminogen before electrophoresis produced plasmin and this in turn converted the HPA52 to HPA33-36 [26] (Fig. 5, lane 3). If the homogenates were treated first with plasminogen and then with minactivin, the HPA52 band normally seen after electrophoresis was greatly diminished and the bands representing HPA33-36 were abolished (Fig. 5, lane 4). Similarly, in the occasional instances in which histologically normal mucosa produced a visible activity band of HPA52, pretreatment with minactivin before electrophoresis resulted in the loss of HPA52 activity.

The enzymes in homogenates of colon cancer tissues which produced bands of M_r 52,000 were therefore markedly affected by incubation with human monocyte minactivin. Direct treatment produced partial inhibition, while minactivin treatment after plasminogen preincubation produced almost total inhibition of HPA52 activity.

Effect of minactivin on colon tumor cell culture plasminogen activator

Cultures of COLO 394 cells grown with serum-free RPMI-1640 medium secreted copious amounts of plasminogen activator activity into culture supernatants (Fig. 6, lane 1; Fig. 7, lane 1+). Zymograms after SDS-PAGE showed that this activity consisted principally of HPA52 with some

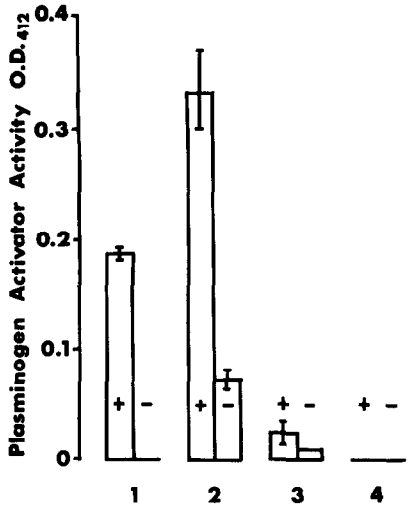

Fig. 7. Colorimetric assays of plasminogen activator (+) and plasmin (−) in the culture supernatants from the same experiments in Fig. 6, showing the effect of minactivin on plasminogen activator activity.

other higher M_r bands (Fig. 6, lane 1), a very similar pattern to the colon cancer tissue homogenates above. Supernatant from cells cultured with minactivin showed no change in this pattern (result not shown), so cultures with plasminogen containing trace amounts of plasmin were set up. When plasminogen alone was included in the culture media, some plasmin was produced (shown by plasmin colorimetric assay, Fig. 7, lane 2−) and this plasmin caused the conversion of some HPA52 to HPA33-36 (Fig. 6, lane 2). If minactivin and plasminogen were added to the media, all the enzyme produced during the culture was inactivated, as shown by both SDS-PAGE (Fig. 6, lane 3) and colorimetric assay (Fig. 7, lane 3+). However, if trasylol, a rapid and potent inhibitor of plasmin, was added to cultures, together with plasminogen and minactivin, no inactivation occurred (Fig. 6, lane 4). Since trasylol does not directly affect the reaction between minactivin and the active form of urokinase [16], these results showed that the HPA52 produced by COLO 394 cells was in the proenzyme form, requiring plasmin for expression of activity, and for reaction with minactivin. In the SDS-PAGE overlay system and in the colorimetric assay, activation of proenzyme occurred due to the traces of plasmin present in the plasminogen substrate. This did not occur when plasmin was inhibited by trasylol. Similar observations were made using a second colon cancer cell line COLO 397 (data not shown).

DISCUSSION

Several major human carcinomas produce amounts of urokinase-type plasminogen activator that are significantly greater than those occurring

in adjacent uninvolved tissue. The recent extensive studies of Markus and co-workers have included tumors of lung, prostate, breast and colon [14, 11, 27, 12] with attention to enzyme content and type, as well as the rate of secretion by tissue explants in short-term culture [28, 29].

In the present study, both the colorimetric assay and the zymograms after SDS-PAGE showed that the amounts of extractible enzyme activity were clearly considerably higher in tumor tissue, compared to normal tissue from the corresponding colons, and this was true for all cancer colons studied. SDS-PAGE analysis of homogenates of normal and tumor tissues agreed completely with Markus' results, showing that the increase in the enzyme content of tumor tissue was attributable to an increase in the urokinase-type plasminogen activator (HPA52). The zymogram method was unable to reproducibly demonstrate the albeit low levels of HPA52 in normal colonic mucosa seen by the colorimetric assay.

