CLOT FORMING AND CLOT STABILIZING ENZYMES FROM THE MOUSE TUMOR YPC-1

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It is generally assumed that for a solid tumor to grow, it has to be supplied with blood vessels. According to a proposition advanced by O'Meara (1958), the blood vessels are guided into the tumor tissue by a fibrin network formed around it as it grows. This theory is supported by a number of observations (Clifton and Agostino, 1962; Larsen, et al., 1964). O'Meara (1958) noticed the presence of a clotting enzyme in tumor tissues.

This idea of the fibrin clot acting as the matrix into which the blood vessels grow is reminiscent of the process of wound healing. In the case of wound healing, the clot is the matrix into which the fibroblasts enter followed by the capillaries. In wound healing, however, the clot can fulfill its role only if it is formed by the action of two enzymes: the clot forming enzyme, and the clot stabilizing enzyme (Beck, et al., 1962). The clot forming enzyme (thrombin) permits the fibrin molecules to establish a network. The clot stabilizing enzyme (Laki-Lorand Factor, Factor XIII, Fibrinase) introduces cross-bonds which connect the fibrin network by chemical bonds (Laki and Gladner, 1964). (As a result, the clot becomes insoluble in concentrated urea solutions).

If a mechanism, similar to wound healing, operates in the growth of a solid tumor, one would expect the tumor to contain not only its own

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clot forming enzyme but also a clot stabilizing enzyme.

The work presented here showed that the tumor investigated contains both clot forming (CFE) and clot stabilizing enzymes (CSE).

A spontaneously developed plasma cell tumor (designated YPC-1) propagated under the skin of mice (BALB/C X A/Ln) F_1 (CAF_1) as a solid tumor (Yancey, 1964) was used in these experiments. The tumors were harvested 10 to 13 days after implantation. About 60 g tumor tissue was collected in one lot and kept frozen from 3 to 15 days in a deep freeze. After thawing, the tissue was cut up with a pair of scissors and washed twice with 200 ml of a 0.25 M cold sucrose solution for about 5 minutes. For each gram of tissue, 4 ml cold sucrose solution was added and the tissue was homogenized in a glass homogenizer.

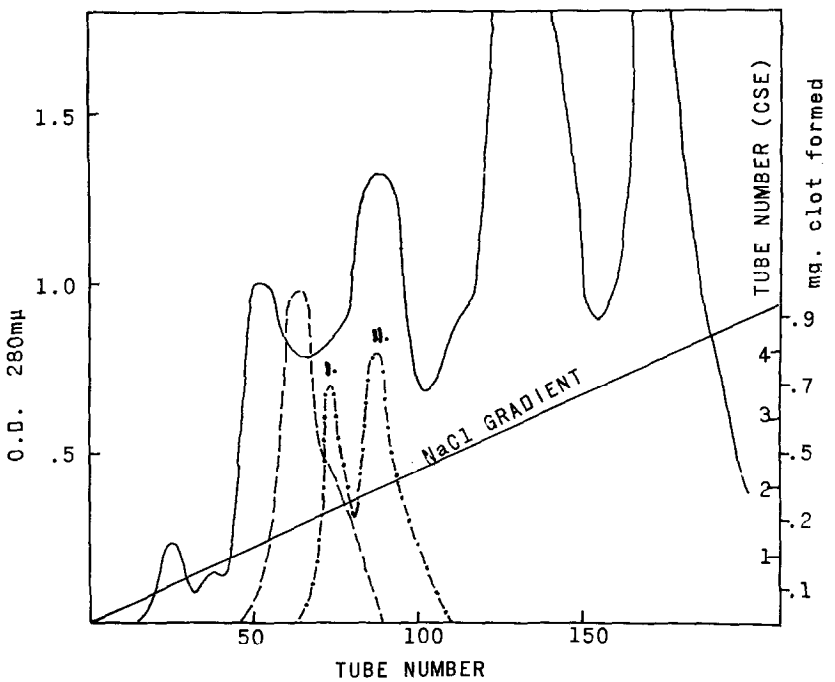
The homogenate was subjected to high speed (30,000 RPM) centrifugation for 90 min. The sediment was discarded and the clear supernatant (250 ml) adjusted to pH 5.0. The precipitate formed was collected by centrifugation and extracted with 18 ml of 0.1 M phosphate buffer, pH 6.3. This extract was dialyzed overnight against 0.005 M TRIS-HCl buffer (pH 7.5) containing 1 mM EDTA.

The dialyzed extract was then subjected to chromatography on a DEAE cellulose column (43x2.2 cm) in the cold using NaCl gradient for elution; 3 ml samples were collected. The samples were then analyzed for clot forming and clot stabilizing activity.

Since it is now known that transglutaminase (an enzyme that catalyzes the transfer of an amine into the γ-carboxyl group of a glutamine residue) can act as clot stabilizing enzyme, the eluates were also tested for the presence of this activity (Tyler and Laki, 1966).

The clot forming activity of 0.1 ml samples was tested on 0.5 ml fibrinogen solution of the following composition: 4.0 ml fibrinogen solution (15 mg/ml in 0.3 M KCl), 2.0 ml EDTA (0.02M),

2.0 ml TRIS buffer (0.5 M, pH 8.3), 1.0 ml $CaCl_2$ (0.1 M) and H_2O to make the total volume 20 ml. The test was carried out at 37°C. After 40 min. incubation, the clots were removed and their protein content determined by the Biuret method. The results are plotted on Fig. 1.



——————— optical densities of the eluates read at 280 mμ.

— — — — — clot forming activities were determined in every 5th tube but in every tube around the peak. The data are plotted as the milligrams of clot formed in 40 minutes. At the peak about 60 percent of the fibrinogen present was clotted.

