Transferrin in the Rat Prostate Dunning Tumor

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ABSTRACT

A major protein of the rat Dunning prostate tumor has been purified. It has physicochemical properties and an amino acid composition similar to that of transferrin. Furthermore, the isolated tumor protein reacts with antiserum to authentic rat transferrin. Immunoperoxidase staining with rabbit anti-rat transferrin localizes transferrin within tumor acinar glands. Rocket immunoelectrophoresis indicates that transferrin constitutes 30 to 40% of tumor fluid protein, but accounts for only ~9% of total serum protein. In normal rat prostate cytosols, the level of transferrin is at least 200 times lower in tumor cytosol. Nevertheless, dorsal and lateral prostate show variable peroxidase staining indicating the presence of immunoreactive transferrin within acinar glands of these normal tissues. While intense staining for transferrin was found in the interstitium of all regions of the normal prostate, transferrin was not detected within acinar glands of coagulating gland, ventral prostate, or seminal vesicle. Immunocytochemical localization of albumin indicates a distribution similar to that of transferrin in normal and neoplastic rat prostate. However, unlike transferrin, the albumin content was lower in tumor fluid than in serum. It is suggested that the high level of transferrin in tumor fluid may be due to selective uptake by the tumor from serum.

INTRODUCTION

The rat Dunning prostate tumor (R3327) is a well-differentiated adenocarcinoma that has remained androgen dependent through more than 15 years of passage at the Papanicolaou Cancer Research Institute, Inc., Miami, Fla. The tumor has a normal high titer of androgen receptor (16, 18, 33) and is believed to have originated spontaneously from the dorsal prostate of an old rat (6). Although the presence of several enzymes supports this theory of origin (19, 28), the major secretory proteins of dorsal prostate are not expressed by the tumor (34). In our search for a major androgen-dependent protein in the Dunning tumor, we have identified transferrin in high concentration in tumor fluid and cytosol. We report here the purification and identification of this major tumor protein and its distribution as revealed by peroxidase immunocytochemistry.

MATERIALS AND METHODS

Rabbit anti-rat transferrin and rabbit anti-rat albumin were purchased from Cappel Laboratories, Cochranville, Pa.; DEAE-Sepharose and Sephadex G-200 were from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; electrophoresis reagents were from BioRad, Rockville Centre, N. Y.; Trisma base, ovalbumin, rat serum albumin, myoglobin type 1 of equine skeletal muscle, and carboxypeptidase B of hog pancreas were from Sigma Chemical Co., St. Louis, Mo.; bovine γ-globulin (Fraction II) was from Miles Laboratories, Elkhart, Ind.; and reagent grade chemicals were purchased from Fisher Scientific Co.

Animals. Copenhagen Fischer rats bearing the Dunning prostate tumor (R3327) were obtained from the Papanicolaou Cancer Research Institute. Tumor tissue was implanted s.c. at the Cancer Institute and was allowed to grow to a tumor diameter of 2 to 4 cm.

Preparation of Cytosols and Fluids. Copenhagen Fischer rats bearing s.c. implants of the Dunning tumor were usually castrated since tumors were also used for studies on the androgen receptor (33). Testes were removed through an abdominal incision under ether anesthesia. After 24 hr, rats were decapitated using a guillotine. The dorsal, lateral, and ventral prostate, coagulating glands, and Dunning tumors were rapidly removed. Fluid was expressed from the luminal compartment of the coagulating gland. Tumor fluid was collected with a Pasteur pipet from fluid-filled cysts of the Dunning prostate tumor that were revealed during surgical removal of the tumor. Tumor fluid was usually straw colored and rather viscous. Tissues were rinsed in 0.9% NaCl solution, quick frozen in liquid N₂, and stored at ~70°C. Cytosols from the various frozen tissues were prepared as described previously (33).

Ejaculated sperm plugs were collected following decapitation and processed as for cytosol preparation. Blood was collected following decapitation and was allowed to clot for 2 hr at room temperature and then overnight at 4°C. Serum was obtained by centrifugation and stored at ~20°C.

