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EVIDENCE FOR THE PRESENCE OF A TRYPSIN INHIBITOR WITHIN RABBIT AND MOUSE TUMOUR CELLS

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Summary

Post-granule supernatant fractions obtained from induced tumour cells in rabbits and from Ehrlich ascites tumour cells in mice have been shown to contain a protease inhibitor, inhibiting trypsin (EC 3.4.21.4) and neutral proteases located in the cytoplasm of the cells. This inhibition was found to be irreversible over the time period studied, independent of the time of enzyme incubation and independent of the extent of trypsin digestion within an insoluble substrate (within the limits of linear enzyme kinetics).

The proliferation of tumours may be dependent upon the presence of neutral proteases [1], particularly those classed as collagenolytic enzymes capable of degrading connective tissue. Recent studies with polymorphonuclear leucocytes have demonstrated the presence of intracellular neutral proteases and collagenase associated with the granule fraction of these cells [2] and a neutral protease inhibitor present in the post-granule supernatant fraction [2]. Further studies [3] with similar fractions employing fluorescein-labelled polymeric collagen fibrils [4] as substrate demonstrated the collagenolytic activity of this neutral protease preparation and the ability of the post-granule supernatant fraction to inhibit both the cellular neutral protease(s) and trypsin (EC 3.4.21.4) [5].

Studies on the activation of a latent form of collagenase [6] present within the cells obtained from tumours induced in rabbits by intramuscular injection of V_2 ascites cell carcinoma [7] suggested that these cells also contained neutral proteases and an inhibitor similar to those found in the polymorphonuclear leucocytes [5]. The present report presents evidence for the presence of the inhibitor in two types of tumour cells; in each case the inhibitor was as-

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sayed by its ability to inhibit the trypsin digestion of fluorescein-labelled peptides from the telopeptide regions of fluorescein-labelled polymeric collagen fibrils [3, 5].

Tumours were induced in rabbits by the method described by McCroskery et al. [7] and the cells collected and washed as previously described [6]. After the cells from a single rabbit tumour had been washed 6 times in isotonic saline to remove serum protease inhibitors, such as α_2-macroglobulin and serum anti-trypsin, the cells were suspended in 0.34 M sucrose containing 50 units* of heparin per ml and two attempts were made to disrupt the cells by repeated passage through a Swinnex filter holder in the manner in which polymorphonuclear leucocytes were disrupted [5]. This treatment caused negligible disruption of the tumour cells but had the advantage that centrifugation after each attempt to disrupt the cells provided a supernatant fraction which was demonstrated to be completely free of trypsin inhibitor activity. It was therefore established that any inhibitor activity obtained in a subsequent post-granule supernatant fraction was derived from within the tumour cells rather than from the serum proteins which might have been present originally within the tumour. The tumour cells were then resuspended in 0.34 M sucrose/heparin and sonicated for two periods of 30 s in an M.S.E. ultrasonicator employing a peak to peak amplitude of 2—4 [3] followed by low-speed centrifugation (600 × *g* for 10 min) to separate the cellular material from the supernatant fraction containing cell sap and cell organelles suspended in sucrose solution. This supernatant fraction was subjected to centrifugation for 15 min at 20 000 × *g* [5] to yield a granule fraction and the first post-granule supernatant. The post-granule supernatant fraction usually measured 50—60 ml of pale yellow fluid, a second sonication of the cellular material followed by a second subcellular fractionation yielded a second post-granule supernatant fraction, also 50—60 ml in volume. Similar preparations of post-granule supernatant were prepared from mouse Ehrlich ascites tumour cells [8] harvested from 10 mice after 8 days of tumour growth.

The assay of the inhibitor depends upon two observations: (a) the ability of trypsin to solubilise two fluorescein-labelled telopeptides per molecule of tropocollagen within polymeric collagen fibrils [3], and (b) the fact that neutral protease inhibitor of polymorphonuclear leucocytes formed an inactive enzyme-inhibitor complex with trypsin in a stoichiometric manner [5]. The assay system used in the study was set up as follows. Weighed samples of fluorescein-labelled polymeric collagen fibrils (7.0 mg) were placed in a series of small plastic screw capped tubes. To each tube was added 1 μg crystalline trypsin pretreated with L-(tosylamino-2-phenyl)ethyl chloromethyl ketone and free of chymotrypsin activity, obtained from Worthington. A known volume of post-granule supernatant was added to each tube followed by additional buffer to adjust the final volume to 5.0 ml. The buffer consisted of 0.5 M KCl/50 mM Tris/40 mM EDTA with a pH of 7.5. The reaction tubes were equilibrated in a shaking water bath at 37°C for up to 24 h, at suitable time intervals (i.e., 0, 1, 2, 3, 5, 7, 10 and 24 h) 100-μl samples were with-

*1 unit = 6.6 μg heparin, sold by Evans Medical Ltd. as Pularin with 1000 units/ml.

