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# Collagenase – A Marker Enzyme in Human Bladder Cancer?

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Summary. Collagenase activity in human bladder carcinomas was measured against $^{14}C$-labeled collagen as substrate. Enzyme activity in vivo increased with the degree of penetration of the bladder wall. It was not detectable or low in the cases of superficially infiltrating tumours (A, B1, B2) and high in the cases of deeply infiltrating tumours (C, D). These results suggest that collagenase activity of advanced tumours is predominantly expressed in the perivesical layer of the bladder wall. A quantitative estimate of this enzyme may thus help to distinguish between Stage B and Stage C tumours.

Key words: Collagenase, Bladder carcinoma, Invasion.

Although little work has been done on the secretion of hydrolases by living cancer cells there are convincing data that suggest a close correlation between the invasive properties of the tumour and the content of degradative enzyme activity (6, 7, 9, 21, 26). Among the proteases, collagenases appear of special interest since cancer cells must often migrate through barriers composed of collagen before they can produce metastases. Indeed, recent reports on elevated collagenase activities in human and animal neoplasms (11, 18, 19, 28, 33) lead to the supposition that these enzymes may not only help to traverse the endothelial basement membrane in vascular invasion but can also destroy the collagen component of massive layers of connective tissue e. g., that of the dermis. Irrespective of the exact origin of collagenase in tumours, progressive levels of tumour invasion should indicate increased levels of collagenase if this enzyme is related to invasion and metastasis. Abramson et al. have found an interrelationship between the clinical agressiveness of epidermoid carcinomas of the neck and the head and the release of tumour collagenase in vitro (2), but the correlation of collagenase activity with staging and grading has not been previously reported. We have now carried out these studies in another invasive human tumour, the bladder carcinoma, and investigated whether collagenase is present and whether the level of collagenase bears any relation to the staging and histological grading of the tumour. Preliminary investigations carried out on collagenase in three other human tumours are also discussed.

## MATERIALS AND METHODS

All the tumours investigated in this study were collected from the Department of Urology and the Department of Gynaecology of Salzburg General Hospital.

Bladder specimens were obtained from 28 patients who underwent partial resection (n = 26) or total cystectomy (n = 2). Representative parts of the tumour were classified by the pathologist. Carcinomas were graded as Grade I - IV which represent in order the increasing grades of malignancy. The method of staging was based on the extent of invasion of the bladder wall according to Jewett and Strong (17). Normal bladder tissue came from patients with resection of the prostate gland (n = 4).

The gyneaecological tumours (n = 3) and the carcinomas of the prostate (n = 6) and kidney (n = 1) represent aggressive tumours.

### Preparation of the Tumour Extracts

In all cases tissue specimens were exhaustively washed with cold 0,15 M NaCl to remove traces of residual blood. They were finely minced,

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Table 1. Distribution of bladder tumours according to stage and grade

| Tumour grade | Pathological stage | | | | | |
|---|---|---|---|---|---|---|
| | A | B1 | B2 | B-C | C | D |
| I | 2 | | | | | |
| II | 2 | 5 | 1 | | | |
| III | 1 | 1 | | 2 | 1 | |
| IV | | | 1 | 2 | 6 | 4 |
| Total | 5 | 6 | 2 | 4 | 7 | 4 |

