ARTICLE

Fibrinolytic Activity of Earthworms Extract (G-90) on Lysis of Fibrin Clots Originated from the Venous Blood of Patients with Malignant Tumors

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u-PA is secreted by the most malignant tumors. As a response to u-PA synthesis surrounding cells synthetize inhibitors of plasminogen activators for tissue protection. Plasminogen activators were found also in earthworm tissue. From the tissue homogenate of earthworm Eisenia foetida the glycolipoprotein mixture named G-90 was isolated. It contains two serine proteases (P I, P II) with fibrinolytic and anticoagulative activities. The fibrinolytic activity of G-90, P I and P II was tested in an *in vitro* euglobulinic test applied to fibrin clot from blood plasma of patients suffered from malignant tumors. G-90 and above-mentioned proteases applied in this study showed euglobulinic time proportionally with the concentrations of added substances. The influence of G-90 on the fibrinolysis rate does not depend only on its concentration, but depends too on histological type of tissue (organ) where the malignant tumors are located. Enzyme P I and P II do not show this activity. (Pathology Oncology Research Vol 4, No 3, 206–211, 1998)

Key words: fibrinolytic activity, earthworms extract, fibrin clot, malignant tumors

Introduction

In homeostasis maintaining a balance between factors – coagulation activators and their inhibitors on one side and fibrinolysis activators and their inhibitors on the other – is important. Malignant cells and their products disturb both activities. With their changed receptors, they activate thrombocytes, which express integrin receptors IIb/IIIa to fibrinogen. Fibrinogen, linked adhesively to thrombocytes, stimulates linking to blood vessel wall, which is the site of thrombus formation. The thrombocyte activation releases tissue factor (TF) thromboplastin stipulating formation of TK-F VII-Ca complex. In addition to this external pathway effect TF has also effects on activation of the internal pathway, started by prekallikrein/kininogen. Both pathways lead to activation of X-Xa factor, which, by the action of thrombin, changes fibrinogen to fibrin dimers, and, further, to insoluble reticulated fibrin. The response to the high activity of thrombocytes is secretion of inhibitor factor of Xa named TFPI (Tissue Factor Pathway Inhibitor). With disturbance of homeostatic balance in the circulation, thrombin-antithrombin III complexes can also be found, and thrombin surplus forms fibrin aggregates, which participate in thrombus formation.[8]

Fibrinolytic activity begins with the activation of epithelial cells, which are stimulated to secrete plasminogen activators (PA), with autocrine effects on receptors of the same cell (PAR).[13,26] In mammalian systems, two plasminogen activators synthesise tissue t-PA and urokinase u-PA, which are coded from different genes. The simple-chain t-PA is active; it lyses fibrin and other proteins of the extracellular matrix and is important in intravascular fibrinolysis.[2] Pre u-PA is activated to form u-PA by splitting two chains linked by disulphide bridge. The activation also occurs with plasmin, kallikrein, coagulation factors XIIa and cathepsin B. u-PA is serine protease of 55 kDa. B-

