CELL STRUCTURE AND FUNCTION 18: 355–362 (1993)


# Regulation of scu-PA secretion and u-PA Receptor Expression in Osteoblast-like Cells

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**Key words:** urokinase-type plasminogen activator (u-PA)/u-PA receptor, osteoblast/cyclic AMP (cAMP)/protein kinase C (PKC)

*ABSTRACT.* **The production of proteolytic emzymes by osteoblasts is considered important for initiating osteoclastic bone resorption. Using the established cell line NY as an example of osteoblast-like cells, the effect of intracellular cyclic AMP (cAMP) and protein kinase C (PKC) on plasminogen activator secretion and its specific binding to the cells were investigated. HT-1080 cells were used as the control. NY cells predominantly secrete single-chain urokinase-type plasminogen activator (scu-PA) and some two-chain u-PA. Both scu-PA and u-PA were present in the cell surface and cell lysate of NY cells, and their distribution in HT-1080 cells was quite similar to that of NY cells. Exposing cells to phorboi myristate acetate (PMA) or dibutyryl cyclic AMP (db cAMP) enhanced the secretion of scu-PA and two-chain u-PA, whereas 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) decreased scu-PA secretion, indicating that it is enhanced by protein kinase C (PKC) as well as by cAMP in NY cells. On the other hand, in HT-1080 cells, PMA decreased the level of two-chain u-PA secretion into the conditioned medium. The binding assay of $^{125}$I-DFP-u-PA to NY cells revealed the presence of a single class of binding sites with a $K_d$ of 2.23 nM and $B_{max}$ of $0.82 \times 10^6$ binding sites/cell. PMA however, altered neither the $K_d$ nor the $B_{max}$. Dibutyryl cAMP increased the $B_{max}$ 1.9 fold. Thus, NY cells secrete u-PA and express specific binding sites on the cell surface, which are modulated by cAMP and PKC. The u-PA/u-PA receptor system may contribute to osteoblastic bone resorption.**

Plasminogen activator (PA) is a serine protease which converts zymogen plasminogen to the active proteinase, plasmin. Plasmin digests fibrin as well as fibrinogen, and can also convert latent collagenase to the active enzyme (1). There are two classes of PA, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). The former has higher affinity for fibrin and enhanced enzymatic activity in the presence of fibrin or fibrin degradation products. Thus, t-PA is a specific thrombolytic agent that lyses intravascular fibrin deposits. On the other hand, u-PA does not have an affinity for fibrin and activates plasminogen regardless of its location.

The role of u-PA in tumor cells has been investigated from many perspectives: cell migration (2), tumor invasion (3, 4), tissue degradation (5), wound healing (6) and cell proliferation (7).

Recently, a specific receptor for u-PA has been demonstrated in several cell types, including endothelial (8) and neoplastic cells (9, 10, 11). The cDNA for the u-PA receptor in U937 cells has been identified (12) as well as the structure of the receptor itself. The receptor recognizes the amino-terminal fragment of u-PA via the first repeated sequence in the receptor (13). Initially, u-PA is secreted from the cells in a single-chain form, which binds to the receptor on the cell surface. After conversion to a two-chain form, u-PA remains on the cell surface by binding to the receptor, thus localizing the enzymatic activity. The localized PA activity is closely associated with cell migration (14), tissue destruction (15), tumor invasion (5) and metastasis (16). However, the precise distribution of u-PA in the intracellular fraction and cellular membrane remains unclear. Furthermore, the signal transduction that modulates the secretion of u-PA and its specific receptor have not been investigated with regard to bone resorption.

Production of proteolytic enzymes by osteoblasts is considered to be important in the initiation of osteoclastic bone resorption (17, 18, 19). We investigated u-PA secretion and its cell-surface specific receptor in osteoblast-like cells using the established NY cell line. The u-PA/u-PA receptor system has been extensively investi-

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gated in U937 cells, suspension culture, but not in adherent cells. Therefore, adherent, HT-1080 cells of fibrosarcoma origin were used as controls since they secrete u-PA and possess u-PA binding sites. Furthermore, the modulation of u-PA secretion and its receptor by protein kinase C (PKC) and cyclic AMP (cAMP) was investigated.