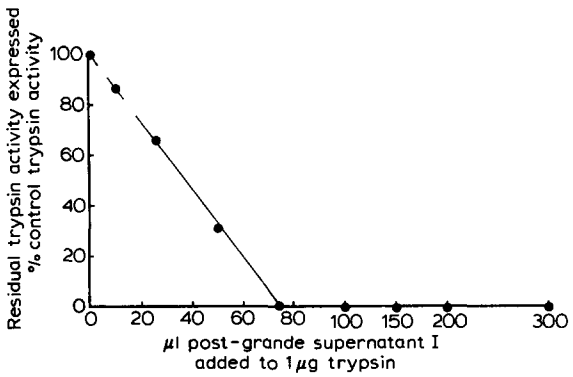

Fig. 1. Inhibition of trypsin digestion of fluorescein-labelled polymeric collagen fibrils by the addition of 0—300 µl post-granule supernatant I to 1 µg trypsin and 7.0 mg substrate in 5 ml of buffer pH 7.5. This is a composite graph of 5 identical graphs obtained by estimating the trypsin inhibition at five different time periods. At very low concentrations of post-granule supernatant I two opposing effects were observed (i) trypsin inhibition and (ii) trypsin activation of a latent neutral protease present in the post-granule supernatant I. In this study only the inhibition of trypsin is shown, dotted line, in the region 0—20 µl post-granule supernatant I.

drawn by micro-syringe from each tube and mixed with 3.0 ml distilled water and the solubilised fluorescein-labelled peptides measured by fluorimetric analysis [9, 10]. The inhibition observed was measured as the percentage control trypsin activity which was present in the absence of any inhibitor. The results of a series of inhibitor studies, with post-granule supernatant I and post-granule supernatant II obtained from rabbit cells, are presented in Figs. 1 and 2, respectively. The composite graph drawn in Fig. 1 represents the degree of inhibition observed when 1 µg of trypsin was incubated for 1—24 h with variable amounts of post-granule supernatant I. The composite nature of this

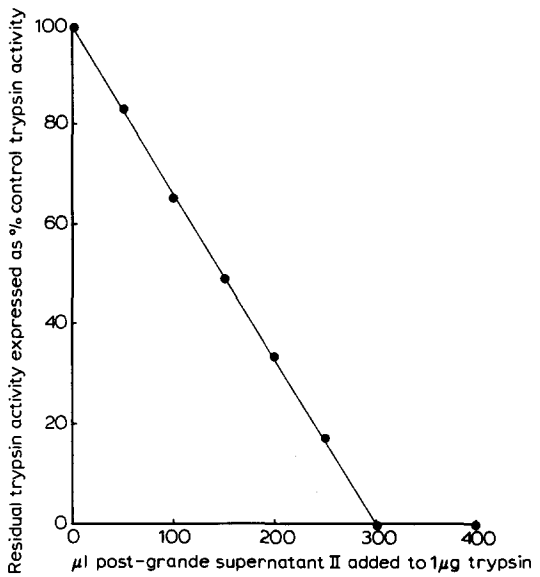

Fig. 2. Trypsin inhibition by post-granule supernatant II. Conditions as for Fig. 1.

graph is a consequence of the superposition of five identical graphs obtained at 1-, 2-, 4-, 6- and 24-h intervals. Since identical graphs were obtained at each of these time intervals the composite graph illustrates three points, (a) inhibition is irreversible over the time period employed in the study, (b) the degree of inhibition produced by addition of a given quantity of post-granule supernatant I is independent of the time of digestion, and (c) the degree of inhibition is independent of the extent of trypsin digestion of the telopeptides within the insoluble substrate. Point (c) is only valid when the relative molecular concentrations of trypsin and substrate are such that linear enzyme kinetics are possible, i.e. at a concentration below the point of equivalence [10]. This condition has been taken into consideration when designing the test system employed in this study.

Post-granule supernatant II exhibited a similar inhibitory action on trypsin (Fig. 2) but in this case 300 μl of the post-granule supernatant II were required to cause complete inhibition of 1 μg trypsin. Studies with post-granule supernatant I and post-granule supernatant II obtained from the mouse tumour cells also showed the presence of a trypsin inhibitor similar to that found in the rabbit tumour cells and the polymorphonuclear leucocytes.

The ability of the tumour inhibitor to form a complex with trypsin was further demonstrated by the removal of the inhibitor from post-granule supernatant I which had been passed through a short affinity column of trypsin linked to Sepharose 4-B. After washing the column, the inhibitor was dissociated from the trypsin-Sepharose 4-B and the eluted inhibitor was shown to be capable of inhibiting trypsin digestion of the fluorescein-labelled polymeric collagen fibrils.

It may be concluded that two types of tumour cells obtained from rabbits and mice both contain an inhibitor for trypsin and neutral proteases within their cytoplasm. This inhibitor can be quantitated by its ability to inhibit trypsin and can also be selectively adsorbed and further purified by trypsin affinity chromatography. When sufficient quantities of the inhibitor have been purified it will be possible to investigate whether the preparation is capable of controlling tumour development in vivo.

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