Inter-α-trypsin inhibitor (ITI), a human serum protease inhibitor of molecular mass 240 kDa which may release physiological derivatives, has been shown to interact with hyaluronic acid (HA), resulting in pericellular matrix stabilization (Chen, L., Mao, S. J. T., McLean, L. R., Powers, R. W., and Larsen, W. J. (1994) J. Biol. Chem. 269, 28282–28287). The purpose of this study is to determine whether ITI binding to tumor cell surface is mediated by urinary trypsin inhibitor (UTI) receptor or cell-associated hyaluronic acid (HA). We demonstrated specific complex formation of the heavy (H) chains of ITI with HA. Binding of the H-chains of ITI to immobilized HA was detected and quantified using colorimetric immunoassays. Binding was time-, temperature-, and concentration-dependent. However, UTI and HI-8 (the carboxyl terminus of UTI) failed to bind to immobilized HA. ITI bound to HA remained functional protease inhibitory activity. After incubation of SMT-cc1 cells with purified biotinylated ITI, biotinylated ITI is bound to the cells, dissociated, and gives rise to the H-chains and UTI on the cell surface. The cell surface receptor-bound UTI derived from ITI may be the result of the limited proteolysis on the cell surface. In the cells treated with hyaluronidase, bound H-chains disappeared from the surface of the cells, while most of the cell surface ITI derivatives was present in deglycosylated UTI (28 kDa). It is suggested that the binding of ITI to the cell surface is mediated by HA on the cells. This was confirmed by the fact that the hyaluronidase-treated cells can abolish the ITI binding. The cell surface UTI formation was inhibited by disopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and eglin C, suggesting that elastase-like enzyme(s) may be responsible for the UTI formation. Preincubation of the cells with UTI did not decrease in exogenously added ITI on the cell surface. A model for cell surface UTI formation is proposed in which ITI binding to cells from serum used for the culture is followed by the limited proteolysis by trace amounts of active serine proteases, to form cell-surface receptor-bound UTI and the H-chains intercalated into cell surface HA. This process is subject to regulation of cell-associated UTI and of stabilization of pericellular matrix.

Inter-α-trypsin inhibitor (ITI) is the precursor of urinary trypsin inhibitor (UTI), which is one of the Kunitz-type protease inhibitors present in human serum and urine (1–4). ITI was found to act as a stabilizer of pericellular matrix. The serum factor, identified as an ITI, is a structural component of the matrix. ITI is required to stabilize the fully expanded cumulus cell-oocyte complexes matrix, thus supporting the process of ovulation (5). Huang et al. (6) showed previously that hyaluronic acid (HA) synthesized by cultured fibroblasts firmly bound 85-kDa proteins, which were derived from serum used for the culture and appeared to be covalently linked to HA. The serum-derived HA-associated protein was confirmed to be the heavy (H)-chains of ITI, suggesting that cooperative binding to HA of the H-chains of ITI is required to stabilize the pericellular matrix (7).

Hyaluronic acid is a glycosaminoglycan of the extracellular matrix in most mammalian tissues. It consists of a linear polysaccharide chain with repeating glucuronic acid-(1→3)-N-acetylglucosamine-(1→4) structure. It is bound by cell surface receptors and extracellular matrix proteins (8). HA is also believed to play an important role in controlling tumor cell growth, migration, invasion, and differentiation. Synthesis and degradation of HA are significantly related to these cellular functions such as cell proliferation. Yoned a et al. (9, 10) have shown that HA added exogenously or supplied endogenously by increased synthesis may act as a modulator of fibroblast proliferation.

We showed previously that highly purified human UTI efficiently inhibits soluble and tumor cell-associated plasmin and subsequently prevents tumor cell invasion and metastasis (11). Inhibition of cell-bound plasmin by UTI is associated with significantly reduced tumor cell invasiveness in vitro and a decreased number of metastasis in vivo (11). Recently we found that some tumor cells have specific binding sites for UTI on the cell surface (12). Exogenously applied UTI may be bound to specific binding sites on the surface of tumor cells. This potentially leads to the build up of a substantial amount of UTI at the surface of the tumor cells (12). There is good evidence that UTI may play an important role in prevention of tumor cell invasion and metastasis (13, 14).

