A pituitary gene encodes a protein that produces differentiation of breast and prostate cancer cells

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A cDNA clone of 1.1 kb encoding a 108-aa polypeptide was isolated from a human pituitary cDNA library by expression cloning. This protein was named tumor differentiation factor (TDF). The recombinant TDF protein and a 20-aa peptide, P1, selected from the ORF of the gene, induced morphological and biochemical changes consistent with differentiation of human breast and prostate cancer cells. Fibroblast, kidney, hepatoma, and leukemic lymphocytic cell lines were unaffected. Breast and prostate cancer cells aggregated in spheroid-like structures within 24 h of exposure to TDF. This effect was abrogated by a specific affinity-purified rabbit polyclonal anti-P1 Ab. E-cadherin expression was increased in a dose-dependent manner by TDF. Treatment of MCF7 cells with TDF led to production of a lactalbumin-related protein. Peptide P1 significantly decreased the growth of androgen-independent DU145 prostate cancer in severe combined immunodeficient mice. The presence of TDF protein in human sera was detected by the anti-P1 Ab, suggesting a role of TDF in endocrine metabolism. The fact that all activities of TDF can be mimicked by a peptide derived from the encoding TDF sequence opens the possibility of therapeutic applications.

Malignant transformation is characterized by the uncoupling of proliferation and differentiation leading to continuing multiplication of cells and the impairment of their ability to progress to complete normal differentiation. Restoration of differentiation of malignant cells has long been considered a potential therapy of cancer (1–5).

We have previously reported that an alkaline extract of rat mammosomatotropic tumor MtTW10 induced morphological and biochemical changes in rat and human breast cancer cells indicative of differentiation. Within 24 h of the exposure *in vitro*, the pituitary tumor extract (PTE) led to aggregation of breast cancer cells, polarization of organelles, the formation of cell junctions and basement membrane, synthesis of mRNA for casein and lactalbumin, and overexpression of E-cadherin (6). The PTE was also effective on prostate cancer cells, inducing cellular changes and overexpression of E-cadherin and prostate-specific antigen.

Because this activity was not reproduced by any of the known pituitary hormones or other growth factors, studied alone or in combination, we assumed that the PTE contained a factor, which we called tumor differentiation factor (TDF). Our attempts to isolate and purify this factor by using conventional chromatographic procedures were unsuccessful.

Now, using expression cloning, we report the isolation of a human cDNA clone encoding a protein responsible for this tumor-differentiating activity. We have sequenced the clone. Computer analysis indicates that it encodes a polypeptide with no significant homology to any other known sequence in the GenBank database. Our results support the proposition that TDF is a previously unrecognized pituitary factor.

Materials and Methods

Pituitary cDNA Library. A cDNA library prepared from a growth hormone-producing human pituitary adenoma (Clontech) was used for sib selection (7–9). This cDNA library was directionally cloned in *Eco*RI and *Hind*III sites of λ Bluemid phage. This vector

includes T3 and T7 RNA polymerase promoter sequences flanking the polycloning region, thus making possible the transcription of the cDNA inserts. By using T3 RNA polymerase, the (+) strand of the cDNA insert could be synthesized.

Sib Selection of the Pituitary cDNA Library. First, we determined the minimum size of the pituitary cDNA library that contained a clone(s) for TDF. The plating and growth of phages on agar plates and their titration followed established procedures (10). Three randomly selected pools of about 200,000, 400,000, and 600,000 phage clones from the pituitary cDNA library were plated. The phage DNA was then prepared; insert transcripts were synthesized with T3 RNA polymerase and were then expressed in *Xenopus* oocytes. Finally, the oocyte lysates were tested for aggregating and differentiating activity on MCF7 cells.

We concluded that the minimum size of the pituitary cDNA library containing a clone for TDF was $\approx 400,000$ plaque-forming units (pfu). Subsequently, we proceeded with the sib selection; $\approx 4 \times 10^5$ plaques were divided into 10 subpools of $\approx 4 \times 10^4$ plaques each, plated separately, and grown until they reached about 1 mm in size. The phage DNA from each pool was prepared; capped transcripts were synthesized with T3 RNA polymerase with the mMessage mMachine kit (Ambion, Austin, TX). The reaction was carried out with 5 μ g of phage DNA linearized by digestion with *Sal*I, following the protocol provided by the manufacturer.

Expression of Transcripts in *Xenopus* Oocytes. Fully grown oocytes (1.2- to 1.3-mm diameter, stages 5 and 6) were isolated from adult *Xenopus laevis* and stored in Barth buffer. The follicular cell layer was removed by incubation of oocytes with 2 mg/ml collagenase type A (Sigma) in Barth buffer for 2 h at 25°C with continuous agitation.