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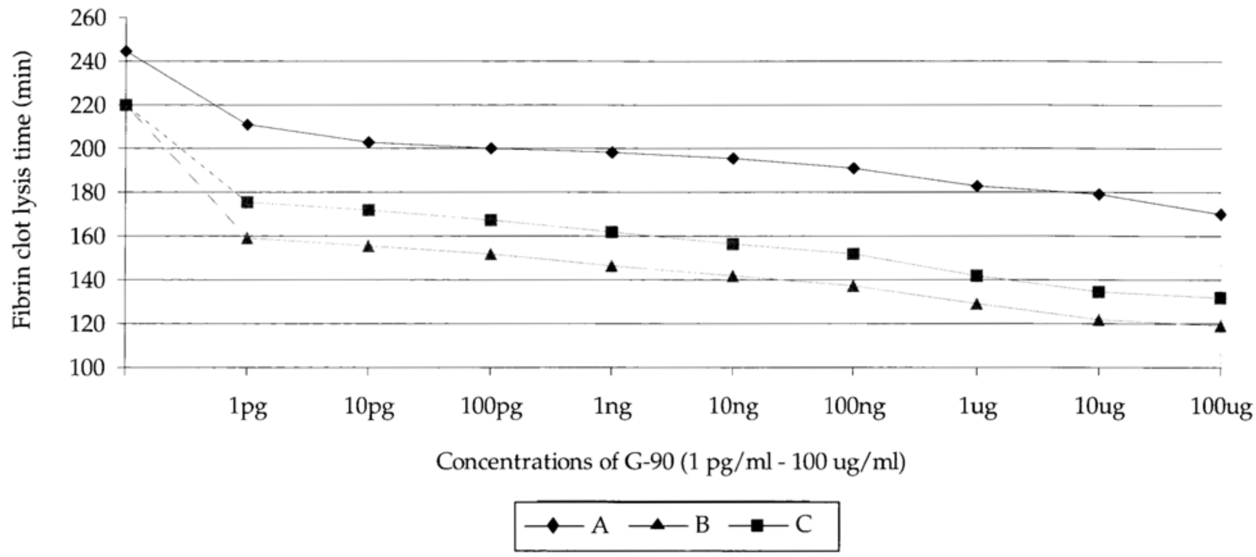

Figure 1. *Effect of G-90 on euglobulinic test. Clot lysis time lines: A- healthy + G-90; B- malignant + G-90; C-* ***imaginary*** *line: malignant + G-90 lower tumor share (10.3%). Interval B-C is the activity of tumor plasminogen activator. On abscissa G-90 concentrations are plotted, and on ordinate time of lysis (min).*

chain has binding determinants for plasminogen and A-chain for growth factors.[3] The plasminogen activators include many other factors such as streptokinase β, vitamin B1, erythrokinases, prekallikrein, Hageman factor, etc. The u-PA activity is 7–10 times stronger if it is linked to the receptors of cells that secrete it. Plasminogen linked to its receptor is converteds to the serine protease plasmin by the action of PA/PAR. Plasmin is active when it is linked to receptors. When free, it links immediately to inhibitors, so that cannot be found free in plasma. Plasmin proteolytically dissolves reticulated fibrin. Increase of fibrinolytic activity accompanies inflammation in the process of wound healing and in patients with malignant tumors.[7,16,23,25] The basic substrate – plasminogen – is present in plasma, but also in other interstitial liquids at 1–2 μM.[17] u-PA is linked to ubiquitin dependent pericellular fibrinolysis.[4] Plasma in patients with malignant tumors, beside physiologically present u-PA, also contains u-PA secreted by most malignant tumors. Thus the increase in plasmin also lyses proteins of extracellular matrix: laminin, collagen IV and other proteins of basal membrane and enables spread of tumor cells. Tumors also secrete insulinases which degrade insulin; cystein proteinases which directly activate X factor causing coagulation; and metalloproteases which also enable dispersion of tumor cells.[5]

In response to higher PA synthesis, surrounding cells synthesise inhibitors of plasminogen activators as a tissue protection. Three inhibitors have been found: PAI-I, PAI-2 and protease neksin which is characteristic of prokaryotes. PAI-I inhibits both u-PA and t-PA, while PAI-2 inhibits only u-PA. The tissue capacity for PAI synthesis is a factor for protection from tissue destruction and metastasis formation.[5] Beside such control of inhibitors of plasminogen activators, there is also plasmin control by α2 antiplasmin and α2 macroglobulin. The consequence is that malignancy threatens both coagulant and fibrinolytic systems. Even if tumors secrete PA who quickens fibrinolysis, this activity is not sufficient for controlling hypercoagulation.[6,7,9,16,27]