## MATERIALS AND METHODS

*Reagents.* The following materials were purchased from the sources indicated: L-Pyroglutamyl-glycyl-L-arginine-p-nitroanilide hydrochrolide (S-2444) (Kabi Vitrum, Stockholm), phorbol-12-myristate-13-acetate (PMA), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7) and 2-*o*-dibutyryladenosine-3,5-cyclic monophosphate sodium salt (dbcAMP) (Sigma, St. Louis, MO), MEM and RPMI-1640 (Nissui Seiyaku, Tokyo), fetal calf serum (GIBCO, Grand), iodogen (Pierce Chemical Co., Rockford, IL), Na-$^{125}$I (Amersham International, Bucks), plasminogen containing bovine fibrinogen (Organon Technika, Bostel), bovine thrombin (Mochida Pharmaceuticals, Tokyo). The purified high molecular weight two-chain urokinase-type plasminogen activator was a gift from Dr. M. Nobuhara (Mochida Pharmaceuticals, Tokyo). Antibodies against u-PA or t-PA were raised in rabbits as described elsewhere (20). The active-site of plasmin was titrated with p-nitrophenyl-p′-guanidinobenzoate as described by Chase and Shaw (21), and the plasmin specific activity was 20 CU/mg. All other reagents and chemicals were of the highest grade available.

*Cell cultures.* The human osteoblast-like cells NY established from osteosarcoma (22) was a gift from the Japanese Cancer Research Resources Bank (Tokyo). The human fibrosarcoma cell line HT-1080 (23) was purchased from the American Type Culture Collection (Rockville, MD, USA). NY cells were cultured in MEM, and HT-1080 cells in RPMI-1640, containing 10% fetal calf serum (FCS) for 48 h 37°C under a 5% $CO_2$ atmosphore.

*Electrophoretic enzymography.* Plasminogen activator activity and its molecular mass were analyzed by electrophoretic enzymography. Samples were obtained from conditioned medium, the cell surface and cell lysates as follows. Cultured NY and HT-1080 cells were washed twice with phosphate buffered saline pH 7.4 (PBS), once with MEM or RPMI-1640 without FCS then incubated with MEM or RPMI-1640 without FCS for 24 h. After harvesting the conditioned medium, u-PA bound to the cell membrane was recovered by incubating the cells with 50 mM glycine/HCl (pH 3.0) for 1 min in NY cells and 3 min in HT-1080, respectively. The pH was brought to 7.4 using 100 mM Tris/HCl (pH 8.0) (24) (This fraction is referred to below as "cell surface"). After removing the cell surface fraction, the cells were rinsed with PBS, lysed with 0.5% Triton X-100, then analyzed by electrophoretic enzymography, as described elsewhere (25).

*Measurements of u-PA activity.* NY and HT-1080 cells were seeded at $2 \times 10^5$ and $1.8 \times 10^5$ cells/well respectively, in 24-well multiplates. The u-PA activity was determined by means of a chromogenic substrate assay using S-2444. Both cell lines were washed with PBS and incubated with MEM or RPMI-1640 medium without FCS for 24 h. After harvesting the conditioned medium, the cell surface was obtained, and the cells were rinsed with PBS and lysed with 0.5% Triton X-100.

Amidolytic activity in the conditioned medium, the cell surface or cell lysate was measured as follows. The sample (80 $\mu$l) was first incubated with 20 ul of plasmin (1.0 ug/ml) or 50 mM Tris/HCl buffer (pH 7.4) with 0.01% (v/v) Tween 80 at 37°C for 30 min. Thereafter, 80 ul of 1 mM S-2444 was added to the reaction mixture. The optical density at 405 nm was measured using a 96 well MTP-120 MICROPLATE READER (Corona Electric, Tokyo, Japan) and recorded in a FTP-140WCL001 (Fujitsu, Tokyo, Japan).

The levels of scu-PA were determined as follows. The S-2444 amidolytic activities in the absence of plasmin and of the plasmin itself, were substrated from the S-2444 amidolytic activity of the sample in the presence of plasmin (1.0 $\mu$g/ml).