In the present study, we investigated whether ITI binding to tumor cell surface is mediated by UTI receptors or by cell-associated HA. Available information on the interaction of ITI derivatives with cells or with immobilized HA is mainly based on the use of biotinylated compounds as ligands. We show that binding of ITI to the cell-associated HA, but not to the UTI receptor, may be essential to the formation of the aggregates comprising the H-chains of ITI and HA which constitutes pericellular matrix.

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†To whom correspondence should be addressed. Fax: 81-53-435-1626.
‡The abbreviations used are: ITI, inter-α-trypsin inhibitor; UTI, urinary trypsin inhibitor (the light chain of ITI); BSA, bovine serum albumin; FCS, fetal calf serum; HA, hyaluronic acid, H-chains, the heavy chains of ITI; HI-8, the domain II of UTI; PBS, phosphate-buffered saline; HLE, human leukocyte elastase; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; HRP-HABP, peroxidase-conjugated hyaluronic acid-binding protein; ELISA, enzyme-linked immunosorbent assay.
MATERIALS AND METHODS

Polyconal Antibody against ITI—Rabbits were immunized with 2 mg of purified ITI in complete Freund's adjuvant followed by booster injections in incomplete Freund's adjuvant every 3 weeks. The serum was purified by protein A chromatography according to the instructions of the manufacturer (Pierce). Polyconal antibody against ITI (anti-ITI antibody) reacts with native ITI and all of its derivatives including H-chains, UTI, and HI-8. This antibody was used in saturating concentrations.

Cells and Cultures—Promyeloid leukemia U937 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) (15). The choriocarcinoma cell line SMT-cc1 was established from a pulmonary metastatic region of human gestational choriocarcinoma (16). These cells were maintained under an atmosphere of 5% CO2 in RPMI 1640 (Nissui, Tokyo) medium supplemented with 10% FCS. SMT-cc1 cells were grown to confluency, removed from the flask by adding 4 mM EDTA and 0.01% DNase solution for 2 min at 23°C, followed by gentle tapping of the tray against the bench. Complete detachment was confirmed by direct visualization. Single-cell suspensions were made by repeated pipettings through a 0.4-mm-diameter canula. After this, the cells could be kept in suspension at 4°C by shaking. The cell viability was determined by trypan blue dye-exclusion prior to use. SMT-cc1 cells were also used for binding studies.

SMT-cc1 cell monolayers or U937 cell suspensions (1 × 10⁶ cells/ml) were grown to confluency, removed from the flask by adding 4 mM EDTA and 0.01%DNase solution for 2 min at 23°C, followed by gentle tapping of the tray against the bench. Complete detachment was confirmed by direct visualization. Single-cell suspensions were made by repeated pipettings through a 0.4-mm-diameter canula. After this, the cells could be kept in suspension at 4°C by shaking. The cell viability was determined by trypan blue dye-exclusion prior to use. SMT-cc1 cells were also used for binding studies.

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Preparation of Inter-α-trypsin Inhibitor (ITI) and Its Derivatives—Human ITI was isolated from serum according to Salier et al. (17, 18). A purified preparation of human UTI with an activity of 2330 units/mg of protein and a molecular mass of 40 kDa (by SDS-PAGE) was kindly supplied by Mochida Pharmaceutical Co., Tokyo, Japan. The covalent structure of the polypeptide chain of the physiological inhibitor ITI has been already determined (2, 4). A trypsin-Sepharose affinity column was used in order to obtain purified HI-8 (domain II of UTI). HI-8 was subjected to preparative gel electrophoresis. Eluted material was determined by amino-terminal amino acid sequence using automated amino acid sequencer (23) and analyzed by SDS-PAGE followed by Western blot using avidin-peroxidase.