Plasmogen activators are found in invertebrates and so also in earthworm tissues. These serine proteases show fibrinolytic and anticoagulative activities. Because of their origin we call them lumbrokinases.[14,18,19,28-30] From the tissue homogenate of earthworms Eisenia foetida and Lumbricus rubellus, the glycolipoprotein mixture named G-90 was isolated. The preparation was neither mutagenic nor carcinogenic[10] and beside growth factors and adhesins,[11,21] it contained two serine proteases with fibrinolytic and anticoagulative activities.[12,20]

Our study shows the effects of new fibrinolytic G-90 and from it isolated enzymes PI and PII on *in vitro* lysis of fibrin clots originated from venous blood taken from the patients suffering from primary malignant tumors of different organs (tissues).

Materials and Methods

Glycolipoprotein compound (G-90) from the tissue homogenate of earthworms Eisenia foetida and Lumbricus rubellus (Annelida, Oligocheta, Lumbricidae) and serine peptidases P I (32 kDa) and P II (23 kDa) isolated from G-90.

Control: fibrin clots of venous blood from clinically healthy persons (20) of both sexes, aged from 25–70 years. Clots of venous blood from the patients suffering from pri-

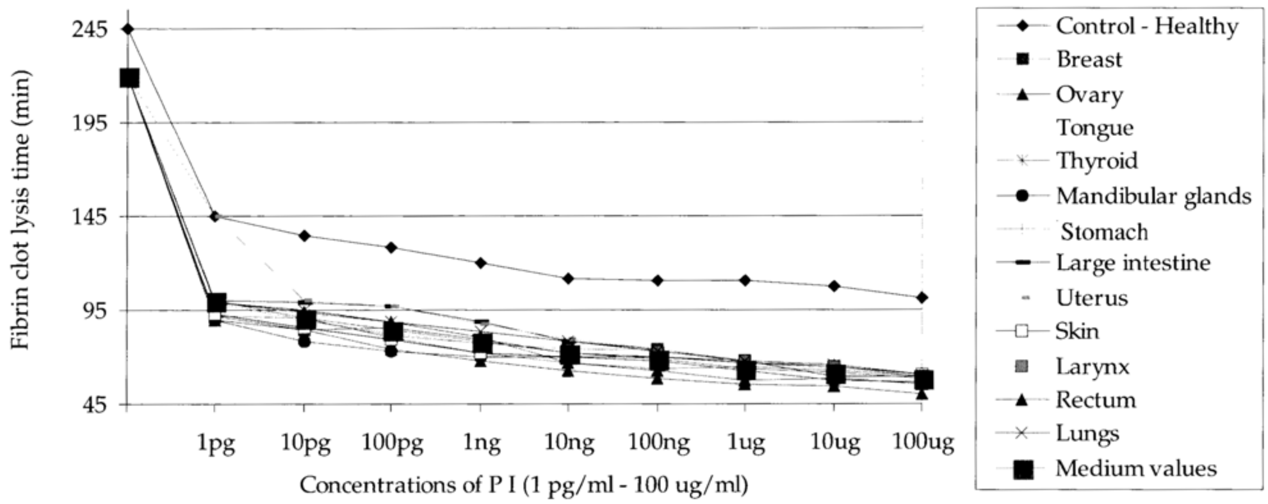

Figure 2. (a–d) *Effects of G-90, PI, PII, PI+PII on fibrin clots lysis from plasma of patients with malignant tumors performed in euglobulinic test. On abscissa concentrations of fibrinolytics are plotted, and on ordinate time of lysis in minutes. Lines are formed by plotting on medium values of time of fibrin clot lysis obtained from 20 patients grouped according to the location (organ, tissue) of tumor. Legends show sites of the primary malignant tumor.*
Figure 2a. *Effects of PI enzyme on results of euglobulinic test of fibrin clots from plasma of patients with malignant tumors.*

mary malignant tumors of different organs: 20 samples were collected from each location: breast, ovary, tongue, thyroid, mandibular glands, stomach, large intestine, uterus, skin, larynx, rectum, lungs.