RNA transcripts from each subpool were injected into 20 fully grown *Xenopus* oocytes (50 ng per oocyte) by using an automatic microinjector. After 3 days of incubation at 18°C, with daily buffer changes, the oocytes were homogenized in 0.15 M NaCl, followed by two centrifugations at $15,000 \times g$ at 4°C for 30 min. The supernatant was then tested for breast cancer cell-aggregating activity by using the bioassay. The subpool displaying the strongest biological activity was selected for further sib selection. Similarly, subpools of 4×10^3 , 400, 40, and 4 pfu displaying aggregating activity were identified. The sib selection continued until a single positive clone was isolated. The phage cDNA clone was converted to pBluescript II SK(+) plasmid clone by digestion of the phage DNA with *Not*I and then circularized with T4 DNA ligase, according to the manufacturer's instructions.

Abbreviations: TDF (previously called PDF), tumor differentiation factor; rTDF, recombinant TDF protein; SCID, severe combined immunodeficient; PTE, pituitary tumor extract; pfu, plaque-forming units; ATTU, average total tumor volume; CBP, calmodulin-binding peptide.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY437503).

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Bioassay. Cultures containing 1×10^5 MCF7 cells in 1 ml of serum-free RPMI medium 1640 were incubated in the presence of various concentrations of oocyte lysate (5–300 μ g of protein per ml) at 37°C in 5% CO₂/95% air. They were assessed for aggregation at different time points by two observers scoring independently. In each bioassay a negative control with lysates from noninjected oocytes and two positive controls with alkaline PTE and with lysates from oocytes injected with pituitary tumor mRNA were included.

DNA Sequencing. The sequencing of TDF cDNA was performed at Rockefeller University Technology Laboratory by using Sanger's dideoxy chain termination method. The sequencing was completed by primer extension strategy with three pairs of primers. The computer program OLIGO primer analysis software, Version 5.1 (NBI/Genovus, Plymouth, MN), was used for selecting the appropriate sequencing primers. The 5' and 3' extremities of TDF cDNA were determined with SMART RACE cDNA amplification kit (BD Biosciences Clontech) and human pituitary poly(A)⁺ RNA, as described (11). The TDF cDNA clone was analyzed with MACDNASIS PRO sequence analysis software, Version 3.6 (Hitachi, San Bruno, CA) and the BLAST program, seeking homology with other known sequences from GenBank database.

Northern Blot Analysis. Poly(A)⁺ RNA was prepared from GH3 rat pituitary tumor cell line by using PolyAtract mRNA Isolation System III kit (Promega). Northern blot analysis was done as described (11, 12).

A multiple normal-tissue Northern blot was obtained from Clontech. The blot was prepared by electrophoresis in a 1.2% denaturing formaldehyde gel and then transferred to nylon membrane of 2 μ g of poly(A)⁺ RNA obtained from eight normal human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas). After prehybridization, the blot was hybridized to TDF radioactive probe as described (11). The probe was the DNA fragment between nucleotides 455 and 665 of TDF cDNA clone, amplified by PCR and labeled with [³²P]dCTP by random primer technique (11). The normalization was done with a 28S rRNA probe.

The autoradiograms were read as described (12).

Peptide Synthesis. Four peptides, each containing 20 amino acids (P1, NH₂-RESQGRVVGQALSFLCKGTA-COOH; P2, NH₂-QNMKHFYLPFPQSLKFTPYF-COOH; P3, NH₂-PDHQWSE-ECCIFLTFRQVWQ-COOH; and P4, NH₂-WHHHCIPMILLI-NAMMLIQL-COOH), were selected from TDF ORF and synthesized (The Lindsley F. Kimball Research Institute of the New York Blood Center) with >95% purity as assessed by HPLC and mass spectrometry.

Antibodies Against Peptide P1. A rabbit polyclonal Ab against peptide P1 was prepared and purified by affinity chromatography by Covance (Denver, PA), at our request. MACDNASIS software was used for selection of peptide P1 as a highly antigenic region in TDF-deduced amino acid sequence.

Western Blot Analysis. Preparation of cellular lysates, SDS/PAGE, and transfer to nitrocellulose paper were performed as described (6). The blots were immunoblotted with anti-P1 Ab (Covance, Denver, PA) with a kit and the instructions from Pierce. The films were read as described (12).

The same procedure was used to analyze the TDF level in human sera. Anti E-cadherin Ab was a mouse monoclonal Ab (Santa Cruz Biotechnology). A rabbit polyclonal anti- α -lactalbumin Ab was obtained from Sigma. Normal rabbit IgG (Santa Cruz Biotechnology) was used as a negative control.