It has been conclusively shown by others that HPA52 is a different gene product [9], not derived by proteolytic or other modification of the "tissue" type plasminogen activator (HPA66) which was seen in homogenates of normal and tumor tissues, probably due to their vascular content. Coupled with the results above showing secretion of HPA52 by cultures of human colon tumor cells, it is clear that an increase in HPA52 proenzyme synthesis occurs in epithelial cells during development of human colon cancer, leading to higher tissue levels of HPA52. It is less likely that a change occurs in the level of inhibitor(s) regulating urokinase activity, since the majority of the enzyme could be extracted as its proenzyme form, which is not affected by inhibitor (see below).

With regard to HPA52 secretion, Markus has reported data showing that human colon tumor explants secrete copious amounts of activator compared to explants of normal colon tissue [29]. However, little is known about the form of the enzyme secreted by human cancer tissues—that is, whether it consists of the proenzyme form, or a mixture of proenzyme and active enzyme. Proenzyme forms of HPA66, HPA52 and the mouse plasminogen activator M_r 48,000 (MPA48) have been characterized as being the major enzyme form of plasminogen activator produced and secreted by cultures of tumor cell lines derived from human melanoma [30], human glioblastoma [31] and virus-transformed murine 3T3 cells [32] respectively. Also, it has been shown recently that proenzyme is the major form of plasminogen activator found in extracts of murine tissues, whether they are of tumor or normal origin [32, 33]. In these studies, formation of active enzyme required proteolytic activation, thought to be mediated by plasmin [22]. Clearly the performance of this conversion is important for extracellular expression of plasminogen activator *in vivo*, as well as for the meaningful assay of tissue enzyme activity.

It is not clear whether the previously reported measurements for normal and tumor tissues actually represent only that enzyme present in the tissue as active enzyme, both proenzyme and active enzyme, or active enzyme with a partial contribution from proenzyme. The results reported in this paper for tissue homogenates and culture supernatant from human colon cancer represent total enzyme since they were obtained with a colorimetric assay procedure under conditions which were highly favorable to proenzyme activation. Plasminogen substrate was used at concentrations 30 times in excess of that used in the standard fibrinolytic assays, and it contained plasmin in sufficient concentration to initiate proenzyme conversion and thus plasminogen activation, to form more plasmin.

In this study, the presence of proenzyme in colon cancer tissues was investigated using the plasminogen activator inhibitor, minactivin. Experiments were peformed in which tissue homogenates were exposed to plasminogen before treatment with minactivin. After SDS-PAGE of these mixtures, it was apparent that inactivation of HPA52 by minactivin following plasminogen treatment was considerably more effective than that observed in untreated homogenates, indicating that the predominant form of the enzyme occurring in tissues is, in fact, the proenzyme, and that reaction with minactivin was dependent on its conversion to active enzyme, probably by means of plasmin. This inference was confirmed by the experiments involving the addition of various combination of plasminogen, trasylol and minactivin to cultures of the human colon cancer cell line COLO 394. The results clearly showed that the major form of plasminogen activator, secreted by these cells was the proenzyme, and that this required the presence of protease activity (in this case provided by plasmin) for its conversion to the active enzyme before it was available to react with minactivin. This has since been confirmed by the use of an inhibitory monoclonal antibody to plasmin [34].

From these results, the conclusion is drawn that in human colon cancer tissue, synthesis of HPA52 is increased and the enzyme is largely presented to the extracellular milieu in its inactive, proenzyme form. Expression of activity, and the consequent initiation of a proteolytic cascade in the intercellular matrix [35], or alternately, inactivation by a specific regulator such as monocyte minactivin, is significantly dependent on the availability of a protease such as plasmin. Whether proteases secreted by commensal bacteria present in the

colonic lumen contribute to the cleavage of the proenzyme remains to be studied. While this condition appears to impose a "closed loop" regulation on the overall system, it nevertheless contains the element of exponential recruitment that can unleash the rapid build-up of proteolytic activity necessary to digest the intercellular matrix, thereby permitting invasion of normal tissue structures by malignant epithelial cells.

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