Mutual Inhibitory Activities of Tumor-Degenerating Factor (TDF) and Fibronectin

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ABSTRACT. It was reported in our previous studies that the “spongy degeneration-like” changes of human tumor cells were detected by coculture with human embryonic fibroblasts *in vitro*. It was discovered that the degenerative changes were mediated by a factor secreted from human embryonic fibroblasts. This factor was named the tumor-degenerating factor (TDF). The present study found that fibronectin inhibited TDF activity while TDF inhibited cell attachment mediated by fibronectin. It was possible that these mutual inhibitions were due to the direct binding of the TDF molecule to the fibronectin molecule. Since it is well known that fibronectin is composed of multiple domains which differ in their biological activities, this study also attempted to clarify which domain(s) inhibit TDF activity, through the use of trypsin, thermolysin and 2-nitro-5-thiocyanobenzoic acid (NTCB). It is concluded that multiple domains of fibronectin are required for the inhibition of TDF activity.

Fibronectin is a key glycoprotein in the interaction of tumor cells with interstitial cells. This substance, produced from fibroblasts and other cells, has various biological actions (23, 26) including cell attachment, cell migration (1, 15) transformation of cells (16, 27), spreading of cells (4), cell differentiation (8), wound healing, enhancement of phagocytosis (4, 11, 12, 13) and metastasis (21). All of these actions influence the interaction of tumor cells with interstitial cells.

A tumor-degenerating factor (TDF) is a glycoprotein produced from human fibroblasts which causes the degenerative changes of human tumor cells (7, 8, 9, 22). The molecular weight of TDF was estimated to be about 26,500–30,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. TDF was relatively stable to heat treatment.

It is known that interstitial cells suppress or regulate the growth of tumor cells, and that TDF may be a mediator in the antitumor action of fibroblasts which are principal elements of interstitial stromas. Thus, fibronectin and TDF are involved in the interaction of tumor cells with interstitial cells consisting of fibroblasts. Therefore, it may be possible that TDF and fibronectin interact.

In the present study, it was found that fibronectin inhibited TDF activity and

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Abbreviations used: TDF, tumor-degenerating factor; NTCB, 2-nitro-5-thiocyanobenzoic acid; MEM, Eagle's minimum essential medium; NCS, newborn calf serum; FBS, fetal bovine serum; EDTA, ethylenediamine tetraacetic acid.

TDF inhibited the cell attachment mediated by fibronectin, and that these mutual inhibitions were due to the direct binding of TDF molecule to the fibronectin molecule.

It is well known that fibronectin is composed of multiple domains. Therefore, it was also attempted, in the present study, to clarify which domains inhibit TDF activity.

MATERIALS AND METHODS

Cells. KB cells from a human nasopharyngeal tumor, grown and maintained in Eagle's minimum essential medium (MEM) (Nissui Co., Ltd., Tokyo, Japan) supplemented with 5% newborn calf serum (NCS; Irvine Scientific, Santa Ana, CA, U.S.A.) were used as target cells. HeLa cells from human cervical cancer, grown and maintained in the same way as KB cells, were used for the cell attachment. Human embryonic fibroblasts (HF8209), grown and maintained in MEM supplemented with 10% fetal bovine serum (FBS; Irvine Scientific, U.S.A.), were used for the production of TDF.

TDF preparations. Human embryonic fibroblasts were grown and maintained in MEM supplemented with 10% FBS in roller bottles. The culture supernatants, harvested 14 days after incubation at 37°C, were partially purified by DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) and by ultrafiltration (Diaflomembrane YM-10; Amicon Corp., Lexington, MS, U.S.A.). These preparations were dialysed against MEM and used as TDF (22). The specific activity of the TDF preparation used was 13 units/mg protein.

Fibronectin. Human plasma fibronectin was supplied by Green Cross Corp. (Osaka, Japan) and purified by affinity chromatography (2) on gelatin-Sepharose 4B.