—·—·—·—· clot stabilizing activities were determined in every 3rd tube as described in the text.

The contents of the tubes containing the clot forming activity (tubes from 50 to 70) were pooled and dialyzed against 10 vol. of distilled water overnight. The preparation was then lyophilized and taken up in about 6 ml of water. 20 μl of this solution

clotted 0.5 ml of the fibrinogen solution within one minute. In terms of NIH units, one milliliter contained about 10 NIH units. The mode of action of this clotting activity is under investigation.

Clot stabilizing activity was assayed by serial, 0.2 ml twofold dilutions of the solution to be tested in borate-saline buffer as described by Tyler and Laki (1966). To these solutions were then added fibrinogen (0.2 ml, 5 mg/ml) and $CaCl_2$ solution (0.1 ml, 0.05 M). Clotting was achieved by the addition of 0.02 ml of thrombin (2 N I H units). After incubation for 15 min. at 37°, urea solution (1 ml, 8 M) was added and the clot stabilizing activity assessed visually one hour later. Control fibrin clots, which contained no enzyme, dissolved in 8 M urea within 20 min. Results are expressed as the number of maximum twofold dilutions of test solution which still produce a urea insoluble clot. It is seen in Fig. 1 that the clot stabilizing activity emerged in 2 peaks.

The contents of the tubes containing the two clot stabilizing activities (peaks I and II) were collected and subjected to ammonium sulfate precipitation (75% saturation). The precipitates formed were collected by high speed centrifugation. After dissolution in 3.0 ml TRIS buffer, the preparations were dialyzed overnight and tested for their ability to catalyze the incorporation of C^{14}-labelled putrescine into fibrinogen. Table I shows that these preparations readily incorporate putrescine into fibrinogen. Under these conditions, 18 to 21 residues became incorporated into one mole of bovine fibrinogen. In similarly conducted experiments, transglutaminase, from guinea pig liver, incorporated only 4 residues of putrescine into fibrinogen.

This difference may be taken as an indication that these two enzymes seek out different sites for amine incorporation. The clot forming peak (tubes from 50 to 70) did not catalyze the incorporation of putrescine into fibrinogen. The amount of the clot stabilizing

Table I

The incorporation of C^{14}-labelled putrescine into fibrinogen by the clot stabilizing enzyme of the mouse tumor YPC-1.

Time of incubation min.	No. of residues incorporated by Peak I	Peak II
5	———	14.7
10	15.4	10.8
20	14.20	18.0
30	———	21.1

The composition of the reaction mixture was the following:

1.0 ml fibrinogen (15 mg protein/ml in 0.3 MKCl)
0.5 ml TRIS buffer/W (0.5M, pH 8.5)
0.5 ml EDTA (0.2 M)
0.1 ml $CaCl_2$ (0.2 M)
0.25 μmoles C^{14} putrescine (100 μM/100 μC/1 ml)
Total volume 5.0 ml

0.5 ml samples were taken out of this mixture and after addition of the enzyme to be tested (0.05 ml), the reaction was allowed to proceed at 37°C for various lengths of time. At the required time, 5.0 ml trichloracetic acid was added to the mixture. The precipitate was centrifuged and was washed several times with TCA. Finally, the precipitates were dissolved in 3 ml alkali (0.1N). Samples from this solution were taken for the counting of the radioactivity in a scintillation counter. The conditions were chosen so that the incorporation of one residue gave ~ 1000 counts per min. With each experiment, a known amount of putrescine was also counted.

The values actually incorporated into fibrinogen are probably about 30 per cent less on account of the self-incorporation of the amide by the enzyme preparation.

Since these preparations were contaminated by the clot forming enzyme which clotted the reaction mixture during incubation with the stabilizing enzyme, the possibility exists that it is actually fibrin which acts as the amine acceptor.

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activity found in the tumor is much greater than could be expected to come from the contaminating blood. Therefore, this activity may be considered as the product of the tumor tissue.

The mode of action of this enzyme is not known yet, but the fact that it catalyzes the incorporation of an amine into fibrinogen indicates that it acts similar to transglutaminase with the exception that it probably utilizes asparagine rather than glutamine residues as the amine acceptors.

The test solution for the transglutaminase activity consisted of a mixture of 0.1 ml CBZ-L-glutaminylglycine and 0.1 ml hydroxylamine solutions made up according to the prescription of Folk and Cole (1965). At varying times after the addition of 0.1 samples to these solutions, the transglutaminase activity was assessed from the purple color that developed when $FeCl_3$ was added to the mixture. In contrast to similar experiments carried out on guinea pig liver extracts (Tyler and Laki, 1966), no transglutaminase activity was found in the eluates.

The finding of both clotting and clot stabilizing enzymes in the tumor is in line with the idea that tumor needs the stabilized clot to grow. More direct proof would be obtained if it could be shown that a specific inhibitor to the clot stabilizing enzyme, or the absence of fibrinogen from circulation, retarded tumor growth.

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References

1. Beck, E., Duckert, F., Vogel, A. and Ernst, M. Z. Zellforsch 57, (1962).
2. Clifton, E. E. and Agostino, D. Cancer 15, 276 (1962).
3. Folk, J. E. and Cole, P. W. J. Biol. Chem., 240, 2951 (1965).
4. Laki, K. and Gladner, J. A. Physiol. Rev., 44, 127 (1964).
5. Larsen, V., Mogensen, B., Amris, C. J. and Storm, O. Dan. Med. Bull., 11, 137 (1964).
6. O'Meara, R. A. Q. Irish J. Med. Sci., 6, 474 (1958).
7. Tyler, H. M. and Laki, K. In print, this Journal (1966).
8. Wood, S. Arch. Path. 66, 550 (1958).
9. Yancey, S. T. J. Natl. Cancer Inst. 33, 373 (1964).