*Stimulation of cells with dbcAMP, PMA, H7 or butyrate.* Confluent NY cells or HT-1080 cells were washed with PBS, then the medium was changed to MEM or RPMI-1640 without FCS, respectively. The cells were stimulated for 16 h at 37°C with 10 mM dbcAMP, 100 nM PMA, 20 $\mu$M H7 or 10 mM butyrate. The u-PA activity and the levels of scu-PA in the conditioned medium, cell surface or cell lysate was measured as previously described. The u-PA activity or the levels of scu-PA was measured in quadruplicate in four or five different experiments, respectively, and the results were expressed as mean values with standard deviation.

*Measurements of u-PA antigen.* The amounts of u-PA antigen were determined using a u-PA ELISA kit (Monozyme, Virum, Denmark).

*Binding assay for u-PA to NY.* Purified high molecular weight two-chain u-PA was treated with diisopropyl fluorophosphate (DFP), and radiolabeled using iodogen and Na-$^{125}$I ($^{125}$I-u-PA). NY cells in 24-well multiplates were stimulated with 10 mM dbcAMP or 100 nM PMA at 37°C for 12 h. Thereafter, u-PA bound to the cell membrane was released using acid as previously described. $^{125}$I-u-PA (0–30 nM) with or without excess unlabeled u-PA (6,000 nM) was added to cells in 24-well multiplates. The binding reaction proceeded at 4°C for 90 min. The radioactivity levels in the conditioned medium and 0.5% Triton X-100 the cell lysate, were measured using a multi-crystal gamma counter LB-2100 (Berthold, Wikdbad, Germany). The u-PA binding to the cells was examined by Scatchard analysis (26).

## RESULTS

*Analysis of PA in NY conditioned medium and cell lysates.* Fig. 1 shows electrophoretic enzymography of the conditioned medium, cell surface and cell lysate of

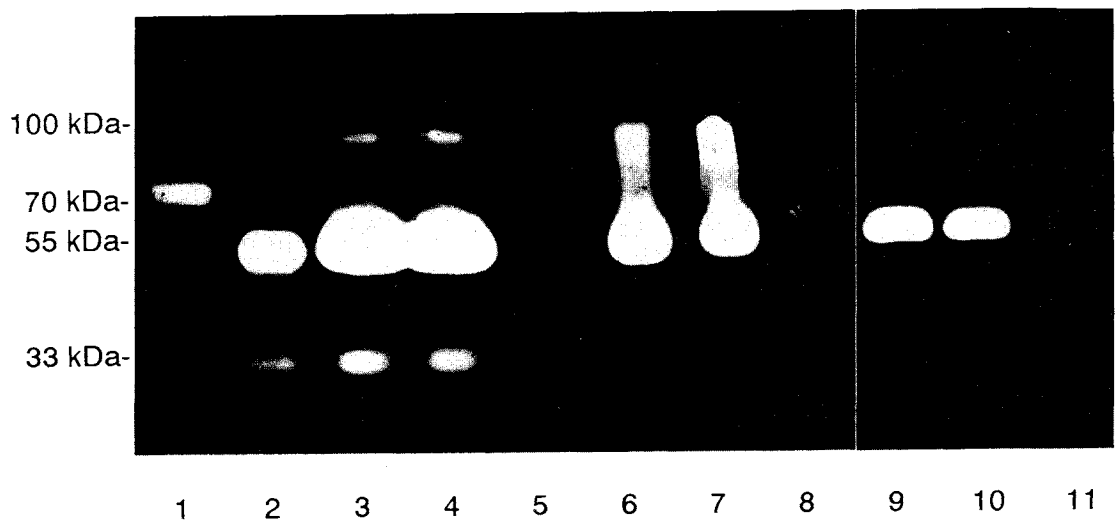


**Fig. 1.** Electrophoretic enzymography of the conditioned medium, cell surface and cell lysate of NY cells. Lane 1, t-PA; lane 2, u-PA; lane 3, conditioned medium; lane 4, conditioned medium treated with anti-t-PA IgG; lane 5, conditioned medium treated with anti-u-PA IgG; lane 6, cell lysate; lane 7, cell lysate treated with anti-t-PA IgG; lane 8, cell lysate treated with anti-u-PA IgG; lane 9, cell surface; lane 10, cell surface treated with anti-t-PA IgG; lane 11, cell surface treated with anti-u-PA IgG.