Reagents. Trypsin was obtained from Worthington Biochemicals (Freehold, NJ, U.S.A.). Soybean trypsin inhibitor and thermolysin were purchased from Sigma, Ltd. (St. Louis, MO, U.S.A.). 2-nitro-5-thiocyanobenzoic acid (NTCB) was obtained from Sigma and recrystallized from ethanol. Gelatin-Sepharose 4B and soybean trypsin inhibitor-Sepharose 4B were prepared according to the method of Cuatrecasas and Anfinsen (3).

Fixation of fibronectin to Sepharose 4B. After Sepharose 4B (40 ml) was suspended in water and its pH was adjusted to 11–12 by 10 N NaOH, CNBr solution was added and the pH was adjusted again to 11–12 on ice. The beads were washed with water and then with borate (0.1 M, pH 10) containing 0.5 M NaCl. These beads (wet volume, 10 ml) and fibronectin (5 mg) dissolved in borate buffer were mixed and left to react for 2 h. The fibronectin-fixed beads were washed with borate buffer and 0.1 M acetate buffer containing 0.5 M NaCl after the removal of active residues by 1 M ethanolamine (pH 8.0).

Binding of TDF to fibronectin-fixed Sepharose 4B. The TDF preparation (60 units/2.5 ml) was dissolved in a buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 mM ethylenediamine tetraacetic acid (EDTA) and 40 mM NaCl, and charged onto the fibronectin-fixed Sepharose 4B column of which the bed volume was 8.2 ml. After the column was washed with the buffer described above, the preparation was eluted with the buffer supplemented with 1 M NaCl and then with the latter buffer further supplemented with 8 M urea at 10 ml/h.

Next, the TDF preparation (60 units/2.5 ml) was dissolved in a buffer consisting of 1 mM sodium phosphate (pH 7.4), 2.5 mM CaCl₂, 1 mM MgCl₂, 4 mM KCl and 140 mM NaCl, and charged onto fibronectin-fixed Sepharose 4B column. Then, after being washed with the same buffer, TDF was eluted with the buffer described above supplemented with 1 M NaCl at a flow rate of 10 ml/h.

Fragmentation of human fibronectin by trypsin. Fibronectin (3 mg/ml) in 1 mM CaCl₂,

30 mM NaCl, and 50 mM Tris-HCl (pH 7.0) was digested at 30°C by trypsin at enzyme/substrate ratios (w/w) of 0.02, 0.06, 0.2 and 2% for 30 min (5). Digestion was terminated by adding soybean trypsin inhibitor at a trypsin inhibitor/trypsin ratio (w/w) of 200%.

Fragmentation of fibronectin by trypsin and purification of trypsin-released fragments. Fibronectin (10 mg), dissolved in 3 ml of 10 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl, was digested by trypsin (1 µg/ml) at 22°C for 15 min (18). The digest was applied to a column (14 ml) of trypsin inhibitor-Sepharose 4B to remove the trypsin. The passed fractions were then applied to a column (10 ml) of gelatin-Sepharose 4B. The fractions bound to gelatin-Sepharose 4B were eluted from the column with 25 mM Tris-HCl (pH 7.6) containing 4 M urea and 0.5 mM EDTA. After being concentrated by a collodion bag, the bound fractions were purified on Sephacryl S-300 (1.6 × 100 cm) equilibrated with 10 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl.

Fragmentation of fibronectin by thermolysin and purification of thermolysin-released fragments. Fibronectin (10 mg) was digested with thermolysin (10 µg/ml) at 22°C for 2 h in 2.5 mM Tris-HCl (5 ml) containing 0.5 mM EDTA, 50 mM NaCl and 2.5 mM CaCl₂ at pH 7.6. The digestion was terminated by adding EDTA (5 mM). The digest was applied to a column (10 ml) of heparin-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) which was equilibrated with 10 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl. The column was washed with the same buffer, and then eluted with the 10 mM Tris buffer containing 0.145, 0.2, and 1 M NaCl, in a stepwise manner (19).