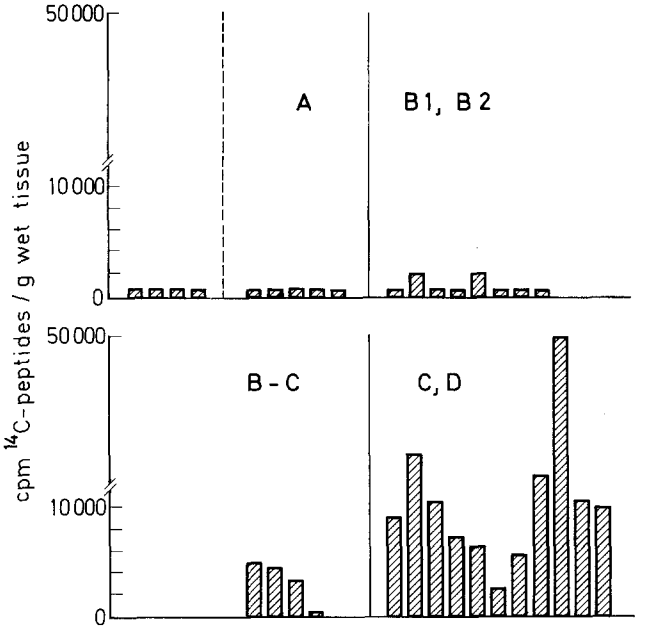


Fig. 1. Levels of sediment-bound collagenase in normal and neoplastic bladder tissues. The sediment obtained after homogenisation and centrifugation was extracted with 5 M urea for two hours. After dialysis and $(NH_4)_2SO_4$ precipitation the enzyme activity was determined, incubating with substrate at 25°C for 8 h. Each value represents one case

blotted on filter paper and weighed. The tissue was then homogenized at 0°C in 10 vol. of 0,4 M sucrose (w/v) in 10 mM Tris-HCl, containing 5 mM $CaCl_2$ (pH 7,6) as described previously (30). Centrifugation at 6000 x g for 20 min. resulted in supernatant fraction I, containing soluble collagenase. Following several washings in 0,15 M NaCl, the 6000 x g pellet was re-extracted for two hours in 5 M urea, dissolved in 50 mM Tris-HCl, containing 0,15 M NaCl and 5 mM CaCl (pH 7,6). After centrifugation and exhaustive dialysis this extract represent the sedimented enzyme (Fraction II).

Both fractions were adjusted to 20-50% saturation with $(NH_4)_2SO_4$(pH 7,2). The precipitate was collected after centrifugation, dissolved in 0,05 M Tris-HCl (0,15 M NaCl and 5 mM $CaCl_2$ pH 7,6) and dialysed. An aliquot of these crude enzyme preparations was examined for collagenase activity by incubation with soluble $^{14}$C-collagen (vide infra). Some tumour tissues were found to contain both active and latent collagenase. In these cases inactive enzyme was activated by 10 min. treatment of fraction I at 25° with 50 µg trypsin/ml. Active collagenase was precipitated between 20 and 50% saturation of $(NH_4)_2SO_4$ and collected as described above.

## Collagenase Assay

Collagenase was assayed by a method as previously outlined (30). This consists of measurement of the release of $^{14}$C-labelled collagen peptides from a mixture 50 µl of 0,2% soluble radioactive guinea-pig skin collagen (13), 25 µl of 0,2 M Tris-HCl buffer, 25 µl of water and 100 µl of enzyme solution. Each assay contained approx. 1800 cpm $^{14}$C-Collagen per tube. Incubation was performed in microtubes at 25°C for eight hours. After addition of 0,4 M EDTA and increase of temperature up to 37°C, soluble collagen peptides were separated from undigested collagen fibrils by centrifugation. From each supernatant, 100 µl were added to 10 ml of Bray's solution and counted. In each case, incubations, together with EDTA served as blanks. Results were expressed in cpm dissolved peptides per gram of wet tumour tissue. Substrate plus 0,01% trypsin (Sigma Chem. Corp.) were included as controls and only values above trypsin control (120-140 cpm above the blank) were registered as due to collagenase.

## Other Assays

For identification of the collagen degradation products the incubation was stopped by adding 10 µl of 2 N HCl instead of 0,4 M EDTA. Following denaturation at 42° C for 10 min. , 100 µl of the reaction mixture was subjected to polyacrylamide disc gel electrophoresis at pH 4.0 as described by Nagai et al. (20).

To measure the collagen content of the tumour tissue, the 6000 x g pellet was dried and extracted in 0.3 M TCA (12). Hydroxyproline was determined by the method of Woessner (31) and the collagen content was calculated (16).