NY cells. The conditioned medium contained a major lysis band at Mr 55 kDa, and small lysis bands at Mr 33 and 100 kDa (lane 3). The cell lysate and cell surface contained a major lysis band at Mr 55 kDa (lanes 6 and 9). In the presence of anti-u-PA IgG, the clear band disappeared from the conditioned medium, cell surface and cell lysate (lanes 5, 8 and 11). Anti-t-PA IgG did not affect the lysis bands induced by the conditioned medium, cell surface and cell lysate of NY cells (lanes 4, 7 and 10).

There was u-PA activity not only in the conditioned medium and cell lysate, but also on the cell membrane of the osteoblast-like NY cells (Table I). After acid extraction, the bound u-PA activity was recovered as "cell surface" fraction. In NY cells, the u-PA activity on the cell surface measured as amidolytic activity, was similar to that of the conditioned medium. Thus, u-PA activity is located on the cell surface. In HT-1080 cells, the u-PA activity on the cell surface was almost half that found in the conditioned medium. The u-PA activity was greatest in the cell lysates of both cell lines.

**Table I.** Distribution of u-PA activity in NY and HT-1080 cells.

| | conditioned medium | cell surface | cell lysate |
|---|---|---|---|
| NY | 0.582±0.012# | 0.478±0.022 | 1.154±0.018 |
| HT-1080 | 0.970±0.023 | 0.465±0.093 | 1.171±0.023 |

# The results are expressed as the mean values with the standard deviation. The values represent the average of three experiments (n=4). Values indicate the optical density at 405 nm after a 24 h incubation.

Fig. 2 shows the time course of u-PA secreted in the conditioned medium. The increase in the level of u-PA activity was initially slow, but a remarkable increase appeared between 12–16 h in both NY and HT-1080 cells. Thereafter, the activity in both cell lines was almost constant.

*Characteristics of the secreted u-PA.* In order to confirm the presence of scu-PA, conditioned media, cell

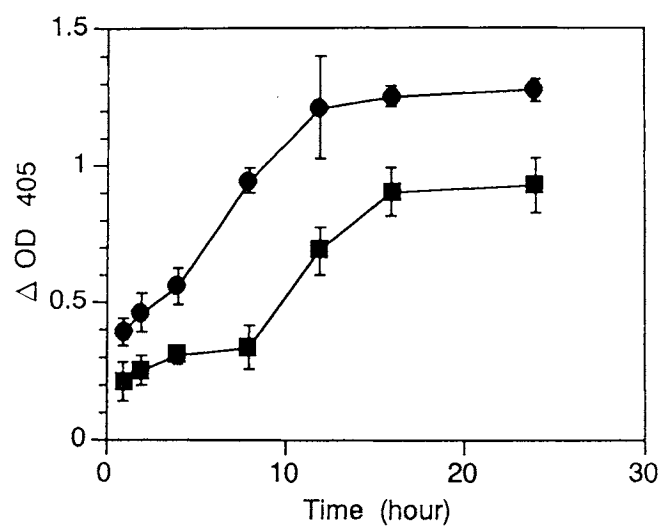


**Fig. 2.** The time course of u-PA secretion of the conditioned medium from NY and HT-1080 cells. NY or HT-1080 cells, $1.8 \times 10^5$ or $2.0 \times 10^5$ respectively, were cultured in MEM or RPMI-1640 medium without FCS for 0 to 24 hours at 37°C. (■) NY cells, (●) HT-1080 cells Ordinate; u-PA activity, abscissa; incubation time (hours).