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RESULTS

Immunoprecipitation of ITI Bound to Tumor Cells—After addition of purified biotinylated preparations of human ITI to cultures of SMT-cc1 cells growing in a medium containing ITI-depleted serum, biotinylated ITI could be recovered as a

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Inter-α-trypsin Inhibitor on the Cell Surface
membrane-associated ITI was dissociated and gave rise to the H-chains of ITI and UTI, were also detected by immunoprecipitation from bound fraction from the cells by an immunoprecipitation method (Fig. 1, lane 1). Some ITI derivatives, which may correspond to the H-chains of ITI and UTI, were also detected after a 12-h incubation with biotinylated ITI at 37 °C (Fig. 1, lane 2). Membrane-associated ITI was dissociated and gave rise to the H-chains and UTI after a 16-h incubation (Fig. 1, lane 3).

SMT-cc1 cell monolayers were treated with ITI-depleted serum containing 2 µM biotinylated ITI and incubated for 16 h at 37 °C. After the cells were washed twice with PBS, 0.1% BSA, the cell monolayers were incubated in serum-free medium for 16 h at 37 °C. Then, the cells were treated with RPMI 1640 medium containing ITI-depleted serum plus biotinylated ITI (2 µM) and incubated for 3 h (lane 1), 12 h (lane 2), and 16 h (lane 3) at 37 °C. Then, the cells were washed and homogenized. Anti-ITI antibody coupled to Sepharose (50 µl) was incubated with cell lysate supernatants (from 5 x 10^6 cells). Immobilized immunocomplexes were analyzed by 12% SDS-PAGE, followed by Western blot using avidin-peroxidase. a, ITI; b, H-chains (HC-1, HC-2, and HC-3); c, UTI. Lane M, molecular masses of standards are indicated.

Hyaluronidase will not digest the whole glycosaminoglycan chain, whose removal results in a shift of the UTI band to 28 kDa as well as minor bands of 70 kDa, 60 kDa, and 50 kDa (Fig. 3). In addition, soluble UTI purified from human urine (25 µg/ml, 30 min, 37°C) was incubated with cell lysate supernatants, and a new band (deglycosylated UTI, molecular mass = 28 kDa) appeared. It has been reported that the 28-kDa form is the deglycosylated UTI. UTI contains a low-sulfated chondroitin 4-sulfate chain, and its apparent molecular mass upon SDS-PAGE shifts from 40 kDa to 28 kDa upon chondroitinase ABC or hyaluronidase treatment (26). SDS-PAGE of UTI yields a molecular mass of 40 kDa, whereas the amino acid sequences yield values of 15–16 kDa. The discrepancy between these value is due to the chondroitin sulfate chain, whose removal results in a shift of the UTI band to 28 kDa. Hyaluronidase will not digest the whole glycosaminoglycan chain but will leave the innermost disaccharide repeat and the linkage region intact.

Although deglycosylated UTI could be recovered from the cell lysate, the H-chains of ITI could not be detected in the immunoprecipitable proteins (Fig. 2). It is likely that the serum-derived HA-associated proteins may be the H-chains of ITI, but without the light chain.

After being treated with hyaluronidase, cell-associated ITI and the H-chains disappeared. This means that H-chains were released into the medium by hyaluronidase treatment. In a parallel experiment, soluble ITI purified from human serum was incubated with hyaluronidase at the same conditions as described above (20 µg/ml, 30 min, 37°C). This treatment did not cause the complete disappearance of the 240-kDa band and the appearance of several new bands, major ones of 80 kDa and 28 kDa as well as minor bands of 70 kDa, 60 kDa, and 50 kDa (Fig. 3). In addition, soluble UTI purified from human urine was incubated with hyaluronidase at the same conditions. This treatment caused the incomplete disappearance of the 40-kDa band and the appearance of new bands (molecular mass of 60 kDa and 28 kDa). Broad staining with several bands shown in Fig. 3 (lane 2, d) may represent the difference in the extent of deglycosylation.

