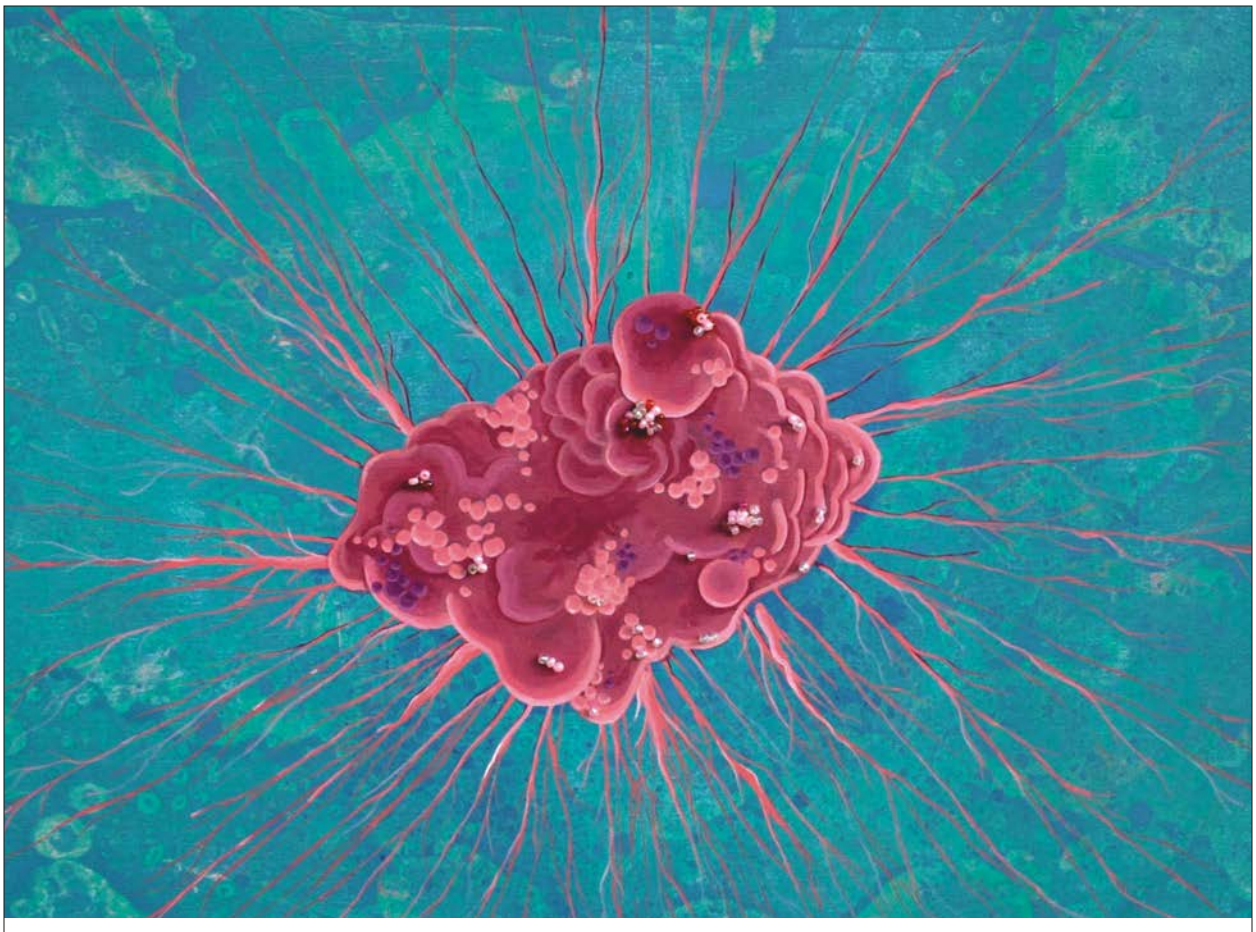

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Zinc Inhibits Tumor Metastasis by Regulating Plasminogen Activation

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Abstract: In this study, we found that zinc exerted a dual inhibition effect on plasminogen activator/plasmin system by inhibiting plasminogen activator activation and down-regulating plasmin activity. Zinc demonstrated significant inhibition effects on plasminogen activator and plasmin induced cell morphology change and movement. These results explain the anti-tumor and metastasis blocking effects of zinc observed in clinical studies. In addition, the strong down-regulation effects of zinc on plasminogen activator/plasmin system also suggest the potential role of zinc in other fibrinolysis associated diseases including neurodegenerative diseases.