For normalization the blots were stripped and then reprobed with anti- β -actin Ab (Sigma).

Cell Proliferation Assay. The following cell lines were used: MCF7 and T47D human breast cancer cell lines, LNCaP and DU145 human prostate cancer cell lines, CAKI 1 human kidney cancer cell line, Hep G₂ human hepatocellular carcinoma cell line, GH3 rat pituitary tumor cell line, NIH 3T3 normal mouse fibroblast cell line, and CEM-C7 (C⁺ and C⁻) acute lymphocytic leukemic cells. All of the cell lines, except CEM-C7 and GH3 (gifts from S. Brower and C. Bancroft, Mount Sinai School of Medicine, New York), were obtained from American Type Culture Collection. Cells were grown in the appropriate media supplemented with 10% FCS (BioWhittaker) at 37°C in 5% CO₂/95% air.

The proliferation assay was performed as described (6). In brief, cultures containing 1×10^5 cells in 1 ml of serum-free medium were incubated in the presence of various concentrations of peptides (1 ng/ml to 50 μ g/ml) or recombinant TDF protein (rTDF; 1 ng/ml to 50 μ g/ml) at 37°C in 5% CO₂/95% air for various times (0–96 h). Control cultures received 0.15 M NaCl. A set of cultures received the P1 at the beginning of the experiment; another set was pulsed every day without change of media. Some cultures received, in addition to the peptide P1, anti-P1 Ab or normal IgG (peptide/Ab ratio, 1:4).

Tumor Growth in Severe Combined Immunodeficient (SCID) Mice. Male SCID mice, 4–6 weeks old and weighing 20–25 g (Charles River Laboratories), were maintained in a special pathogen-free facility in Mount Sinai Animal Care Facilities. This research was conducted in conformity with the regulations of the Institutional Review Board and the Institute Animal Care Committee. All animals were treated in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Five million DU145 cells in 75 μ l of medium and 75 μ l of Matrigel were injected s.c. into the right flank of each animal. After tumor appearance, the animals were assigned at random to control or experimental groups. The animals in experimental groups were injected i.p. with doses of 2.5, 25, and 250 μ g of P1 peptide in 100 μ l of 0.15 M NaCl twice a day for the duration of the experiment. Control animals received an identical volume of 0.15 M NaCl. Tumors were measured twice a week with a caliper. Tumor volume was calculated by the formula $0.523 \times (\text{length}) \times (\text{width})^2$ (13). When the tumors reached 1 cm in diameter, the mice were killed. The experiment was repeated three times. Five-micrometer sections of control and treated tumors were stained with methylene blue, azure II, and basic fuchsin. MIB1 immunostaining was used to determine the Ki-67 labeling index. MIB1 monoclonal Ab raised against Ki-67 antigen were purchased from Santa Cruz Biotechnology.

Statistical Methods. To compare mean tumor volumes between groups at each time point of tumor measurement, the *t* test for independent groups was used. To obtain an overall assessment of tumor volume throughout the time-vs.-tumor-volume curve, the average total tumor volume (ATTV) from the first day of tumor measurement to the last day of measurement was calculated for each animal. The ATTV equals the area under the curve of the tumor volumes over time divided by the number of days. The ATTV represents the average height of the volume–time curve, thereby giving an overall index of tumor size. Statistical comparison of ATTVs between groups across all four experiments was performed by a pooled analysis with two-way ANOVA.

Determination of TDF Glycosylation. Treatment with glycosidase F and H was done with the reagents and the protocol from New England Biolabs. Aliquots of lysates prepared from GH3 rat pituitary tumor cell line were denatured for 10 min at 100°C in the presence of 0.5% SDS/1% mercaptoethanol/40 mM EDTA either in 100 mM phosphate buffer (pH 6.1) for *N*-glycosidase F or 50 mM citrate buffer (pH 5) for endoglycosidase H. Nonidet P-40 was added to a final concentration of 1% for glycosidase F digestion. Then, glycosidase F or H was added for a 2-h incubation at 37°C in

TAA AAT ATG GCT TTC GCC CGA AAA CGG CCT TTT GTT TCC GAC CCT AAC CAG TAA CCA GAA 61
 AGG CAG ACA GGA AGA AAC CAG AGA CAC ACC TCA AAC CAG CAT TTC TTC ATT GGT TTA CTT 121
 TTC TGA CCA GAC ATA TTT CCT ATA GAG CTA TTT CAT ATG ATC TTC TCG AAG AAT ATT CAA 181
 CTG ATC AAA TTA TCA TCC TTA CAG AAC ACT ATT ATC TTA CTA CTG CTA CCA ACT CGG GAC 241
 CAT GCC ATC GAC ATG GAT ACT TTG ATG GAC CTT TTC TAT GTC TAA AGA ATG ACA AGT GGT ATG 301
 TTT GGA TAA ATA GAT AAT TAC AAG CAT TGG GTA Met Arg Glu Ser Gln Gly Thr Arg Val 361