We speculated that the cell surface UTI might have been derived from ITI bound on the cell surface, but not from UTI formed in the medium and subsequently bound to the cells. It was confirmed by Western blot analysis that biotinylated ITI does not contain UTI fragments. In a parallel experiment, the cells were treated with the biotinylated ITI (0.2 µM) in the presence of an excess of unlabeled ITI (2 µM). Under this condition, H-chains and UTI could not be detected in the immunoprecipitable proteins (Fig. 4).

To test which enzymes were responsible for the cell surface ITI cleavage, we added biotinylated ITI to the serum-free culture medium of SMT-cc1 cells in the presence or absence of several protease inhibitors including diisopropyl fluorophosphate, PMSF, aprotinin, leupeptin, pepstatin, cystatin, eglin C, and E64. Several ITI derivatives were found even in the presence of aprotinin, leupeptin, pepstatin, cystatin, and E64. How-
H-chains of ITI appear to bind specifically to HA.

In the next experiment, we investigated whether ITI bound to HA has functional protease inhibiting activity. The relative enzyme activity obtained when trypsin, plasmin, or elastase was titrated with ITI bound to immobilized HA was measured (Table 1). ITI bound to HA also strongly inhibited trypsin, plasmin, and elastase activities. Inhibition constant (K_i), a measure of the stability of protease inhibition, varied with a difference of more than 10 between trypsin and plasmin. Trypsin exhibited the most stable inhibition as well as the greatest association rate (Table 1).

Recently, the heavy chain 2 of ITI (HC2) has been shown to be easily degraded by HLE whereas HC1 is more resistant (27). The molecular mass of fragments derived by limited proteolysis of UTI with HLE was 22 kDa (13). The 22-kDa protein does not appear to be degraded further in lower molecular mass forms, despite prolongation of the incubation time or increased protease concentrations, indicating that the 22-kDa protein is resistant to further degradation (13). The 22-kDa protein inhibited HLE with essentially the same affinity as native UTI. ITI-enzyme complexes can dissociate, which allows for a cleavage of

FIG. 4. Immunoprecipitation of ITI in cells saturated with biotinylated ITI in the presence of an excess of unlabeled ITI. The experiment was performed as described in the legends to Figs. 1 and 2. SMT-cc1 cell monolayers were incubated with biotinylated ITI (0.2 μM) in the presence of unlabeled ITI (0, 0.5, and 2.0 μM) in RPMI 1640 medium containing ITI-depleted FCS for 16 h at 37°C. Anti-ITI antibody-coupled Sepharose 4B was incubated with the supernatants of cell lysates (16 h, 4°C) and immunoprecipitated.

FIG. 5. Immunoprecipitation of ITI in SMT-cc1 cell layers saturated with biotinylated ITI in the presence of protease inhibitors. SMT-cc1 cell monolayers were treated with 2 μM biotinylated ITI and incubated for 16 h at 37°C in the presence or absence of diisopropyl fluorophosphate (1 mM), PMSF (1 mM), apronit (1 μM), leupeptin (10 μM), pepstatin (1 μM), cystatin (1 μM), eglin C (5 μg/ml), or E64 (10 μM). a, ITI; b, H-chains; c, UTI. SDS-PAGE and Western blot were carried out using avidin-peroxidase, followed by enzyme substrate.

FIG. 6. Specific cleavage of ITI by elastase. ITI (5 μg) was treated with leukocyte elastase (0–2 μg) for 30 min at 37°C. a, ITI; b, H-chains; c, UTI; d, 22-kDa UTI fragment. The protein bands were detected as described in Fig. 3. Although lanes 2 and 3 appear to have much more protein compared with lane 1, the same amount of ITI (5 μg) was used for each lane.