Key words: Metastasis, zinc, cancer therapy, plasminogen activator

Introduction

Zinc, an essential trace element for growth, is required for the normal functioning of a large number of enzymes and is a structural component of many proteins. Zinc deficiency has a profound adverse effect on cellular biochemistry including the impairment of memory, growth retardation, frequent infections, delayed wound healing and depressed immune function (Shankar and Prasad, 1998). Zinc has also been reported to inhibit the growth of prostate (Shankar and Prasad, 1998; Huang *et al*., 2001; Uzzo *et al*., 2002; Liang *et al*., 1999), lymphoblastoid (Prasad *et al*., 2001) and colon (Jaiswal and Narayan, 2004; Park *et al*., 2002) tumors both *in vitro* and *in vivo*. For example, the malignant prostate tissue is characterized by a dramatic decrease in zinc level (~85%) as compared to extremely high zinc levels in normal and hyperplastic glands and zinc can inhibit growth and invasive capabilities of malignant prostate (Zowczak *et al*., 2001). Now it is clearly that intracellular zinc depletion results from down-regulated zinc uptake transporter (*ZIP1*) expression on prostate tumor cells and zinc inhibits the proliferation of malignant cells predominantly through suppression of NF-κB signaling (Jaiswal *et al*., 2004; Costello and Frankin, 2006). However, the possible mechanism for zinc blocked metastasis in prostate cancer is nevertheless not fully understood. On the other hand, it has been found that the activity of tissue-type plasminogen activator or plasmin correlates well with a poor prognosis in several cancers and inhibitors to plasminogen activators or plasmin can restrain tumor growth and improve the prognosis of cancer treatment (Baker and Leaper, 2003; Gonzalez-Gronow *et al*., 2005). Effects of zinc on plasminogen activator and plasminogen/plasmin system have not been investigated yet. The objective of this study is to test the effects of zinc on plasminogen activation and examine if zinc blocked tumor metastasis comes from inhibited plasminogen activation or plasmin activity.

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Materials and Methods

Materials

Human lys-plasminogen, plasmin, α-casein, H-D-Val-Leu-Lys p-nitroanilide (S-2251), H-D-Ile-Arg p-nitroanilide (S-2288) and Coomassie blue R-250 were purchased from Sigma (St. Louis, MO, USA). Two-chain t-PA was obtained from Genentech Inc. (South San Francisco, CA, USA).

Amidolytic Activity of t-PA

Amidolytic activity of t-PA was determined in wells of microtitration plates by using S-2288 as the chromogenic substrate. S-2288 was prepared in TBS buffer (10 mM Tris-HCl, 0.15 M NaCl, pH 7.4) containing 0.01% Tween 80. Unless otherwise stated, the final chromogenic substrate concentration in the assay mixture was 1.2 mM. To examine the effects of zinc on tPA activity, $ZnCl_2$ and t-PA were mixed in wells and then S-2288 was added. The initial rates of substrate hydrolysis at 25°C were determined by measuring the absorbance at 405 nm (A_{405nm}) at various time intervals by using a microplate reader (BioRad Laboratories, Richmond, CA, USA) and they were expressed as A_{405nm}/min.

Plasminogen Conversion Activity of t-PA

The t-PA mediated plasminogen activation was determined using chromogenic substrate 2251 as described previously. Briefly, plasminogen, t-PA and $ZnCl_2$ were all prepared in TBS buffer (10 mM Tris-HCl, 0.15 M NaCl, pH 7.4). Zinc solutions of various concentrations were mixed with t-PA in wells. After 10 min incubation at room temperature, S-2251 and plasminogen were added. The final concentrations of S-2251, plasminogen and t-PA were 0.8 mM, 0.05 U mL^{-1} and 10 μg mL^{-1}, respectively. The initial rates of substrate hydrolysis at room temperature were determined by measuring the absorbance at 405 nm using a microplate reader (Biorad, Richmond, CA, USA) at various time intervals. The results were expressed as ΔA_{405} min^{-2}. Conversion of plasminogen to plasmin was further confirmed by 10% SDS-PAGE.