## RESULTS

The relationship between staging and grading of the bladder carcinomas is shown in Table 1. In 11 cases tumours were more often superficial extending to the submucosa (Stage A) and the

Table 2. Collagenase activity of two Grade IV tumours. Both tumours were removed by two steps. Tissue extracts were prepared as described in the text. The enzyme mixture was incubated for 8 hours at 25°C. Collagenase activity was expressed in cpm per gram tumour tissue

| Patient | Submucosa and muscularis | | | Muscularis and perivesical layer | | |
|---|---|---|---|---|---|---|
| | Fract. I | Fract. I +trypsin | Fract. II | Fract. I | Fract. I +trypsin | Fract. II |
| 21/77 | No activity[a] | | | 1,520 | 5,500 | 7,530 |
| 23/78 | No activity[a] | | | 4,000 | 11,500 | 6,350 |

[a] No activity above the trypsia blank of 130-150 cpm/tube (25 µg trypsia/ml)

internal layer of the muscularis (Stage B1). In two cases the tumours proceeded through submucosa and muscularis (Stage B2) while 15 cases were more or less deeply invasive carcinomas infiltrating the perivesical layer to different degrees (Stages C and D). The four cases classified as Group B-C were borderline tumours between B2 and C.

As can be seen, Stage C and Stage D tumours were highly dedifferentiated carcinomas while the majority of the A and B1 tumours were tumours of Grade I and Grade II. Thus it seems evident, that infiltration of the perivesical tissue and severe atypia of the tumour cells are well correlated.

The data described in Fig. 1 clearly show that the sediment-bound collagenase is a characteristic of both C and D tumours. In this group there is only one case below the majority of cases which fall between 6,000 and 12,000 cpm dissolved $^{14}$C-collagen/g wet tissue and two cases reaching 26,000 and 50,000 cpm respectively. In the group including both B1 and B2 tumours only two from eight cases showed collagenolytic activity. In the group B-C, intermediate levels of collagenase were found. However, one sample showed no acitivity and we believe that this failure to detect the expected collagenase most likely resulted from overstaging this tumour. Additionally, neither the five cases of the A Group nor the control tissues demonstrated collagenase activity. Soluble collagenase was only found in the majority of tumours comprising Groups C and D (Fig. 2) but was generally lower then the sediment-bound collagenase.

Following activation with trypsin collagenolytic activity was detected in the sucrose extract of the borderline group B-C and markedly enhanced activity was found in C and D tumours. Treatment of the sucrose extracts from Stage A and B tumours and the control group with trypsin, failed to elicit collagenolytic activity.

In order to explore the possibility that the grade of anaplasia may account for the high activity in C and D tumours we examined two deeply

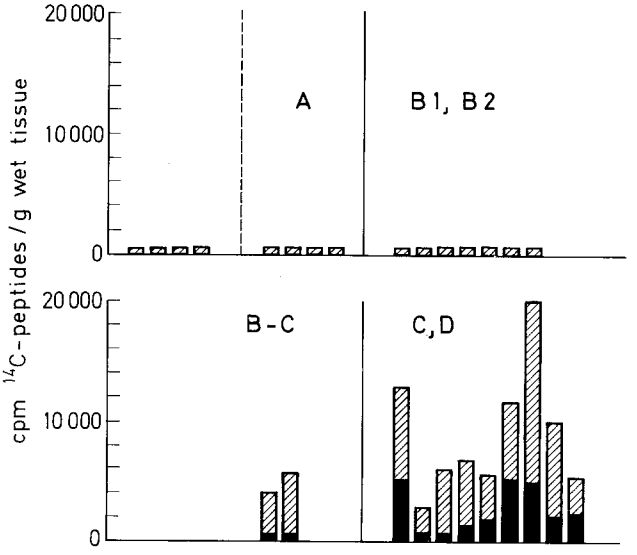


Fig. 2. Levels of soluble collagenase in normal and neoplastic bladder tissues. Collagenase activity was determined before (■) and after treatment of fraction I with trypsin (▭). Each column represents a different case

invasive carcinomas of grade IV. In these cases, the papillary and more superficial part of the tumour was excised in the first session while the deeper portions were removed two weeks later. In all the cases the neoplastic epithelium showed atypia of grade IV. As expected from our previous results, active and trypsin-activatable collagenase were only detected when the tissue material included muscle fasicles and the perivesical fat (Table 2). The number of cases is insufficient for valid conclusions but it is interesting to note that the depth of infiltration may determine whether or not extractable collagenase is produced.