Fragmentation of fibronectin by s-cyanilation and purification of s-cyanilation-cleaved fragments. Fibronectin (10 mg) dissolved in 0.2 M Tris acetate (pH 8.0) containing 1 mM EDTA and 8 M urea was reacted with 0.5 mM 2-nitro-5-thiocyanobenzoic acid (NTCB) at 37°C for 30 min (17). The reaction was quenched by adjusting the pH to 4 with acetic acid. The modified fibronectin was desalted by passage through PD-10 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.1% acetic acid and then lyophilized. For cleavage, it was dissolved in 0.2 M Tris acetate (pH 9.0) containing 8 M urea and incubated at 37°C for 20 h. The cleavage products were desalted by passage through PD-10 equilibrated with 10 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl, and then applied to a column of gelatin-Sepharose 4B. The bound fractions were eluted with 10 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA, 50 mM NaCl and 4 M urea. After being concentrated by a collodion bag, the bound fractions were purified on Sephacryl S-300 (1.6 × 100 cm) equilibrated with 10 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl.

Assay for TDF activity. TDF activity was assayed according to the method described elsewhere (22). In brief, TDF preparation (50 µl) was serially twofold diluted on the microplate (Nunc Micro Test Plate; A/S Nunc, Denmark). KB cell suspensions (50 µl), of which cell density was adjusted to 4×10^5 cells/ml with MEM containing 10% NCS, were put into each well of the microplate. After 3 days of incubation at 37°C in a 5% CO₂ atmosphere, KB cells were observed under a light microscope. The maximum dilution of the TDF preparations, which induced the degenerative changes in KB cells, was expressed as TDF units.

Assay method for the inhibitory activity of TDF. The inhibitory activity of fibronectin was assayed by the method of box titration. In brief, serial twofold dilutions (50 µl) of TDF and serial twofold dilutions (50 µl) of fibronectin were placed in the wells of the columns and the rows of the microplate, respectively. KB cell suspensions (50 µl), of which cell density was adjusted to 4×10^5 cells/ml in MEM supplemented with 5% NCS, were then added to the mixture in each well of the microplate. After 3 days of incubation at 37°C in an atmosphere of 5% CO₂ in air, the degenerative changes of KB cells were observed under a light microscope, and the inhibitory activity was determined.

Cell attachment. In the first coating, fibronectin solution (100 μ l) was placed into each well of the microplate (Microtest III flexible assay plate, Falcon 3912, Becton-Dickinson, Co., Oxnard, CA, USA) at 20 μ g/ml and incubated at room temperature for 4 h. In the second coating, MEM containing 8 units/ml of TDF or bovine serum albumin (BSA) with a protein concentration equal to that of TDF, was placed into each well of the microplate and incubated at room temperature for 4 h. Finally, the wells were washed with MEM, and 100 μ l of HeLa cell suspension was placed into the wells at 4×10^5 cells/ml. HeLa cells, usually used for cell attachment, were also proved to be as sensitive to TDF as KB cells. The cells were incubated at 37°C in a 5% CO₂ atmosphere for 2 h. The cells attached to the substrate were dissociated into single cells by 0.25% trypsin solution, and the viable cells were counted using the trypan blue dye exclusion test.

In the experiments performed without the second coating, 8 units/ml of TDF or BSA were placed into the wells together with the HeLa cell suspension and incubated at 37°C in a 5% CO₂ atmosphere for 2 h. The cells attached to the substrate were dissociated into single cells, and the viable cells were counted.

RESULTS

Effect of human plasma fibronectin on TDF. Human plasma fibronectin was tested for inhibition of TDF activity. TDF activity was inhibited by human plasma fibronectin (Table 1). The concentrations of fibronectin in the TDF preparation used in this experiment and in the supernatant of the KB cell culture for this assay were 0.16 and 0.20 μ g/ml, respectively, which did not influence the TDF activity.