GGA CAA GCA TTA AGT TTT CTC TGC AAA GGT ACT GCT GTT CAA GAC ACA CAC ACA CAC ACA 421
 Gly Gln Ala Leu Ser Phe Leu Cys Lys Gly Thr Ala Val Gln Asp Thr His Thr His Thr

CAC ACA CAC ACA CAC ACA CAC ACA CAC ACA CAC ACA CAC ACA CAC ACA CAC ACA CAC ACA 481
 His Thr His Thr His Thr His Thr His Thr His Thr His Thr His Thr His Thr His Thr

TTT CTT TAT GGT CCC TTT CAA TCC CTC AAA TTT ACC CCC TAT TTT TGG CAT CAT CAT ATC 541
 Phe Leu Tyr Gly Pro Phe Gln Ser Leu Lys Phe Thr Pro Tyr Phe Trp His His His Ile

TGT CCA ATG ATC ATT TTA ATC AAT GCA ATG ATG CTG ATA CAA TTA TTA TTA GAG GGC CCG 601
 Cys Pro Met Ile Ile Leu Ile Asn Ala Met Met Leu Ile Gln Leu Leu Leu Glu Gly Pro

GAT CAT CAA TAC TCA TTA GAA TGC TGT ATC TTT CTT ACA TTC AGA CAA GTG TGG CAA TGA 661
 Asp His Gln Tyr Ser Leu Glu Cys Ile Phe Leu Thr Phe Arg Gln Val Trp Gln ***

TGC GAA AGA AAG GAC TAG TAC AAG CAA AAT TTC TAG GGA GAA AGT ACA TGT ATC ACA ATT 721
 TAG TGT GCC AGC GAA GGG TCA GTG TCA AAG AAC AGT TTC AAA CCA GGG GAC AGA GAA ATG 781
 AAA TGG GGA CTT TAA CTC ATG TAA TGG GCA GGG CTT TGA ACA AAA CAT ATA CAT CTT GAA 841
 ACT TCA GTT CTG ACG TGA AAG GTG AAT TTT AAG TCT CAT TAG ACA TAG ATG CTA AAG ATT 901
 GTG AAA ACA ATT TCC TTT ACT TTT CTA TTC ATT GCA AAA AAT CTA ATG ATG TTC CCA TAA 961
 GAT AAC ACA TGT ATT TAA AAG AGT AAT AAA ATT ACT GTG TAA TTG TAT ACT CTT ACC ATC 1021
 AGT AGT TAG GCA AAT TAA TAA AAG TGA TAT TTT TGA AAG TGA TTG TAG AAT GAA TTT TCT 1081
 TAG AAT GTT GAA GAA AGG GAA TAA AAA TGA CGC AAC CGA CTG GCA CCA AGC CGA AAA AAA 1141
 AAA AAA AAA AAA AAA 1156

Fig. 1. TDF cDNA sequence and its deduced amino acid sequence (accession no. AY437503).

a 30- μ l final volume. Finally, the samples were analyzed by Western blotting.

rTDF Expression in *Escherichia coli*. rTDF was synthesized in *E. coli* and purified by using the affinity ligation-independent cloning and protein purification kit according to the manufacturer's protocol (Stratagene). The procedure is based on subcloning of the TDF encoding sequence in pCAL-n-FLAG vector by ligation-independent cloning. After nonenzymatic subcloning the vector containing the TDF encoding sequence was transfected into BL21 *E. coli* strain. Then, three ampicillin-resistant clones were selected, and the DNA was purified and sequenced with the primers from the vector. All three clones contained the TDF encoding sequence in the proper reading frame with the vector. One clone was grown in liquid culture and induced with isopropyl-1-thio- β -D-galactopyranoside. Because the TDF protein was expressed as a hybrid with a calmodulin-binding peptide (CBP) and FLAG epitope the hybrid protein could be identified on Western blots with anti-FLAG Ab from the kit. The CBP-TDF hybrid was purified from large amounts of bacterial lysate by affinity chromatography by using calmodulin bound to resin. Finally, the rTDF was released from the hybrid by a digestion with enterokinase, which has the target sequence [(Asp)₄-Lys] located between the FLAG epitope and the N terminus of the TDF protein. The availability of anti-P1 Ab facilitated the rTDF purification. Partial amino acid sequencing of rTDF was done at The Lindsey F. Kimball Research Institute of the New York Blood Center.