All of the sera were taken from venous blood of the patients submitted to usual routine tests at the Clinic for Tumors and Allied Diseases (Zagreb, Croatia), according to ethical committee rules. The sera were kept at –20°C until used.

Euglobulinic test on fibrinolytic activity: 1 ml of fibrinolytics in concentrations from 1 pg/ml to 100 pg/ml in saline was added to fibrin clot obtained from 1 ml of plasma. Time of fibrin clot lysis was noted.

Homeostasis tests as routine parameters: prothrombin time; fibrinogen (mg/dl); APTV-test; thrombosis time.

Statistic evaluation was performed by Kruskal-Wallis test; $p<0.001$.

Results

Euglobulinic test results for physiological time of fibrin clot lysis from the venous blood of the control group was 245 minutes (medium values), which is similar to data quoted in the literature. The time of fibrin clot lysis from venous blood of patients with maligna.nt tumours, regardless of location, was shortened to 220 minutes (medium

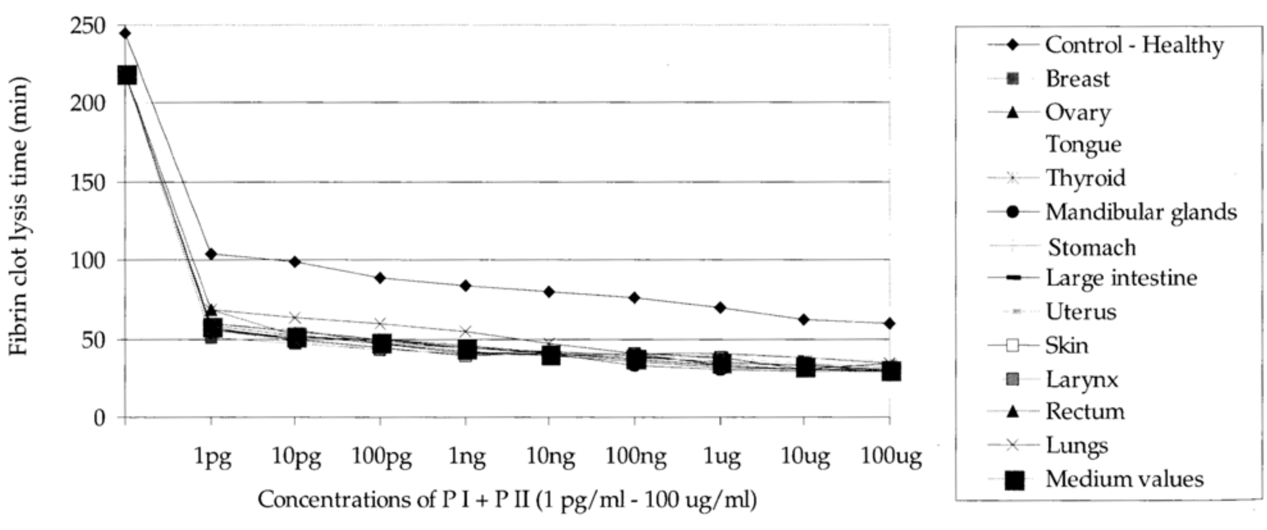

Figure 2b. *Effects of PI+PII enzymes on results euglobulinic test of fibrin clots from plasma of patients with malignant tumors.*