Inhibition of fibronectin-mediated cell attachment by TDF. In the preliminary experiment, the appropriate concentration of fibronectin and the appropriate incubation time were examined. It was found that when the substrate was coated with 20 μ g/ml of fibronectin, and the cells were incubated for 2 h, the cell attachment was maximum. Therefore, in the subsequent experiments, these two conditions

TABLE 1. EFFECT OF HUMAN PLASMA FIBRONECTIN ON TDF ACTIVITY

FN μ g/ml	TDF U/ml	32	16	8	4	2	1
250		+	—	—	—	—	—
125		+	—	—	—	—	—
63		+	+	—	—	—	—
31		+	+	—	—	—	—
16		+	+	+	+	—	—
8		+	+	+	+	+	+
4		+	+	+	+	+	+
2		+	+	+	+	+	+

Serial twofold dilutions (50 μ l) of TDF and those of the inhibitor (50 μ l) were placed into the wells of the columns and rows of the microplate, respectively.

KB cell suspensions (50 μ l), of which cell density was adjusted to 4×10^5 cells/ml in MEM supplemented with 10% NCS, were then added to the mixture in each well of the microplate. After 3 days of incubation at 37°C in an atmosphere of 5% CO₂ in air, the degenerative changes of KB cells were observed under a light microscope, and the inhibitory activity was determined.

+ indicates the degenerative changes by TDF. FN: fibronectin

were repeated.

The degree of cell attachment to the substrate of the fibronectin-uncoated well was low (8%), while that to the substrate coated with fibronectin was high (42%). After the substrate was coated with fibronectin, HeLa cells suspended in MEM containing 8 units/ml of TDF or BSA were placed into the well. The degree of attachment of the cells suspended in MEM containing TDF was lower than that of those suspended in MEM containing BSA (Fig. 1). Furthermore, when the substrate was coated with TDF alone, the cell attachment was unchanged (data not shown). When the substrate was further coated with TDF after the coating with fibronectin, the cell attachment was reduced (Fig. 1). Thus, it is concluded that TDF inhibited the attachment of HeLa cells mediated by fibronectin.

Binding of TDF to fibronectin. The TDF preparation dissolved in the buffer containing 25 mM Tris-HCl, 0.5 mM EDTA and 40 mM NaCl was charged onto the fibronectin-fixed Sepharose 4B. TDF activity was not found in the fractions washed out with the buffer used for charging, while 100% of TDF was recovered in the fractions eluted with the buffer supplemented with 1 M NaCl (Fig. 2).

Next, TDF preparation (60 units) dissolved in the buffer consisting of 1 mM

Fig. 1. Inhibitory effect of TDF on the cell attachment mediated by fibronectin. In the first coating, fibronectin solution (20 μ g/ml) was placed into the wells of a microplate and incubated at room temperature for 4 h. In the second coating, MEM containing 8 units/ml of TDF or BSA was placed into each well of the microplate and incubated at room temperature for 4 h. HeLa cell suspension was placed into the wells at 4×10^5 /ml together with TDF or BSA, or without these agents after being washed with MEM, and incubated in a 5% CO₂ atmosphere at 37°C for 2 h. The cells attached to the substrate were dissociated into single cells and counted. The ratio was calculated by the following formula:

$$\text{Ratio (\%)} = \frac{\text{numbers of cells attached to the substrate}}{\text{numbers of cells put into the well}} \times 100$$

Fig. 2. Fibronectin-fixed Sepharose 4B column chromatography of TDF. Fibronectin was fixed to Sepharose 4B by the method described in the MATERIALS AND METHODS. TDF preparation (60 units/2.5 ml) was charged onto fibronectin-fixed Sepharose 4B and washed with the buffer containing 40 mM NaCl. It was then eluted with the buffer supplemented with 1 M NaCl at 10 ml/h.

sodium phosphate (pH 7.4), 2.5 mM CaCl_2 , 1 mM MgCl_2 , 4 mM KCl and 140 mM NaCl was charged onto fibronectin-fixed Sepharose 4B, in order to examine the binding of the TDF molecule to the fibronectin molecule under the physiological condition. Then, it was eluted with the buffer described above supplemented with 1 M

Fig. 3. Binding of TDF to fibronectin molecule under the physiological condition. TDF preparation was charged onto fibronectin-fixed Sepharose 4B and washed out with the buffer containing 140 mM NaCl. It was then eluted with the buffer containing 1 M NaCl at 10 ml/h.