Our observations of the presence of collagenase in highly malignant bladder tumours prompted us to examine three other human carcinomas for the ability to produce this enzyme in vivo. Each group represented only a small number of specimens. The results of this survey are summarised in

Table 3. Levels of extractable collagenase in three human carcinomas. While normal samples were not available in cases in which cervical cancers and cancers of the prostate were obtained, kidney samples included normal, neoplastic and mixed tissue. Extracts were prepared as described under "Material and Methods". The incubation time in the assay was 8 h. For collagenase determinations in urea extracts from cervix carcinomas, the enzyme solution was diluted 5-10 fold to obtain linearity in the enzyme assay

| Specimen | N | mg collagen/g Dry sediment | Collagenase activity | | |
|---|---|---|---|---|---|
| | | | Fract. I | Fract. I +trypsin | Fract. II |
| Cervical carcinoma | 3 | 2 - 5 | 4,520 | 6,200 | 48,300 |
| | | | 10,700 | 17,380 | 64,000 |
| | | | 18,300 | 27,100 | 180,500 |
| Carcinoma of the prostate | 6 | 100 - 150 | No activity[a] | | |
| Hypernephroma | | | | | |
| Normal tissue | 1 | 60 | No activity[a] | | |
| Mixed tissue | 1 | 150 | No activity[a] | | |
| Carcinoma | 1 | 120 | No activity[a] | | |

[a] No activity above the trypsia blank of 130-150 cpm/tube (25 µg trypsia/ml)

Table 3. Malignant invasive neoplasms of the cervix uteri clearly produced collagenase in vivo. On the other hand, adenocarcinomas of the prostate and invasive carcinoma of the kidney did not contain appreciable amounts of collagenase.

Collagenases derived from bladder tumours and from cervical cancers attacked soluble collagen in an identical manner, resulting in a single cleavage of the molecule across the triple helix and the formation of two products called $TC_A$ and $TC_B$ as are those produced by other mammalian and human collagenases.

## DISCUSSION

The discovery of collagenase, an enzyme capable of degrading native collagen under physiological pH and temperature conditions in two human carcinomas, supports our previous conclusions (29) that these enzymes may be involved in cases where microdestruction zones surrounding parts of cancer cells have been established by light and electron microscopic studies (5, 8, 24, 27, 32). The reasons for failure to detect collagenase in tumours of the prostate and kidney are not clear at present.

In a human bladder wall affected by cancer, the muscularis, in comparison to the perivesical layer, demonstrated either no frequency or a low frequency in the lysis of collagen substrate if both layers were consecutively invaded by cancer cells. Collagenolytic activity did not appear to originate from Stage A tumours and was not found in normal bladder tissue. Tentatively we conclude that the observed collagenase activity may be a product of the host in response to the tumour and not a product of the tumour itself. This interpretation would be consistent with immunocytochemical studies that indicate that in vivo collagenase is found almost exclusively in stromal elements rather than in the tumour cell itself. Connective tissue elements such as fibroblasts (4) subjacent to epithelial skin tumours, and macrophages (1) seem to play an important role in collagenase production. Bladder tumour cells may release an as yet unidentified diffusable factor capable of stimulating collagenase production in unknown cells in the perivesical tissue. Recent findings of prostaglandin E (PGE) synthesis in vitro by breast cancer (23), renal cortical carcinoma (3) and some invasive mammalian tumours (14, 22) suggest that this factor might be PGE-like in nature. Thus prostaglandin synthesis by deeply infiltrating cancers of the bladder is probably a necessary intermediate step in collagenase stimulation as was shown for collagenase-induced bone resorption (10).

Whether collagenase exists in the tissue primarily as a latent form and therefore needs limited activation by proteolytic enzymes, released by the tumour cells, is not known. At present we do not know whether the latent enzyme, generally found in extracts of Stage C und D tu-

mours, represents procollagenase (15) or results from formation of enzyme-inhibitor complexes (25).

Nevertheless it is also possible that tumour cells infiltrating the perivesical layer of the bladder secrete great amounts of either active or latent collagenase. If this is correct, and if the results from Table 2 are representative, the tumour cells entering this layer may be a subpopulation of Grade IV cells and may thus have special biological and immunogenic properties. On the other hand, Grade IV cells may have been conditioned by their environment to produce collagenase. These possibilities would help to explain why collagenase was found also in the borderline tumours and in minor amounts in two of eight B-staged tumours, graded as III and IV tissues. Differences in the ability of tumour cells to release collagenase may thus be due to differences in their intrinsic qualities.