**Table II.** LEVELS OF SCU-PA IN NY AND HT-1080 CELLS INCUBATED WITH PMA, H7 AND DBCAMP.

| | | conditioned medium | cell surface | cell lysate |
|---|---|---|---|---|
| NY | control | 0.143±0.016# | 0.022±0.003 | 0.135±0.007 |
| | PMA | 0.180±0.012** | 0.024±0.003 | 0.121±0.005* |
| | H7 | 0.088±0.007** | 0.025±0.002 | 0.143±0.006* |
| | dbcAMP | 0.188±0.013** | 0.045±0.004** | 0.138±0.006 |
| HT-1080 | control | 0.114±0.010 | 0.053±0.008 | 0.352±0.008 |
| | PMA | 0.125±0.050 | 0.050±0.004 | 0.366±0.004 |
| | H7 | 0.101±0.016 | 0.054±0.004 | 0.374±0.021 |
| | dbcAMP | 0.113±0.008 | 0.055±0.004 | 0.369±0.008 |

# The results are expressed as the mean values with the standard deviation. The values represent the average of three experiments (n=4). Values indicate the optical density at 405 nm after a 6 h incubation.
* $p<0.05$ ** $p<0.01$

surface or cell lysates were treated with and without plasmin and the u-PA activity was measured using S-2444 (see Materials and Methods for details). Single-chain u-PA was detected in the conditioned medium, cell surface and cell lysates of NY cells and HT-1080 cells (Table II). The levels of scu-PA in the conditioned medium in NY cells were higher than that of HT-1080 cells. However, on the cell surface and cell lysate, the levels of scu-PA in NY cells were less than half those of HT-1080 cells. Thus, scu-PA was detected in all three samples from both NY cells and HT-1080 cells.

*The effect of cAMP or PKC on secretion of u-PA.* To clarify the effect of cAMP or PKC on the secretion of u-PA, the cells were incubated with dbcAMP, PMA, H7 or butyrate, and the results were expressed as a ratio of the control level (Fig. 3). In NY cells (A), dbcAMP remarkably enhanced the secretion of u-PA into the conditioned medium. Butyrate did not affect u-PA secretion. PMA, an activator of PKC, enhanced the secretion of u-PA. However, H7, an inhibitor of PKC, did not inhibit the secretion of u-PA. The amount of u-PA bound on the cell surface was increased by dbcAMP and not affected by PMA, H7 or butyrate. The intracellular u-PA was decreased remarkably by dbcAMP and slightly by PMA. H7 and butyrate had no effect on intracellular accumulation of u-PA.

In HT-1080 cells (Fig. 3B), PMA significantly decreased u-PA activity in the conditioned medium, whereas H7, dbcAMP or butyrate has no significant effect. On the other hand, dbcAMP significantly decreased intracellular u-PA accumulation. PMA inhibited the intracellular accumulation of u-PA, but H7 did not affect it.

The effects of these reagents on the secretion of scu-PA were then investigated (Table II). In NY cells, the levels of scu-PA in the conditioned medium was significantly increased by stimulation with PMA and dbcAMP, but decreased significantly with H7. On the cell surface, scu-PA was increased two fold by dbcAMP. In the cell lysate, PMA significantly decreased scu-PA and H7 increased it. In HT-1080 cells, the levels of scu-PA were not changed by stimulation with PMA, H7 or dbcAMP in the conditioned medium, cell surface or cell lyaste.

The u-PA antigen in the conditioned medium was measured by means of an ELISA using an antibody that reacts with both u-PA and scu-PA (Table III). In NY cells, the amount of u-PA antigen was significantly increased by stimulation with PMA or dbcAMP, but significantly decreased by H7. In HT-1080 cells, u-PA antigen was decreased by PMA stumulation, but H7 and dbcAMP had no effect.

*Binding assay.* The binding of u-PA to NY cells was analyzed using $^{125}$I-DFP-u-PA (Fig. 4). The specific binding of u-PA to the cells was obtained by substract-

**Table III.** AMOUNTS OF U-PA ANTIGEN IN NY AND HT-1080 CELLS INCUBATED WITH PMA, H7 AND DBCAMP.

| | control | PMA | H7 | dbcAMP |
|---|---|---|---|---|
| NY | 10.09±0.05# | 11.91±0.41* | 9.40±0.13* | 14.50±0.06** |
| HT-1080 | 23.44±1.22 | 20.06±1.61* | 22.89±0.20 | 22.85±1.72 |