Amidolytic Activity Assay for Plasmin

Plasmin, $ZnCl_2$ were mixed in wells first and S-2251 were added. The final concentrations of plasmin and S-2251 were 5 μg mL^{-1} and 0.8 mM. And the Zn 2+ concentrations were 0, 25, 50, 100, 200 μM, respectively. The initial rates of substrate hydrolysis at room temperature were expressed as ΔA_{405}/min. Amidolytic activity of plasmin was also measured by a pretylatic assay using α-Casein as substrate. In that experiment, α-Casein, plasmin and $ZnCl_2$ were mixed in eppendorf tubes and incubated at 37°C for 2 h. After that, reaction mixtures were analyzed using 10% SDS-PAGE.

Cell Scatter Assay

HT-29 cells (4×10^4 cm^{-2}) were seeded in 24-well plates and cultured in complete medium for 24 h. After that, cells were fed with serum-free medium. After 24 h culture, cells were treated with t-PA/plasminogen or plasmin in the presence or absence of Zn^{2+} for another 16 h. Cell morphology changes were then examined under microscopy and average cell cluster sizes were calculated.

Results and Discussion

Effects of Zn^{2+} on t-PA/plasminogen System

The overall effect of zinc on plasminogen/plasmin system was first examined *in vitro* using a small substrate system containing t-PA, plasminogen and small substrate S-2251. The reaction conditions had been optimized in our previous studies (Liang *et al*., 2000). Zinc concentrations

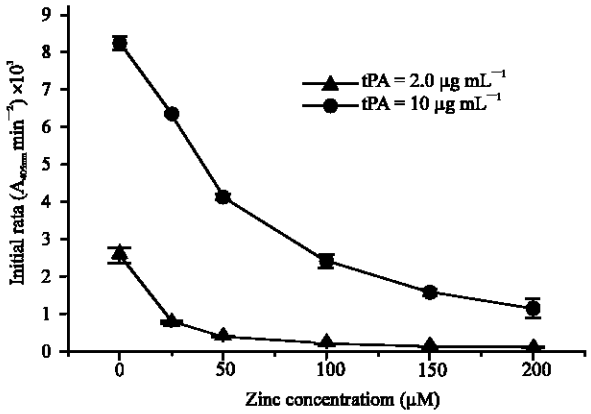

Fig. 1: Effect of Zn^{2+} on the tPA-mediated plasminogen activation. Amidolytic activities of plasmin produced by the reaction of tPA with plasminogen were measured using S-2251 as the substrate in the presence of various concentrations of zinc

(0 ~ 400 μM) in these experiments was determined by the zinc concentrations used in most clinical studies. Zn^{2+} exerted a profound inhibitory effect on S-2251 conversion even at very low concentration (25 μM) (Fig. 1). As t-PA concentration was increased from 2.0 to 10 μg mL^{-1}, the IC_{50} value of zinc was increased from 16 μM to about 45 μM correspondingly. Therefore, the possibility of inhibited amidolytic activity of t-PA/plasminogen system from plasminogen/zinc interaction could be excluded. Although S-2251 is a substrate of plasmin, the inhibited S-2251 conversion can also come from reduced plasmin production as a result of zinc inhibited tPA activity.

The possible interaction between t-PA and zinc was studied using a t-PA specific substrate, S-2288. Zinc clearly inhibited t-PA mediated S-2288 substrate conversation and dose-dependent inhibition curves could be obtained. However, inhibition curves from t-PA activity (S-2251) and amidolytic activity assay (S-2251) were different. Increasing zinc concentration above 100 μM would not result in further decrease in t-PA activity (Fig. 2A). This result was confirmed in followed plasmin production assays using SDS-PAGE (Fig. 2B). High concentration of zinc (higher than 200 μM) hardly produced any additional effects on t-PA mediated plasmin production, as justified from unchanged plasmin band intensity on the gel. Since reducing SDS-PAGE was used, two plasmin chains, which were linked by two disulfide bonds under physiological condition, were visualized as two close bands on the gel.

The interaction of plasmin and zinc was significant. Zinc demonstrated profound inhibitory effects on plasmin's activity in tested concentration ranges (Fig. 3). In the amidolytic activity assay using protein substrate, α-casein, α-casein degradation by plasmin could be totally abolished (99%) in the presence of 400 μM zinc (Fig. 3B). Obviously, zinc can down-regulate plasminogen activator/plasmin system through its interaction with both t-PA and plasmin. However, the inhibitory effect of zinc to plasmin is more profound and effective.