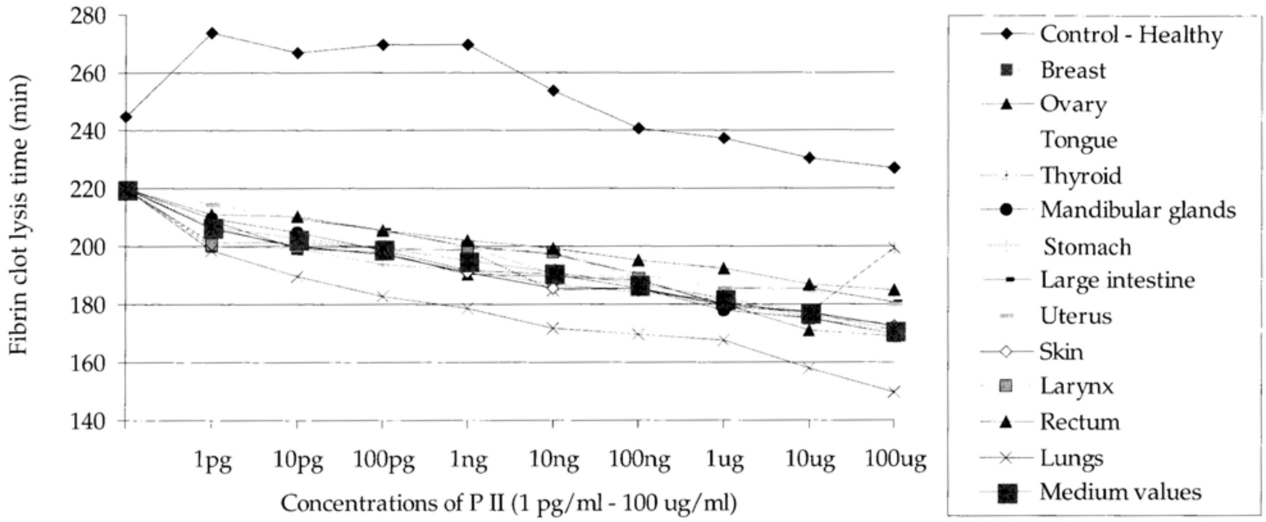

Figure 2c. *Effect of PII enzyme on results euglobulinic test of fibrin clots from plasma of patients with malignant tumors.*

values). Fibrinolysis was quicker by 10.3%. Euglobulinic test performed on clots of the same sources, but with G-90 added in increasing concentrations (1 pg/ml – 100 µg/ml) additionally shortened lysis time, depending on the concentration of G-90 added (from 36 to 44% more). Lysis time reduction after the highest concentration of 100 µglml G-90 was in total 55% *(Figure 1)*. *Enzyme PI* in clots from healthy blood origin increases lysis proportionally with concentration: from 145 minutes in concentration 1 pg/ml to 100 minutes in concentration 100 µg/ml. The same concentrations of PI in clots from patients' blood reduced lysis time from 98 to 60 minutes or for 54 to 76% *(Figure 2a)*. *Enzymes PI+PII* reduce lysis time of venous blood clots in healthy individuals to 105 min at the lowest concentration (1 pg/ml) and to 60 min at the highest concentration (100 µg/ml). The effect on the clots from the patients' blood reduced from 70 to 30 minutes or 77 to 86% *(Figure 2b)*. *Enzyme PII* effects fibrin clot lysis, in healthy individuals, in two ways. Concentrations from 1 pg/ml to 100 ng/ml slow down lysis by 8% in average, while concentrations from 100 ng to 100 µg increase by 7% (245 min) on average. PII added to fibrin clots of patients with malignant tumors does not slow down, but quickens lysis by 11 to 13% *(Figure 2c)*. *Figure 2d* shows the G-90 effect on clot lysis considering the malignant tumor location. Legend marks lines all starting from the value of 220 minutes in the case when G-90 was not added. With G-90, fibrinolysis lines show significant differences. The values depend not only on G-90 concentrations, but also on the organ involved in malignant process. Even with the lowest concentration (1 pg/ml) the lysis lines are spread around the mean value.