# The results are expressed in ng/ml/$2.0\times10^5$ cells for NY and ng/ml/$1.8\times10^5$ cells for HT-1080 cells. These are mean values with the standard deviation. The values represent the average of three experiments (n=4). * $p<0.05$ ** $p<0.01$

**Table IV.** THE BINDING PARAMETERS OF $^{125}$I-DFP-U-PA TO NY CELLS INCUBATED WITH PMA OR DBCAMP.

| | $K_d$ (nM) | $B_{max}$ (binding sites/cell) |
|---|---|---|
| control | 2.33±0.61# | $(0.82\pm0.08)\times10^6$ |
| PMA | 2.15±0.89 | $(0.98\pm0.19)\times10^6$ |
| dbcAMP | 2.24±0.54 | $(1.54\pm0.17)\times10^{6*}$ |

# The results are expressed as the means of values with the standard deviation. The values represent the average of five experiments (n=3). * $p<0.05$

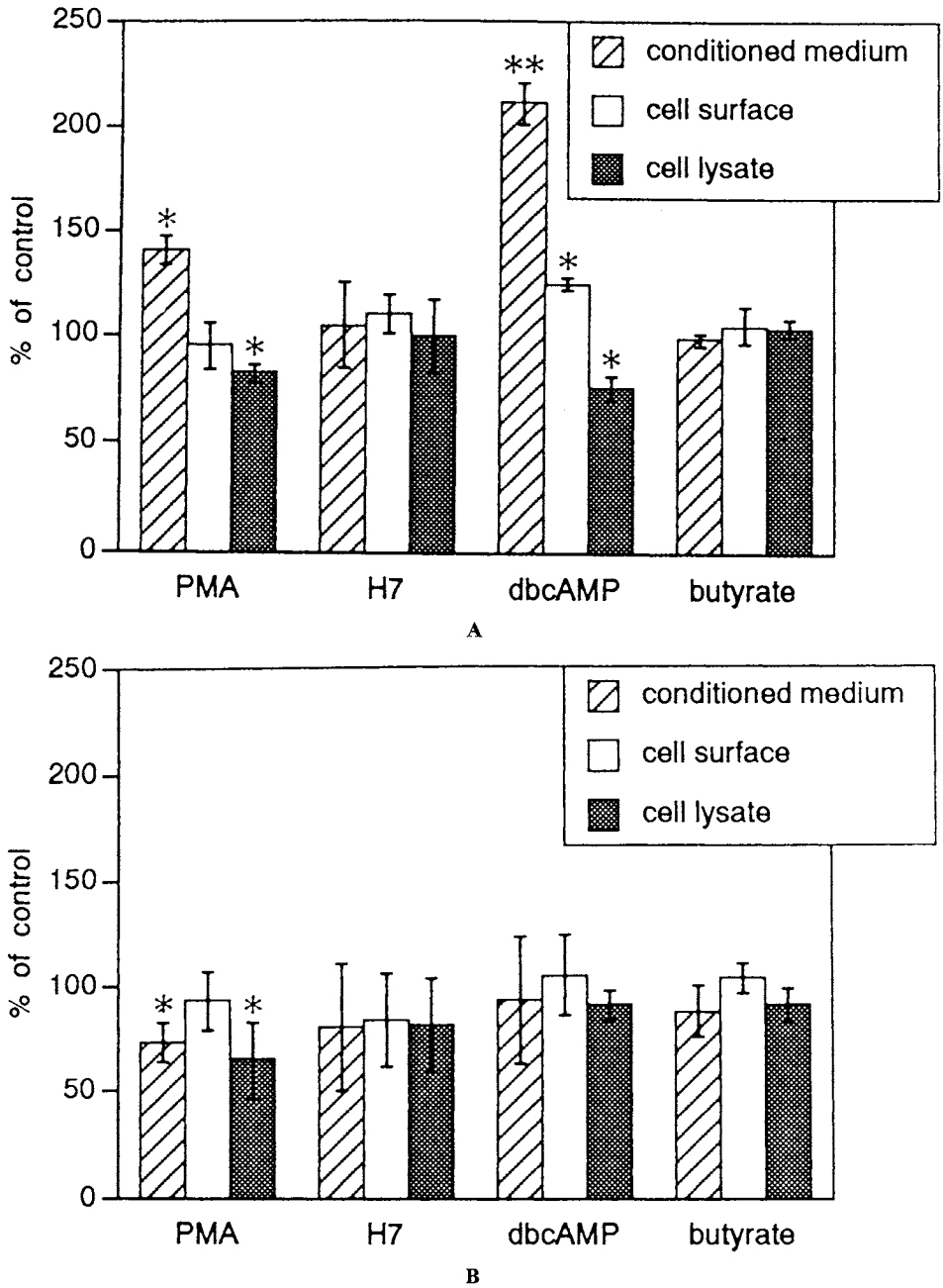


**Fig. 3.** The u-PA levels in the conditioned medium, cell surface and cell lysate of NY (Fig. 3A) and HT-1080 cells (Fig. 3B). Ordinate, Values in the presence of reagents are expressed as % control. (* $P<0.05$, ** $P<0.01$)

ing the non-specific binding from the total binding and the nature of the binding was investigated by means of a Scatchard analysis. Since a single line was obtained, this indicated that NY cells possess one type of