Effects of Zn^{2+} on Cell Morphology and Movement

Plasmin, through the direct degradation of proteins of the basement membrane and the extracellular matrix or the activation of zymogen forms of ECM-degrading metalloproteases such as interstitial procollagenase (matrix metalloproteinase-1) and prostromelysin (matrix metalloproteinase-3), plays a crucial part in tissue remodeling (Tsirka, 2002; Seeds *et al*., 1999; Indyk *et al*., 2003; Kim *et al*., 1999), angiogenesis and tumor metastasis (Baker and Leaper, 2003;

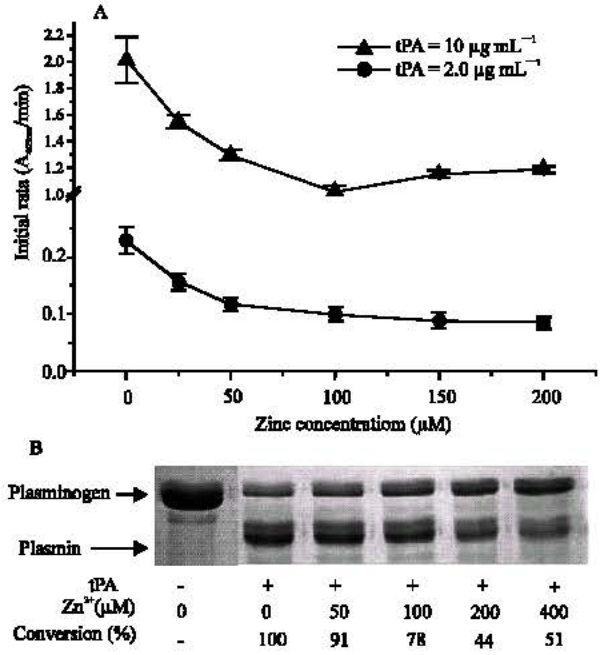

Fig. 2: Effect of Zn^{2+} on t-PA activity. The activity of t-PA was measured using small substrate 2288 (A) and by examining t-PA mediated plasminogen conversion into plasmin using reducing SDS-PAGE (B) in the presence of various concentrations of zinc

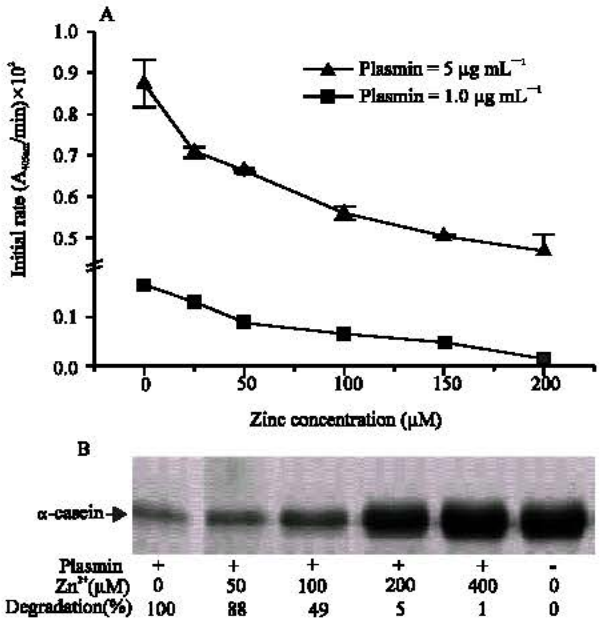

Fig. 3: Effect of Zn^{2+} on plasmin activity. The midolytic activity of plasmin was measured using small substrate 2251(A) and by examining the α-casein degradation using reducing SDS-PAGE (B) in the presence of various concentrations of zinc