Results

To isolate the tumor differentiation factor we used expression cloning in *Xenopus* oocytes (14, 15) and a biological assay based on the aggregating and differentiating effect of the PTE on breast carcinoma cells in culture (6). To determine whether this assay was sensitive enough to detect the protein encoded by the TDF cDNA clone isolated from a human pituitary adenoma library, we first determined the effect of the lysate prepared from oocytes injected with mRNA from a human pituitary tumor on MCF7 cells.

A definite, but weak aggregating effect was observed after 72 h of incubation of MCF7 cells with these lysates (5–300 μ g of protein per ml), but not with lysates from oocytes injected with rat liver mRNA (data not shown). This result validated the bioassay.

Isolation of the TDF cDNA Clone. We next determined whether the pituitary cDNA library selected by us contained the TDF clone. The pools of 4×10^5 and 6×10^5 pfu displayed the TDF-aggregating activity.

The pool of 4×10^5 pfu was used for sib selection. This pool was divided in 10 subpools of $\approx 4 \times 10^4$ clones each and tested on MCF7

cells for aggregating effect. After seven sib-selection steps we identified a single clone showing aggregating activity. During sib selection, as the subpools became smaller, the aggregating effect on MCF7 cells became progressively stronger, reflecting the increased concentration of TDF in oocyte lysate.

Analysis of TDF cDNA Sequence. The cDNA clone isolated contains a cDNA insert of ≈ 1.1 kb. The complete TDF cDNA sequence (Fig. 1) contains 1,150 bp, including a poly(A) tail at the 3' end and a polyadenylation signal. The TDF cDNA contains a continuous ORF, starting with the ATG initiation codon at position 335. The deduced amino acid sequence of TDF ORF includes 108 amino acids with an estimated molecular mass of 12,740 Da. It has several motifs for phosphorylation by PKC, casein kinase II, and cAMP-dependent protein kinases. The search with BLAST program revealed no significant nucleotide homology with any other genes from the GenBank database, indicating that TDF cDNA encodes a hitherto undescribed protein.

Expression of TDF Gene in Various Tissues. We studied the expression of the TDF gene in various human tissues by using a multiple normal-tissue Northern blot. TDF mRNA was expressed only in normal brain tissue as a single transcript of 1.1 kb (Fig. 2). The normalization with 28S rRNA probe showed an equal amount of poly(A)⁺ RNA in all lanes.

Northern blot analysis of TDF mRNA from GH3 rat pituitary tumor cells also showed a single band of 1.1 kb (data not shown).

Determination of Biological Activity of P1 Peptide. Next, we confirmed that the TDF cDNA clone encoded a factor responsible for the biological activity found in the PTE. For this purpose, four peptides, P1–P4, each containing 20 amino acids selected from the TDF ORF, were synthesized and tested for their biological activity. Peptide P1 induced morphological changes similar to, although less intense than those produced by the PTE on breast MCF7 and T47D and prostate DU145 and LNCaP cancer cells. P1 had no effect on the other cell lines studied.

We present here only the effect on DU145 cells as representative of P1 effect on breast and prostate cancer cells. DU145 cells treated

Fig. 2. TDF expression in mRNA from various normal human tissues as determined by Northern blot analysis. Lanes: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas. As described in *Materials and Methods*, 2 μ g of poly(A)⁺ RNA from each tissue was hybridized to TDF probe.

Fig. 3. Morphology of DU145 cells after 72 h of culture in serum-free RPMI medium 1640 in the absence (A) or presence of 10 ng/ml peptide P1 (B), 1 µg/ml peptide P1 (C), or 1 µg/ml peptide P1 and 4 µg anti-P1 Ab (D).

with P1 (1 ng/ml to 50 µg/ml) aggregated and formed large spheroids, whereas the untreated control cells remained as isolated single cells (Fig. 3). P1 was active at 10 ng/ml, as early as 24 h after addition. A dose response was seen. The effect was greater in cultures receiving the peptide every day than in cultures receiving a single dose.

The aggregation effect was abolished by simultaneous treatment of DU145 with peptide P1 and the anti-P1 Ab in a 1:4 ratio (Fig. 3). Normal rabbit IgG had no effect on aggregation.

P1 increased the level of E-cadherin in breast MCF7, prostate LNCaP, and DU145 cancer cells compared with untreated control cells as determined by Northern and Western blot analyses. Western blot analysis of lysates of untreated DU145 cells or cells treated with several concentrations of peptide P1 showed that the intensity of E-cadherin bands was proportional to the concentration of P1 peptide used (Fig. 4).