Finally, we cannot exclude the possibility that the failure to detect collagenase in controls, in A tumours and in most of Stage B tumours resulted from collagenase inhibitors present in the tissue under investigation (18). It should be emphasized that both tumour tissue and normal tissue close to the tumour were utilised in this study. In a general sense our study demonstrated a high frequency of collagenolysis in deeply invasive tumours, in sharp contrast to the slight activity found in some Stage B tumours.

These observations lead to the supposition, that the increases in collagen degradative activity may permit the tumour cell to penetrate connective tissue barriers including that of the blood vessels, thus allowing the cancer cells to enter the circulation. It is well known that when invasion is limited to the area between basement membrane and external muscle fibres, more than 80% of the tumours are curable. But as soon as the tumour has reached the perivesical tissue, where lymphatics are large and numerous the cure rate drops sharply to approx. 25% (17). Thus our data would suggest that patients whose tumours demonstrate high levels of collagenase, have a lower rate of survival.

From the clinical standpoint our findings would also appear to emphasize the importance of collagenase as an effective parameter to distinguish between deeply and more superficially infiltrating tumours. At the moment collagenase estimations do not allow differentiation between B1 and B2 tumours and the statistical significance of our observations must await larger series and a longer follow-up. However, enzyme assays should help to improve the accuracy of clinical staging in borderline cases between B2 and C tumours. The presence of more than 2000 cpm $^{14}C$-collagen peptides/g tumour in our system would suggest that the tumour had entered the perivesical tissue.

REFERENCES


1. Abramson, M., Huang, C.: Localization of collagenase in human middle ear cholesteatoma. The Laryngoscope 87, 771 (1977)
2. Abramson, M., Schilling, R. W., Huang, C., Salome, R. G.: Collagenase activity in epidermoid carcinoma of the oral cavity and larynx. Annals of Otology, Rhinology and Laryngology 84, 1 (1975)
3. Atkins, D., Ibbotson, K. J., Hillier, K., Hunt, N. H., Hammonds, J. C., Martin, T. J.: Secretion of prostaglandins as bone-resorbing agents by renal cortical carcinoma in culture. British Journal of Cancer 36, 601 (1977)
4. Bauer, E. A., Gordon, J. M., Reddick, M. E., Eisen, A. Z.: Quantitation and immunocytochemical localization of human skin collagenase in basal cell carcinoma. Journal of Investigative Dermatology 69, 363 (1977)
5. Birbeck, S. M. C., Wheatly, D. N.: An electron microscopic study of the invasion of ascites tumour cells into the abdominal wall. Cancer Research 25, 490 (1965)
6. Blackwood, E. C., Mandl, I., Long, M. E.: Proteolytic enzymes and their inhibitors in human gynecological tumors. American Journal of Obstetrics and Gynecology 91, 419 (1965)
7. Bosman, B. H., Hall, T. C.: Enzyme activity in invasive tumors of human breast and colon. Proceedings of the National Academy of Science 71, 1833 (1974)
8. Chowaniec, J., Hicks, R. M.: Ultrastructural changes at the epithelialmesenchymal interface in experimentally induced bladder tumours. British Journal of Cancer 35, 254 (1977)
9. Dano, K., Reich, E.: Serine enzymes released by cultured neoplastic cells. Journal of Experimental Medicine 147, 745 (1978)
10. Dowsett, M., Eastman, A. R., Easty, D. M., Easty, G. C., Powles, T. J., Neville, A. M.: Prostaglandin mediation of collagenase-induced bone resorption. Nature 263, 72 (1976)
11. Dresden, M. H., Heilman, S. A., Schmidt, J. D.: Collagenolytic enzymes in human neoplasms. Cancer Research 32, 993 (1972)
12. Fitch, S. M., Harkness, L. R., Harkness, R. D.: Extraction of collagen from tissues. Nature 176, 163 (1955)
13. Gross, U.: Studies on formation of collagen. I. Properties and fractionation of neutral salt extracts of normal guinea pig connective tissue. Journal of Experimental Medicine 107, 247 (1958)
14. Galasko, C. S. B., Bennet, A.: Relationship of bone destruction in skeletal metastases to osteoclast activation and prostaglandins. Nature 263, 508 (1976)