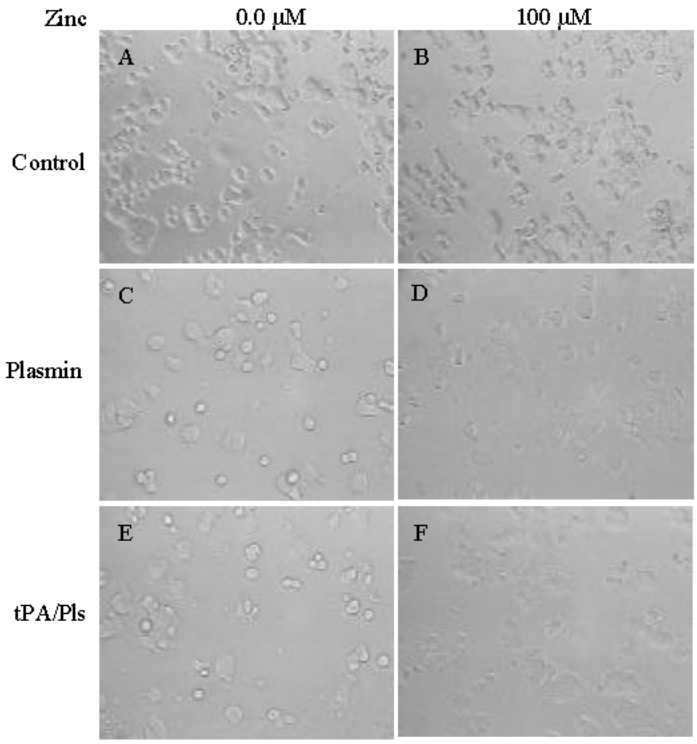

Fig. 4: Plasmin or t-PA/plasminogen induced cell morphology changes. Cells were incubated with blank medium (A, B), culture medium with plasmin (C, D) and culture medium with t-PA/plasminogen (E, F) in the absence (A, C, E) or presence (B, D, F) of 100 μM zinc for 16 h. Cell morphology was observed under microscopy

Gonzalez-Gronow *et al*., 2005). Effects of zinc on cell morphology and cell mobility were tested on cultured human colon carcinoma cells in the presence of plasmin or t-PA/plasminogen. In agreement with previous experiments, zinc did induce measurable and observable morphology changes, suggesting that zinc concentrations used in this experiment are safe to cells (Fig. 4A and 4B). In the presence of plasmin or plasminogen/t-PA, cells demonstrated a significant morphology change, from plat (Fig. 4A) to round (Fig. 4C and E) shapes. Such cell morphology changes were associated with significantly increased cell motility/scatting, as reflected by decreased cell numbers in fixed area (Fig. 5). Addition of zinc reversed plasmin or plasminogen/t-PA caused cell morphology change, from round to plat (Fig. 4D and F) shapes. In agree with this result, no significant cell cluster size changes could be observed if zinc was added with plasmin or plasminogen/t-PA, suggesting that plasmin or plasminogen/t-PA increased cell mobility was blocked by zinc (Fig. 5).

Taken together, the dual inhibition effect of zinc on plasminogen activator/plasmin system, inhibiting plasminogen activator activation and blocking plasmin activity, make zinc become a strong and effective inhibitor to plasminogen activator/plasminogen system. Present finding can explain the anti-tumor and metastasis blocking effects of zinc observed in clinical studies. In addition, the strong down-regulation effects of zinc on plasminogen activator/plasmin system suggest the potential roles of zinc in fibrinolysis and associated diseases including neurodegenerative diseases (Kim *et al*., 1999; Liang *et al*., 2005).

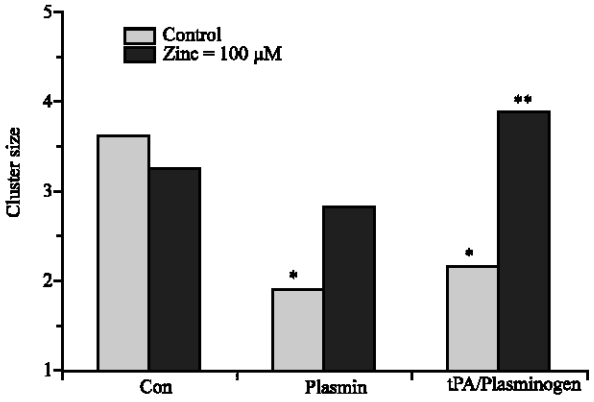

Fig. 5: Inhibition effect of Zn^{2+} on plasmin or t-PA/plasminogen induced cell mobility. Cells were incubated with blank medium, culture medium with plasmin and culture medium with t-PA/plasminogen in the absence (light grey) or presence (black) of 100 μM zinc. Cell clusters were counted and average cluster sizes were calculated after 16 h incubation. * $p<0.05$ compared to the control group without zinc; ** $p<0.05$ compared to the same treated group without zinc

Acknowledgment

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