In contrast, the other peptides tested in a wide range of concentrations for 96 h induced no morphological or biochemical changes on the cells studied.

Peptide P1 was given i.p. at doses of 25 µg twice daily to SCID mice carrying s.c. DU145 tumors. Control tumors ($n = 12$) grew rapidly. In contrast, in animals treated with 25 µg of P1 ($n = 13$), the growth of tumors was continuously inhibited. By day 18, the difference between the tumor volumes in control and experimental groups was statistically significant ($P < 0.031$; Fig. 5). The experiment was repeated three times with a smaller number of animals in each group ($n = 4-7$ mice). Statistical comparison of the ATTVs for control animals across all four experiments (mean ATTV = 617

Fig. 4. Immunoblot analysis for E-cadherin of lysates of DU145 cells treated with P1. (A) Cell lysates (15 µg of protein) from DU145 control, untreated cells (lane 1), and treated with 1 µg (lane 2) or 10 ng/ml P1 (lane 3), were separated on SDS/PAGE and then immunoblotted with anti E-cadherin Ab. (B) Actin determination by Western blot. (C) Densitometric reading (%) of normalized E-cadherin as determined from A and B.

Fig. 5. Growth of DU145 tumors in SCID mice treated with 25 µg of peptide P1 twice daily.

mm³, $n = 26$ mice) with animals treated with 25 µg of P1 twice daily (mean ATTV = 443 mm³, $n = 30$ mice) revealed a statistically significant difference ($P < 0.002$, $F = 10.4$, $df = 1.51$) by pooled analysis using two-way ANOVA. Doses of P1 of 2.5 µg and 250 µg per mouse by using the same regimen did not inhibit the growth of DU145 tumors (data not shown).

We examined the tumors from control animals and animals treated with 25 µg of P1 at the 18th day of the experiments for the Ki-67 (MIB1) labeling index by immunohistochemical staining. The Ki-67 index was lower in tumors from animals treated with 25 µg of P1 (10%) than in control tumors (30%) (Fig. 6). These experiments were repeated twice and yielded similar results.

Polyclonal Antibodies Against P1 Peptide. Affinity-purified rabbit polyclonal antibodies raised against peptide P1 reacted against the peptide P1, extracts of rat and bovine pituitary, and lysates from GH3 rat pituitary tumor cell line and produced a band with a molecular mass of ≈45 kDa, higher than the calculated molecular mass of rTDF protein expressed in *E. coli* (Fig. 7). No activity was found against BSA, protein standards, or lysates from mouse normal fibroblast NIH 3T3 cells.

Experiments were conducted to determine whether the differences in molecular mass are due to posttranslational modification, like glycosylation. Fig. 8 shows that the treatment of GH3 lysate with glycosidase F or H reduced the TDF size from ≈45 kDa to ≈35 kDa. These results demonstrate glycosylation of TDF protein.

rTDF Expression in *E. coli*. We next prepared the rTDF in *E. coli*. Translation of the TDF cDNA resulted in a predicted protein of 108 amino acids with a relative molecular mass of ≈12,740 Da. A band at ≈17 kDa representing the CBP-FLAG-rTDF protein was revealed by Western blot analysis by using, first, anti-P1 Ab (Fig. 7, lane 4) and, then, anti-FLAG Ab (not shown). The successful isolation of the rTDF was confirmed by partial amino acid sequencing.

In Vitro Effect of rTDF. The same bioassay described (6) was used for the assessment of the *in vitro* effect of rTDF on MCF7 and DU145. The rTDF protein induced morphological changes similar to, although weaker than those produced by the PTE on these cancer cells. The cells aggregated and formed spheroid-like structures within 24 h (Fig. 9).

Fig. 6. Immunohistochemical staining with MIB1 for the Ki-67 of DU145 tumors from untreated control SCID mice (A) and mice treated twice daily with 25 µg of peptide P1 (B) for 18 days.

Fig. 7. Immunoblot analysis for TDF. Lysates (15 μ g of protein) from rat pituitary tumor MtTW10 (lane 1), bovine pituitary extract (lane 2), GH3 rat pituitary tumor cells (lane 3), and CBP-FLAG-rTDF protein (lane 4) were separated on SDS/PAGE and then immunoblotted with anti-P1 Ab.

Biochemical changes paralleled the morphological changes. Western blots prepared with lysates of MCF7 cells treated with different concentrations of rTDF showed a positive dose-response correlation with E-cadherin levels (Fig. 10). Similar changes occurred with DU145 cells (data not shown).