FIG. 7. Quantitative assessment of specific binding of ITI derivatives to hyaluronic acid immobilized to microtiter plate wells. 96-well microtiter plates were coated with hyaluronic acid or BSA by incubation of 100-μl aliquots of each solution (0.1 mg/ml hyaluronic acid or 2% (w/v) BSA). After washing the wells, nonspecific binding sites were blocked with 200-μl aliquots of PBS containing 2% BSA, pH 7.4. ITI and its derivatives (0–100 nM) diluted in 100 μl of PBS, 2% (w/v) BSA was allowed to bind for 2 h at 23°C. Then the plates were washed three times with PBS. Wells were allowed to react with anti-ITI antibody (1 μg/ml, 1 h, 23°C). This polyclonal antibody reacts with ITI, H-chains, UTI, and HI-8. Specific binding of ITI derivatives (total binding (HA-coated wells) – nonspecific binding (BSA-coated wells)) approached saturation at approximately 100 nM ITI or H-chains. Bar, S.D. The experiments were performed at least three times.
We demonstrated that cell-associated HA binds to the H-chains of ITI, but not to UTI or HI-8. They are consistent with the reports in which it has been demonstrated that HA synthesized by cultured fibroblasts firmly bound 85-kDa protein and the serum-derived HA-associated protein is confirmed to be the H-chains of the ITI. The H-chains could be involved in the formation of HA-rich extracellular matrix through their calcium-dependent HA binding activity (5–7, 28, 29). Our results are also supported by a recent study showing that pre-α-inhibitor stabilizes the expanding cumulus extracellular matrix by binding directly with HA and that pre-α-inhibitor may serve as a structural protein to organize the function of the cumulus extracellular matrix (28, 29). It was also shown that binding of pre-α-inhibitor and HA is thought a stereospecific charge interaction. The stabilizing action of pre-α-inhibitor may not be indicated through protease inhibiting activity (28, 29).

It was reported that stabilization of the pericellular matrix may require more than a simple binding interaction between pre-α-inhibitor (or ITI) and HA (22–24). We found that ITI and UTI interact strongly with hyaluronic acid-binding protein (HABP) (data not shown). This binding interaction was relatively fast. We propose that HABP may further strengthen or stabilize the expanding cumulus extracellular matrix by binding directly with HA and that pre-α-inhibitor may serve as a structural protein to organize the function of the cumulus extracellular matrix (28, 29). It was also shown that binding of pre-α-inhibitor and HA is thought a stereospecific charge interaction. The stabilizing action of pre-α-inhibitor may not be indicated through protease inhibiting activity (28, 29).

**DISCUSSION**

We demonstrated that cell-associated HA binds to the H-chains of ITI, but not to UTI or HI-8. They are consistent with the reports in which it has been demonstrated that HA synthesized by cultured fibroblasts firmly bound 85-kDa protein and the serum-derived HA-associated protein is confirmed to be the H-chains of the ITI. The H-chains could be involved in the formation of HA-rich extracellular matrix through their calcium-dependent HA binding activity (5–7, 28, 29). Our results are also supported by a recent study showing that pre-α-inhibitor stabilizes the expanding cumulus extracellular matrix by binding directly with HA and that pre-α-inhibitor may serve as a structural protein to organize the function of the cumulus extracellular matrix (28, 29). It was also shown that binding of pre-α-inhibitor and HA is thought a stereospecific charge interaction. The stabilizing action of pre-α-inhibitor may not be indicated through protease inhibiting activity (28, 29).

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More recently, it has been reported that the carboxyl-terminal Asp of H-chains was esterified to the C6-hydroxyl group of an internal N-acetylgalactosamine of hyaluronic acid chain, demonstrating the covalent binding of proteins to hyaluronic acid (30).