NaCl (Fig. 3).

The first peak of protein was located in the washed-out fractions (fractions 3 to 10); about 75% of the TDF was found in these fractions. In fractions 39 to 42, where the protein was eluted by the buffer, the low peak of protein was noticed; about 25% of TDF was found in these fractions.

Furthermore, the TDF preparation dissolved in the buffer containing 25 mM Tris-HCl, 0.5 mM EDTA and 40 mM NaCl was charged onto the plain Sepharose 4B in order to exclude the possibility that TDF was bound simply to untreated Sepharose 4B beads. The TDF activity was found at more than 90% in the fractions washed out with buffer used for charging and at less than 10% in those eluted with buffer supplemented with 1 M NaCl (data not shown). When the TDF preparation dissolved in the buffer containing 140 mM NaCl was charged onto Sepharose 4B, the TDF activity was found at 100% in the fractions washed out with the buffer for charging. Thus, it was proved that TDF was not bound simply to Sepharose 4B.

Inhibitory effect of various domains of fibronectin on TDF activity. Mixtures of fibronectin fragments were produced by incubating human plasma fibronectin with trypsin at enzyme/substrate ratios of 2, 0.2, 0.06 and 0.02% for 30 min at 30°C. After termination of digestion, the digests were analysed on a 2.5/5/15% polyacrylamide-SDS gel following reduction by 2-mercaptoethanol (Fig. 4A). The number of fragments produced was proportional to the enzyme/substrate ratios. The mixtures of fragments were assayed for the inhibition of TDF activity. The degrees of inhibition of TDF activity were sharply decreased, corresponding to the progression of digestion of fibronectin (Fig. 4B).

Human plasma fibronectin received limitative digestion by trypsin, thermolysin and 2-nitro-5-thiocyanobenzoic acid. Fragments of fibronectin obtained by weak trypsinization were purified on gelatin-Sepharose 4B and Sephacryl S-300. These fragments were analysed on 2.5/3/15% polyacrylamide-SDS gel after reduction by 2-mercaptoethanol. Fragments obtained by degradation of fibronectin with thermolysin were purified by heparin-Sepharose 4B and Sephacryl S-300. These fragments were analysed on 2.5/9% polyacrylamide-SDS gel after reduction by 2-mercaptoethanol. Fragments obtained by the degradation of fibronectin with 2-nitro-5-thiocyanobenzoic acid were purified by gelatin-Sepharose 4B and Sephacryl S-300. These fragments were analysed on 2.5/9% polyacrylamide-SDS gel after reduction by 2-mercaptoethanol. The effect of the fragments on TDF activity was examined by the method mentioned above. Only large fragments obtained by trypsinization of fibronectin inhibited TDF activity, but those obtained from the degradation of fibronectin with thermolysin or 2-nitro-5-thiocyanobenzoic acid did not (Fig. 5). It was also recognized that the small fragments obtained from the degradation of fibronectin with trypsin and thermolysin did not inhibit the activity (data not shown).

DISCUSSION

It was reported in our previous studies that the "spongy degeneration-like" changes of human tumor cells and continuous cell lines were detected when they were cocultured with human embryonic fibroblasts *in vitro* (7, 8). Further it was determined that the degenerative changes were mediated by a factor secreted from human embryonic fibroblasts, because the supernatant of human embryonic fibroblast culture caused the same degenerative changes as those observed in the

Fig. 4. Inhibitory effect of tryptic fibronectin on TDF activity. Intact fibronectin (o) was digested by trypsin at enzyme/substrate ratios of 2 (a), 0.2 (b), 0.06 (c), and 0.02 (d)% for 30 min at 30°C. The digests were analysed on a 2.5/5/15% polyacrylamide-SDS gel (Fig. 4A) following the reduction. The inhibitory effect of the digests on TDF activity was examined by the method described in "MATERIALS AND METHODS" (Fig. 4B).