15. Harper, E., Gross, J.: Collagenase, procollagenase and activator relationships in tadpole tissue culture. Biochemical and Biophysical Research Communications 48, 1147 (1972)
16. Jackson, D. S., Cleary, E. G.: Determination of collagen and elastin. In: Methods of biochemical analysis. Glick, D. (ed.). New York: Interscience Publishers 1967
17. Jewett, H. J., Strong, G. H.: Infiltrating carcinoma of the bladder: Relation of depth of penetration of the bladder wall to incidence of local extension and metastases. Journal of Urology 55, 366 (1946)
18. Kuettner, K. E., Soble, L., Croxen, R. L., Marczynska, B., Hiti, J., Harper, E.: Tumor cell collagenase and its inhibition by a cartilage-derived protease inhibitor. Science 196, 653 (1977)
19. McCroskery, P. A., Richards, J. F., Harris, E. D.: Purification and characterization of collagenase extracted from rabbit tumors. Biochemical Journal 152, 131 (1975)
20. Nagai, Y., Gross, J., Piez, K. A.: Disc electrophoresis of collagen components. Annals of the New York Academy of Science 121, 494 (1964)
21. Nagy, B., Ban, J., Brdar, B.: Fibrinolysis associated with human neoplasma: Production of plasminogen activator by human tumours. International Journal of Cancer 19, 614 (1977)
22. Powles, T. J., Clark, S. A., Easty, D. M., Easty, G. C., Neville, A. M.: The inhibition by aspirin and indomethacin of osteolytic tumour deposits and hypercalcaemia in rats with walker tumours, and its possible application to human breast cancer. British Journal of Cancer 28, 316 (1973)
23. Powles, T. J., Dowsett, M., Easty, G. C., Easty, D. M., Neville, H. M.: Breast cancer osteolysis, bone metastases, and anti-osteolytic effect of aspirin. Lancet, 608 (1976)
24. Rubio, C. A., Biberfeld, P.: The basement membrane of the uterine cervix in dysplasia and squamous carcinoma: an immunofluorescent study with antibodies to basement membrane antigen. Acta Pathologica et Microbiologica Scandinavica; Section A 83, 744 (1975)
25. Sellers, A., Cartwright, E., Murphy, G., Reynolds, J. J.: Evidence that latent collagenases are enzyme-inhibitor complexes. Biochemical Journal 163, 303 (1977)
26. Sylven, B., Bois-Svenson, I.: On the chemical pathology of interstitial fluid. I. Proteolytic activities in transplanted mouse tumors. Cancer Research 25, 458 (1965)
27. Tarin, D.: Sequential electron microscopical study of experimental mouse skin carcinogenesis. International Journal of Cancer 2, 195 (1967)
28. Usuku, G., Honya, M., Oyamada, I., Ogata, T.: Studies on animal collagenase. Acta Pathologica Japonica 24, 1 (1974)
29. Wirl, G.: Extractable collagenase and carcinogenesis of the mouse skin. Connective Tissue Research 5, 171 (1977)
30. Wirl, G.: Extraction of collagenase from the 6000 x g sediment of uterine and skin tissues of mice. A comparative study. Hoppe-Seyler's Zeitschrift für Physiologische Chemie 356, 1289 (1975)
31. Woessner, J. F. Jr.: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Archives of Biochemistry and Biophysics 93, 440 (1961)
32. Woods, D. A., Smith, C. J.: Ultrastructure of the dermal epidermal junction in experimentally induced tumors and human oral lesions. Journal of Investigative Dermatology 62, 259 (1969)
33. Yamanishi, Y., Dabbous, K. M., Hashimoto, K.: Effect of collagenolytic activity in basal cell epithelioma of the skin on reconstituted collagen and physical properties and kinetics of the crude enzyme. Cancer Research 32, 2551 (1972)

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