binding site for u-PA. The binding parameters are shown in Table IV. After stimulating NY cells with dbcAMP, the $K_d$ was not changed, but the $B_{max}$ increased 1.9 fold. When NY cells were stimulated by PMA, both the $K_d$ and $B_{max}$ tended to increase, but not significantly so.

## DISCUSSION

Several cytokines or growth factors are involved in bone resorption (27, 28). Some of them enhance PA production, and others are protease inhibitors. Thus bone resorption is regulated by the balance between PA and protease inhibitors (18, 19, 29). Advances in pericellular proteolysis have identified a regulatory mechanism based upon a specific u-PA receptor (10, 12, 14, 30).

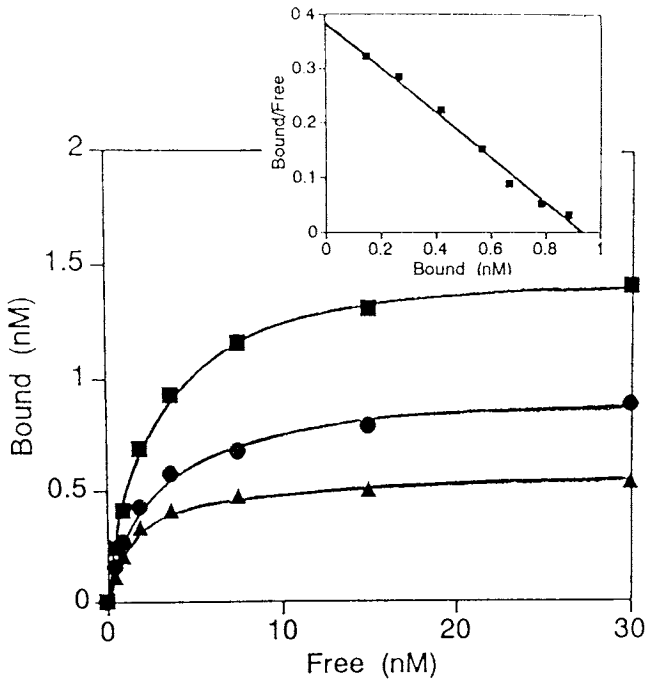


**Fig. 4.** Binding of $^{125}$I-DFP-u-PA to NY cells. NY cells, $1.8 \times 10^5$, were cultured in MEM medium without FCS for 24 hours at 37°C. (■) total binding, (▲) non-specific binding, (●) specific binding The inset shows a Scatchard analysis.

Therefore, as a model of bone resorption, the mode of production as well as secretion of PA and its receptor were investigated in the osteosarcoma cell line, NY. HT-1080 cells were used as the control, as they are adherent and they secrete u-PA.