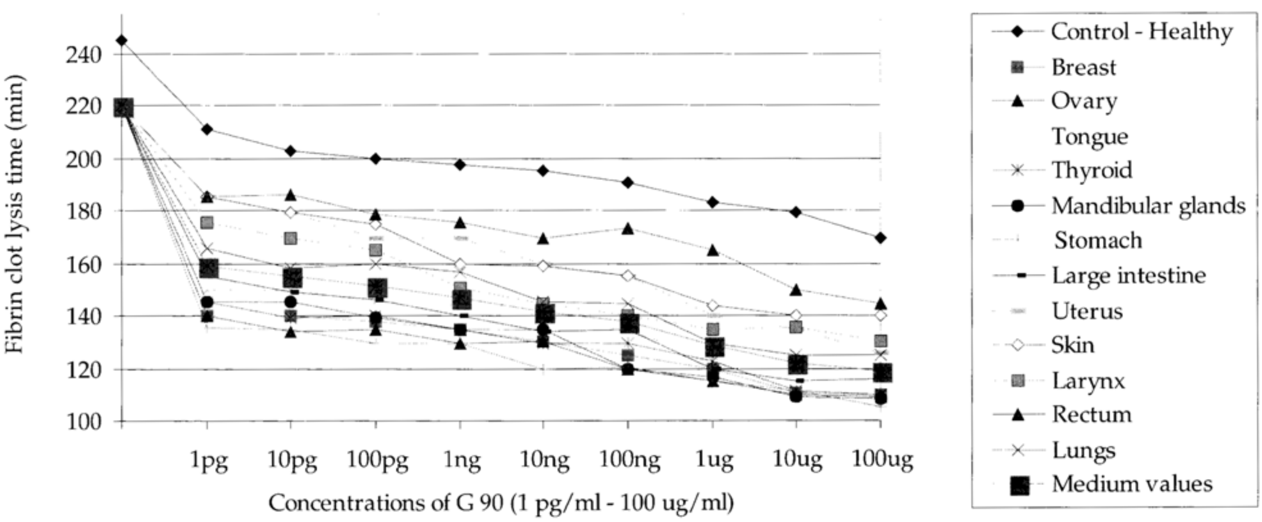

Figure 2d. *Effect of G-90 on results euglobulinic test of fibrin clot from plasma of patients with malignant tumors.*

Discussion

Each plasma sample in the *in vitro* euglobulinic test contains physiologically present u-PA, which lyses fibrin clot in a time that is constant for a particular species. As inhibitors of u-PA, stimulated by tissue factors from pathologically changed cells are synthesised, we do not expect the additional influence of plasminogen inhibitors; so fibrinolysis occurs within physiological time of lysis. However, u-PA synthesised and secreted in circulation by malignant tumors shortens significantly euglobulinic time.[5,7,22,25] Fibrinolytic activity factors applied in this study (G-90, PI, PII, and PI+PII), additionally quicken lysis and euglobulinic time is reduced proportionally with the concentration of added substances.[12,20] Quantity, and therefore respectively concentration, of the tumor u-PA depends on the type of cells that synthesise it. With this additional activator fibrinolysis is equally quicker for tumors of different organs and tissues. We suppose that this effect occurs due to the presence of inhibitors (PAI 1, PAI 2) which, in order to preserve homeostasis, link partly to u-PA in dissolvable complex u-PA/PAI. **Law** u-PA concentration search **laws** PAI and indicates good prognosis.[7] The additional lysis quickening followed the addition of fibrinolytics. The addition of PI, PII, (PI+PII) to fibrin clots from patients with carcinoma shortened clot lysis times, but they didn't differ according to location of pathologic process. However, by using G-90 as fibrinolytic we found significant differences in Iysis times, depending on the location of primary tumor process. Lysis time differences in euglobulinic test are the same as those quoted in the literature which shows, for instance, that a breast tumor secretes less u-PA than a large intestine tumor; and that the latter tumor secretes 10 times more u-PA than a healthy mucosa, but its activity is only five times higher. The u-PA concentration is not the same as its activity.[15] That means that the great part of tumor u-PA remained free and active, with lysis time shortening and bad prognosis as results.[5,28] In *in vitro* trials it was found that each of three types of cell lines of stomach carcinoma synthesised different quantities of u-PA.[26] Skin melanoma synthesises less u-PA than other tumors, and also in the euglobulinic test within this study it shows the longest lysis time.[28] Another interesting fact is that low PII concentrations inhibit fibrinolysis, while high concentrations quicken it.