●: intact fibronectin, ○: 0.02%, ▲: 0.06%, △: 0.2%, ■: 2%.

Fig. 5. Effect of partially purified fragments on TDF activity. Large fragments released from human plasma fibronectin by trypsin (185 and 215 K; a), thermolysin (145 and 155 K; b) and 2-nitro-5-thiocyanobenzoic (160 K; c) acid were obtained by the method described in "MATERIALS AND METHODS" (5A). The effect of the fragments on TDF activity was examined by the method described in "MATERIALS AND METHODS" (5B).

●: intact fibronectin, ○: 185 K & 215 K fragments, ▲: 145 K & 155 K fragments, △: 160 K fragment.

coculture of human tumor cells and continuous cell lines with human embryonic fibroblasts (22).

Fibronectin is produced by fibroblasts (24) and other cells. This substance affects a variety of properties of cells (23, 26) including cell attachment, adhesion, spreading (4), transformation (16, 27), differentiation (25), oncogenesis, and metastasis (6, 21). These biological functions have been attributed to a number of specific binding sites, such as on the fibronectin molecule, of collagens (gelatin), heparin and other glycosaminoglycans, fibrin, complement component C1q, actin, DNA, and surface components of eukaryotic cells (10) and certain bacteria. Fibronectin is composed of multiple domains (14) which differ in their biological functions.

As mentioned above, it was recognized in the present study that TDF activity was inhibited by fibronectin. It was also found that TDF inhibited the attachment of HeLa cells to the substrate coated with fibronectin. It was possible that these inhibitions were due to the direct binding of the TDF molecule to the fibronectin molecule. TDF will bind to the fibronectin molecule partly under the physiological condition in the presence of 140 mM NaCl. This may suggest that the TDF molecule is bound to the fibronectin molecule and inhibits fibronectin activity *in vivo*.

Furthermore, it was attempted to clarify which domain(s) interact with the TDF molecule and inhibit TDF activity. It is well known that the domains of fibronectin can be separated by proteolytic and chemical fragmentation (5, 18, 20). The mixtures of human plasma fibronectin fragments were produced by trypsin at different enzyme/substrate ratios and the inhibitory activity of these were determined. The

Fig. 6. Schematic drawing of fibronectin domains for the proteolytic and chemical fragmentation (according to Sekiguchi *et al.*, (20), with a slight modification). Fib-1, fibrin binding domain; Hep-1, heparin binding domain; S. aur, *Staphylococcus aureus* binding domain; Actin, actin binding domain; Gel, gelatin binding domain; Col, collagen binding domain; Cell, cell surface binding domain. A, Fragments by trypsin; B, Fragments by thermolysin; C, Fragments by 2-nitro-5-thiocyanobenzoic acid.

degree of inhibitory activity was sharply decreased in the progression of digestion of fibronectin. Thus, it was suggested that the large fragments trypsinized at low enzyme/substrate ratios (0.02 and 0.06%) inhibited TDF activity.

It is necessary to fractionate the large fragments produced by some agents in order to identify the fragment(s) involved in the inhibition. Therefore, human plasma fibronectin was limitatively fragmented by trypsin, thermolysin and 2-nitro-5-thiocyanobenzoic acid (NTCB). The fragments were then fractionated by chromatographies. The results show that TDF activity was inhibited only by the trypsin-released large fragments (185 K, 215 K), but not by the trypsin-released small fragments (domain VI of α chain and domain I), the thermolysin-released large fragments (145 K, 155 K), thermolysin-released small fragments (domain I, domain II, and domain VI) or the NTCB-released large fragment (160 K) (Fig. 6). It was concluded from these results that domain IV, the domain for cell attachment, did not individually inhibit the TDF activity, and that multiple domains were required for the inhibition of TDF activity.

Thus, TDF may influence fibronectin functions as an extracellular matrix, and *vice versa*, while it consequently regulates or suppresses the function of tumor cells.

In any case, it must be considered that TDF and fibronectin are important substances for an analysis of the interaction of tumor cells with interstitial cells.

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