**TABLE I** Kinetic constants for ITI inhibition

| Enzyme  | K<sub>i</sub> (nM) | k<sub>i</sub> (M<sup>-1</sup> s<sup>-1</sup>) | k<sub>-1</sub> (s<sup>-1</sup>) |
|---------|------------------|------------------------------|-------------------------------|
| Trypsin | 2.9              | 5.0 × 10<sup>5</sup>          | 1.5 × 10<sup>4</sup>          |
| Plasmin | 32               | 3.5 × 10<sup>4</sup>          | 1.1 × 10<sup>3</sup>          |
| HLE     | 20               | 4.2 × 10<sup>3</sup>          | 8.4 × 10<sup>2</sup>          |

**FIG. 8.** Quantitative assessment of specific binding of hyaluronic acid to immobilized ITI derivatives. 96-well plates were coated with ITI derivatives (2 μg/ml, 16 h, 4°C). HA specifically bound to ITI derivatives was detected with HRP-HABP (0.5 μg/ml; 1 h, 23°C). It was confirmed previously that HRP-HABP can bind to HA (see Refs. 19–21).

**FIG. 9.** Time- and temperature-dependent binding of ITI to immobilized HA. 96-well plates were coated with HA (0.1 mg/ml, 16 h, 4°C). Biotinylated ITI (0.1 μM, 100 μl/well) was added to HA-coated wells and incubated at 37°C (●), 23°C (○), or 4°C (■) for the various times indicated. Biotinylated ITI specifically bound to HA was detected with avidin-peroxidase. 2 μl ITI was added to one sample prior to incubation (37°C, 48 h).

**FIG. 10.** Specific binding of ITI to the surface of tumor cells (cell ELISA). A, in cell ELISA, SMT-cc1 cells (●) cultured in 96-well microtiter plates were incubated with varying concentrations of biotinylated ITI (0–200 nM, 1 h, 23°C). SMT-cc1 cells preincubated with 200 nM biotinylated ITI in the presence of increasing concentrations of UTI (○) or ITI (■).
Expression of CD44 in lymphocytes or tumor cells and HA-ITI complex in the matrix may mediate cell-matrix interactions in inflammation and tumor invasion (22). HA has been found in tissues and body fluids, as part of larger molecular structures as in aggregates with proteoglycans and as a coat attached to cell surfaces (22). Some blood proteins such as fibronectin, collagen type IV, fibrinogen, IgG, or IgM have been shown to possess HA binding ability. Lymphocyte CD44 may recognize HA linked to ITI-H-chains located on the cell surface. Our results strongly support the hypothesis that serum factor, which has been identified recently as a protein belonging to the ITI family, acts as a structural component of the close environment of cell surface and showed that specific binding of ITI to HA is essential for successful organization of the matrix around the cell surface.

We demonstrated that specific receptors for UTI have been determined on the surface of certain tumor cells (12). However, ITI can bind to HA strongly but not to the UTI receptor. The receptor binding domain within the UTI has been localized to the amino acid sequences 1–79 (domain I) of UTI. Domain I and the domain II (79–143, HI-8) are the result of extensive treatment of UTI with trypsin-like enzyme (31). HI-8, lacking the domain I, does not bind to the cells (31). We speculate that, in a complete ITI molecule, domain I of UTI may be masked by the H-chains of ITI. The HA-bound ITI may be cleaved into the active UTI and the H-chains of ITI by elastase-like protease(s) on the cell surface (27). It is likely that the UTI moiety binds to the UTI receptor after ITI is processed on the cell surface. Recently, possible implications for the physiological function of the ITI family were discussed. Mast cell protease inhibitor, trypstatin, was found to be a fragment of ITI light chain (32).

Plasma UTI fragment is taken up into mast cells and stored in cytoplasmic granules, since UTI fragment may not be transported across the mast cell membrane. On the other hand, receptor-bound UTI may act as a membrane-associated protease inhibitor (12). Besides protease inhibiting activity, UTI has additional functions including prevention of cytokine production or inhibition of cellular calcium influx (33). This attractive hypothesis will be confirmed by the demonstration of ITI-cleaving enzyme(s) as well as of UTI receptors on the cell surface.

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J. Biol. Chem. 1996, 271:11362-11367.
doi: 10.1074/jbc.271.19.11362

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