Among the usual homeostasis tests on the material studied the prominent one is the fibrinogen (mg/dl) which is 15% higher. It was interesting that the increase of fibrinogen occurred in spite of *in vitro*, performed quicker lysis of clots from venous blood of patients with malignant tumors. It is obvious that the influence of G-90 on fibrinolysis rate does not depend only on its concentration, but the degree of fibrinolysis depends on histological type of tissue with the malignant tumor. We have every reason to suppose that the original G-90 contains also the structure with high affinity for PAI inhibitor. By linking it and separating from the complex uPAIPAI, u-PA is being released depending on G-90 concentration as same as on the degree of u-PA/PAI available. This activity seems to be specific for each tumor type, respectively each tissue involved in pathological process.

References

1. *Brommer EJP, Emeis JJ, Verheijen JH, et al:* Progress in Clinical Fibrinolysis. In: 7 Recent Advances in Blood Coagulation. (Eds. Poller L and Ludlam CA), Churchill Livingstone, 1997, pp. 161-182.
2. *Bugge TH, Flick MJ, Danton MJ, et al:* Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci USA 93:5899-5904, 1996.
3. *Cooper DL, Sandler AB, Wilson LD, et al:* Disseminated Intravascular Coagulation and Excessive Fibrinolysis in a Patient with Metastatic Prostate Cancer. Cancer 70:656-658, 1992.
4. *Driscoll J and Goldberg AL:* The proteasome (multicatalytic protease) is a component of the 1500 kDa proteolytic complex which degrades ubiquitin-conjugated proteins. J Biol Chem 265:4789-4792, 1990.
5. *Gandolfo GM, Conti L and Vercillo M:* Fibrinolysis Components as Prognostic Markers in Breast Cancer and Colorectal Carcinoma. Anticancer Res 16:2155-2160, 1996.
6. *Gabazza EC, Taguchi O, Yamakami T, et al:* Coagulation-Fibrinolysis System and Markers of Collagen Metabolism in Lung Cancer. Cancer 70:2631-2636, 1992.
7. *Gabazza EC, Taguchi O, Yamakami T, et al*: Evaluating prethrombotic state in lung cancer using molecular markers. Chest 103:196-200, 1993.
8. *Gailani D and Broze GJ:* Regulation of coagulation by tissue factor pathway inhibitor. In: 7 Recent Advances in Blood Coagulation. (Eds. Poller L and Ludlam CA), Churchill Livingstone, 1997, pp. 1-17.
9. *Hanss M, Bonvoisin C, Patouillard B, et al*: Increased plasma levels of urokinase type plasminogen activator during hepatocellular carcinoma. Fibrinolysis 8:255-260, 1994.
10. *Hrženjak T, Hrženjak M, Kašuba V, et al*: A new source of biologically active compounds-earthworm tissue (Eisenia foetida, Lumbricus rubelus). Comp Biochem Physiol 102A:441-447, 1992.
11. *Hrženjak M, Kobrehel D, Levanat S, et al*: Mitogenicity of the earthworm's (Eisenia foetida) insulin-like proteins. Comp Biochem Physiol 104B:723-729, 1993.
12. *Hrženjak T, Popović M, Božič T, et al*: Fibrinolytic and anticoagulative activities from the earthworm Eisenia foetida. Comp Biochem Physiol, in press, 1998.
13. *Inndorf S, Bechtel MJ, Reinartz J, et al:* Cell density-dependent downregulation of urokinase-type plasminogen activator in normal but not in ransformed human epidermal keratinocytes. Arch Dermatol Res 288,783-785, 1996.
14. *Jeon OH, Moon WJ and Kim DS:* An anticoagulant/fibrinolytic protease from Lumbricus rubellus. J Biochem Mol Biol 28:138-142, 1995.
15. *Kramer MD, Schaefer B and Reinartz J*: Plasminogen activation by human keratinocytes: molecular pathways and cell-biological consequences. Biol Chem 373:131-141, 1995.
16. *Massignon D, Lepape A, Bienvenu J, et al*: Coagulation fibrinolysis balance in septic shock related to cytokines and clinical state. Haemostasis 24:36-48, 1994.