Western blot analysis for lactalbumin in the lysates of MCF7 cells treated with several concentrations of rTDF for 48 h showed a band at 35 kDa. A very strong band with an expected molecular mass of 17 kDa and a weak band at 35 kDa were present in the lactalbumin standard (Sigma). Both bands were abolished by preincubation of the antibodies with lactalbumin or when the specific antibodies were substituted by normal IgG. Lysates from untreated control MCF7 cells did not react with anti-lactalbumin antibodies (Fig. 11).

TDF Is Present in Human Sera. Sera from several healthy adult men and women and from prostate and breast cancer patients reacted with anti-P1 Ab. A band of $\approx$ 45 kDa was detected in all sera tested, showing different levels of expression (Fig. 12). This band is highly specific because preincubation of the Ab with the peptide P1 abolished the signal. Preliminary data show that, in 66% of the normal sera ($n = 25$), the TDF level was higher than in sera from breast cancer patients ($n = 25$).

Discussion

We reported that an alkaline extract from mammosomatotropic tumor MtTW10 induced the aggregation and differentiation of rat and human breast cancer cell lines (6), manifested by polarization, formation of cell junctions and basement membrane, milk protein synthesis (16, 17), and overexpression of E-cadherin (18–22), which are all markers of differentiation. Similar effects were obtained on human prostatic carcinoma cells, which showed increased E-cadherin (23–25) and prostate-specific antigen (26, 27) levels, but not on fibroblasts, hepatoma, kidney cancer, or leukemic cells. It is possible that this differential response to TDF is due to the presence or absence of TDF receptors. The differentiation was not reproduced by any of the known hormones or growth factors produced by the pituitary (prolactin, growth hormone, thyroid-stimulating hormone, luteinizing hormone, corticotropin, oxytocin, vasopres-

Fig. 8. Effect of glycosidase treatment on the electrophoretic mobility of TDF. Thirty micrograms of protein of GH3 lysate, untreated (lane 1) or treated with glycosidase H (lane 2) or F (lane 3), was subjected to SDS/PAGE and then immunoblotted with anti-P1 Ab as described in *Materials and Methods*.

Fig. 9. Morphology of DU145 cells after 72 h of culture in serum-free RPMI medium 1640 in the absence (A) or presence (B) of 10 ng/ml rTDF.

sin, epidermal growth factor, platelet-derived growth factor, transforming growth factors, and insulin growth factors), tested alone or in combination, over a wide range of concentrations, suggesting that this is a previously unrecognized pituitary factor. We called the molecule responsible for this activity TDF.

Because we were not able to purify the factor responsible for the biological activity from the PTE by using the conventional chromatographic methods, we selected expression cloning. This method has been used for cloning and sequencing of numerous proteins (9, 28–30). It does not need purification of the product or any knowledge of the amino acid sequence of the protein to be cloned. However, it requires a biological assay to detect the presence of the target protein in an expression system. In our studies we used *Xenopus* oocytes as an expression system (14, 15, 31), because the proteins synthesized in oocytes undergo posttranslational modifications and then are included in the oocyte membrane or secreted into the medium, similarly to the cells of mRNA origin. Production of a protein that causes aggregation and differentiation of breast and prostate cancer cells was assessed by a bioassay that has been shown to be satisfactory. The TDF clone isolated was then sequenced and found to have only a distant homology with other sequences from the GenBank database, indicating that TDF is the product of a hitherto undescribed gene.

Northern blot hybridization analysis with a TDF probe showed that TDF mRNA was detected as a transcript of 1.1 kb only in human brain tissue, but not in other organs. We assume that TDF is produced in the pituitary, because we isolated the TDF clone from a pituitary cDNA library and TDF mRNA and TDF protein were found in the pituitary. The presence of TDF mRNA in the brain could be explained by contamination of the brain with the pituitary or because the TDF is also present in some parts of the brain.

P1, a peptide consisting of 20 amino acids selected from the TDF ORF sequence, induced changes similar to those produced by the PTE on MCF7 breast cancer and LNCaP androgen-sensitive and DU145 androgen-independent prostate cancer cells. P1 (25 μ g per mouse) also inhibited the growth of DU145 cells in SCID mice. Doses of 250 μ g of P1 per mouse were ineffective, possibly because P1, like estrogens (32, 33), exhibits a biphasic dose response.

The Ki-67 mitotic index, which is a useful prognostic indicator of biologic aggressiveness in malignancies (34, 35), was significantly lower in tumors from animals treated with peptide P1 than in control tumors, suggesting that TDF acts also on cell proliferation.