The distribution of u-PA was examined not only in the conditioned medium and cell lysate, but also on the cell surface, to clarify its production and secretion. The u-PA bound to the cell membrane was released by acid treatment of the cells, and the recovered u-PA designated "cell surface" was measured. As shown in Table I, u-PA activity was observed in the conditioned medium, cell surface and cell lysate. Therefore, it is assumed that u-PA is produced intracellularly, transported to cell surface and/or secreted into the conditioned medium. Since u-PA is in the two-chain form, the presence of its precursor, single-chain u-PA (scu-PA) was examined. As shown in Table II, scu-PA was identified in the three fractions. These results indicate that NY cells produce scu-PA and store it. Some scu-PA is transported to the membrane fraction, and released into the conditioned medium. Some scu-PA in all fractions is activated into the two-chain form. Activation of intracellular scu-PA to u-PA is induced by intracellular proteases including plasmin, cathepsins B/D, kallikrein and others (31). The production and transport of scu-PA in NY cells in not different from that of HT-1080 cells.

The intracellular signal transduction involved in u-PA secretion was investigated by altering the levels of PKC or cAMP. PMA, which elevates intracellular PKC (32), increased the secretion of u-PA in NY cells. This phenomenon has been observed in other cell lines (33, 34, 35). H7 decreased u-PA secretion in NY cells. This also occurs in human renal epithelial cells (36). Similarly, exposing human umbilical vein endothelial cells to H7 decreased t-PA levels (37). Since H7 inhibits protein kinases C and A, two protein kinases may be involved in the inhibition of u-PA secretion. However, as the elevated PKC induced the increased secretion of u-PA, it is assumed that PKC is deeply involved in the modulation of u-PA secretion. In control HT-1080 cells, PMA inhibited the secretion of two-chain u-PA, but other reagents had no effect. Therefore, the secretion of u-PA in HT-1080 cells is not regulated by the same mechanism as in NY cells. The increase in intracellular cAMP by dbcAMP (38) enhanced u-PA secretion in NY cells. PA is induced by increasing levels of cAMP in the mouse mammary carcinoma cell line, SC115 (35) and in LLC-PK1 (39), but not in granulosa cells from hen ovary follicles (40). Thus, scu-PA secretion in NY cells is regulated by PKC and cAMP, but that of scu-PA in HT-1080 cells is regulated by a system other than that of PKC and cAMP.

$^{125}$I-DFP-u-PA was incubated with the cells after acid treatment, which exposes whole receptors on the cell membrane. $^{125}$I-DFP-u-PA bound NY cells specifically and saturably (Fig. 4), indicating the presence of a specific u-PA receptor. As a single straight line was obtained by the Scatchard analysis, this indicates that one type of receptor is present on the cell membrane. The number of receptors for u-PA was not affected by PMA, as seen for U937, but enhanced 1.9 fold by dbcAMP. The $K_d$ value was not changed by either PMA or dbcAMP. Therefore, PMA enhances scu-PA secretion, but does not affect the u-PA receptor. In HT-1080 cells, the $K_d$ value was close to that of NY cells, but the $B_{max}$ was about three times larger than that of NY cells ($K_d$ was $2.97 \pm 0.91$ nM, and $B_{max}$ $(2.15 \pm 0.42) \times 10^6$ binding sites/cell). In U937 cells, PMA increased the number of scu-PA receptors about 10 fold (41, 42). This difference in the induction of u-PA receptor between NY and U937 cells may be derived from the difference in the cell types; the former is of osteosarcoma origin in adherent culture, and the latter, a monocyte/macrophage cell line in suspension culture. Thus, in the osteosarcoma cell line, pericellular enzymatic activity is enhanced by binding scu-PA to the specific receptor expressed on the cell surface.

Several reports (18, 19, 29, 43) have indicated that the results obtained from osteosarcoma cell lines were similar to those from osteoblast-like cells from calvaria. Therefore, the regulation of scu-PA and u-PA receptor

in NY cells may represent the physiological mode of their regulation in osteoblasts. Thus, the osteosarcoma cell line NY secretes scu-PA, which binds to u-PA receptor on the cell membrane and expresses u-PA activity. This enzymatic activity on the cell membrane may contribute to bone resorption.

*Acknowledgments.* The present studies were supported in part by the Ministry of Education (03670045 and 04670057), the Yasuda Research Foundation and the Osaka Cancer Society.

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*(Received for publication, June 7, 1993*
*and in revised form, October 27, 1993)*