17. *Mihara H, Sumi H, Mizumoto H, et al:* Oral administration of erthworm power as a possible thrombolytic therapy. Recent Advances in Thrombosis and Fibrinolysis (Ed Tanaka K): 287-298. Academic Press New York 1990.
18. *Mihara H, Sumi H, Yoneta T, et al:* A Novel fibrinolytic enzyme extracted from the earthworm Lumbricus rubellus. Jap J Physiol 41: 461-472, 1991.
19. *Nakajima N, Mihara H and Sumi H:* Characterisation of potent fibrinolytic enzymes in earthworm, Lumbricus rubellus. Biosci Biotech Biochem 57:1726-1730, 1993.
20. *Popović M, Tiska-Rudman LJ and Hrženjak T:* Tissue extract of earthworm Eisenia foetida (G-90) as a blood anticoagulant and fibrinolytic. Vet Arhiv 66:161-167, 1996.
21. *Popović M, Grdiša M, Vuković S, et al:* Adhesins of immunoglobulin-like superfamily from earthworm Eisenia foetida. Gen Pharmacol 30:795-800,1997.
22. *Prasad KSM, Sharma BS, Marwaha N, et al:* Haemostatic derangement in patients with intracranial tumours. Br J of Neurosurg 8:695-702, 1994.
23. *Sagripanti A, Capri A and Zacharski LR:* The pathophysiology of the haemostatic system in cancer patients: Insights gained from studies using plasmatic markers of haemostatic system activation. In: Cancer and Blood Coagulation: basic and clinical aspect. (Eds: Sagripanti A, Carpi A and Zacharski LR), Ets Editrice, 1990, pp 69-92.
24. *Schaefer BM, Stark HJ, Fusenig NE, et al:* Differential expression of urokinase-type plasminogen activator (uPA), its receptor (uPA-R), and inhibitor type-2 (PAI-2) during differentiation of keratinocytes in an organotypic coculture system. Experimental Cell Res 220:415-423, 1995.
25. *Salgado A, Boveda JL, Monasterio J, et al*: Inflammatory Mediators and Their Influence on Haemostasis. Haemostasis 24:132-138, 1994.
26. *See WA, Yong X, Crist S, et al:* Diversity and modulation of plasminogen activator activity in human transitional carcinoma cell lines. J Urology 151:1691-1696, 1994.
27. *Van-Duijnhoven EM, Lustermans FAT and Van-Wersch JWJ:* Evaluation of the coagulation/fibrinolysis balance in patients with colorectal cancer. Haemostasis 23:168-172, 1993.
28. *Wojtukiewich MZ, Zacharski LR, Memoli VA, et al*: Interaction with Coagulation and Fibrinolysis Pathways In Situ. Am J Clin Path 93:516-521, 1990.
29. *Zhu CL, Zhang GH, Duan Y, et al:* A clinical observation on the treatment of cerebral infarction with Eisenia foetida enzyme. Acta Acad Med Shanghai 20:447-450, 1993.
30. *Yang JS and Ru BG*: Purification and characterisation of a SDS-activated fibrinolytic enzyme from Eisenia fetida. Comp Biochem Physiol 118B:623-631, 1997.