Fig. 10. E-cadherin expression level in MCF7 cells treated with rTDF. Cell lysates (15 μ g of protein) from MCF7 untreated control cells (lane 1) and cells treated with 10 ng (lane 2), 50 ng (lane 3), 500 ng (lane 4) of rTDF per ml were separated on SDS/PAGE and then immunoblotted with anti-E-cadherin Ab (Upper) and with β -actin Ab (Lower) as described in *Materials and Methods*.

Fig. 11. Immunoblots for lactalbumin. Cell lysates (15 μ g of protein) of MCF7 untreated control cells (lane 2) or cells treated with 10 ng (lane 3) or 100 ng (lane 4) rTDF per ml and lactalbumin standard (Sigma) (lane 1) were separated on SDS/PAGE and then immunoblotted with anti-lactalbumin Ab (Sigma) as described in *Materials and Methods*.

A polyclonal rabbit Ab raised against peptide P1 and purified by affinity chromatography reacted not only with peptide P1, but also with the PTE and with the rTDF expressed in *E. coli*. In addition, serum samples from healthy men and women and from breast and prostate cancer patients reacted with this Ab. This band was highly specific, because it was abolished when the Ab was blocked with peptide P1. This Ab was also used to demonstrate that the aggregation of breast and prostate tumor cells is specifically due to the treatment with TDF. Simultaneous treatment of breast or prostate cancer cells with TDF and anti-P1 Ab prevented their aggregation. Translation of the TDF cDNA in *E. coli* resulted in a protein with a predicted relative molecular mass of ≈ 12.7 kDa, which is less than the molecular mass observed for the native TDF from GH3 rat pituitary tumor cells and rat and bovine pituitary extract or serum (≈ 45 kDa) by SDS/PAGE. The differences in the actual molecular mass of a protein and its electrophoretic mobility in SDS/PAGE could be due to either posttranslational modifications (e.g., glycosylation and/or phosphorylation) and/or to specific regions that bind SDS anomalously and affect electrophoretic mobility (36, 37). The expression of rTDF in *E. coli* typically leads to the production of proteins that are not modified posttranslationally. Therefore, analyses of the mass of recombinant forms by SDS/PAGE often reveal proteins of lower molecular mass than the native proteins.

Our data show that the treatment of GH3 extract with glycosidase F or H reduced the TDF size from ≈ 45 to ≈ 35 kDa. These results demonstrate that the TDF protein undergoes posttranslational glycosylation. Because TDF protein contains three asparagine residues, it is possible that glycosylation of these residues could explain the increase in the size of TDF from the calculated molecular mass of 12.7 kDa to the found molecular mass of 45 kDa.

MCF7 and DU145 human cancer cells underwent morphological and biochemical changes in response to rTDF. Cells aggregated and

Fig. 12. Immunoblot analysis for TDF of human sera. Sera (1.5 μ g of protein) from healthy individuals (lanes 4, 5, 8, and 9), women with breast cancer (lanes 1–3), and men with prostate cancer (lanes 6 and 7) were electrophoresed on a SDS/PAGE gel and immunoblotted with anti-P1 Ab as described in *Materials and Methods*.

formed spheroid-like structures. The lack of posttranslational changes of rTDF could explain its weaker effect on breast and prostate cancer cells as compared with the effect obtained with the PTE. These changes could be involved in the biological function of the protein.

Biochemical changes paralleled the morphological changes. Western blots prepared with lysates from MCF7 cells treated with rTDF showed E-cadherin expression increasing in linear correlation with rTDF concentration. Lactalbumin-related protein was also detected by Western blot analysis in TDF-treated MCF7 cells, but not in untreated control cells. The size of the detected lactalbumin band was higher than the expected molecular mass. The lactalbumin control, in addition to the expected band at 17 kDa, showed a weaker band at 35 kDa. Both the 17- and 35-kDa bands were absent when the antiserum was saturated with lactalbumin or when specific antibodies were replaced with normal IgG, suggesting that they are immunologically closely related to lactalbumin. Because lactalbumin is present only in normal breast epithelial cells, and a decreased E-cadherin level is usually associated with a more malignant phenotype, the increase of E-cadherin (18–22), aggregation of cells (38–40), and synthesis of lactalbumin (16, 17) suggest the conversion to a more benign, less aggressive growth pattern of breast cancer cells on treatment with rTDF. Taken together, these data indicate that a hitherto undescribed pituitary gene codes for a protein that can partially restore the differentiation of breast and prostate cancer cells.

The TDF protein is found in the serum of men and women. TDF levels in normal sera are higher than in sera from breast cancer patients. The fact that all activities of the tumor differentiation factor can be mimicked by a peptide derived from the ORF of its cDNA opens the possibility of further therapeutic investigation.

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