Purification of Transferrin from the Dunning Tumor. A major tumor protein identified as transferrin (see “Results”) was purified from Dunning prostate tumor cytosol. The pellet of a 40 to 60% (NH₄)₂SO₄ fraction of tumor cytosol was resuspended in one-tenth the original volume of cytosol in 50 mM Tris (pH 7.5):1 mM EDTA:10 mM KCl; following dialysis, the pellet was applied to a DEAE-Sepharose column (1.6 x 15 cm) equilibrated in the same buffer. The flowthrough fraction was dialyzed against 2 mM Tris (pH 7.5) overnight at 4°C and applied to a second DEAE-Sepharose column equilibrated in 2 mM Tris (pH 7.5). A 200-ml gradient from 0 to 0.2 M KCl in 2 mM Tris (pH 7.5) was applied. The major peak eluting at approximately 0.1 M KCl was pooled, dialyzed, and lyophilized. The protein was resuspended in 50 mM Tris (pH 7.5), 1 mM EDTA and chromatographed on a Sephadex G-200 column (64 x 2.6 cm) while collecting fractions of 3.3 ml. The major peak eluting at 38 Å was pooled, dialyzed against distilled H₂O, and lyophilized. The resuspended protein was rechromatographed on DEAE-Sepharose equilibrated in 2 mM Tris (pH 7.5), and was eluted with a KCl gradient as described above. Protein recovered from the peak eluting at about 0.1 M KCl was found to be pure by SDS²: polycrylamide gel electrophoresis, as shown in Fig. 1, Gel 1. When lyophilized, the protein appeared white, suggesting the absence of iron in the preparation.

Preparation of Antiserum. The antisera used to detect transferrin by rocket immunoelectrophoresis and immunocytochemistry was purchased from Cappel Laboratories. We also prepared an antiserum to the purified tumor protein (identified as transferrin). Two rabbits were immunized with 150 μg purified tumor protein emulsified by homologization in 2 ml sterile 0.9% NaCl solution, 2 ml complete Freund’s
adjuvant, and 20 mg *Microbacterium butyricum* as described previously (30). Two months later, one rabbit showed an antibody reaction by double diffusion. At this time, the rabbit received a booster injection of 120 µg tumor protein. Antiserum collected 8 days later produced a precipitin band on double diffusion, and faint rockets when assessed by rocket immunoelectrophoresis as described below. This antibody was used for some immunocytochemistry studies (see Fig. 3B). The staining reaction was similar for the prepared antibody and for that purchased from Cappel Laboratories.

Rocket Immunoelectrophoresis. Rocket immunoelectrophoresis was carried out as described previously (3, 34). Lyophilized rabbit anti-rat transferrin was restored in 2 ml sterile distilled water. An aliquot of 75 µl was added to 35 ml of a 1% agarose solution at 55°. Protein samples in 5 µl were applied under 50 V. Electrophoresis was for 4 hr at 200 volts while cooled with circulating water at 10°. A rocket height of 42 mm corresponded to approximately 1 µg purified tumor protein (transferrin) when measured by the assay of Lowry et al. (17).

Immunocytochemical Localization. Tissues were removed and fixed in phosphate-buffered formalin (10%) for 48 hr or by perfusion as described previously (20). Rabbit antiserum to rat transferrin obtained from Cappel Laboratories was used to localize transferrin in sections of the prostate and the Dunning tumor as described previously using the peroxidase-immunoglobulin bridge technique (21, 22).

**RESULTS**

Biochemical Studies. Fluid collected from cysts in the rat Dunning prostate tumor had a protein concentration ranging from 5 to 14 mg/ml. SDS:polyacrylamide gel patterns of tumor fluid revealed 2 major proteins (Fig. 1, Gel 3, Fig. 2) which were invariant despite differences in fluid protein concentration. Both proteins were also present as major bands on SDS:polyacrylamide gels of Dunning prostate tumor cytosol (Fig. 1, Gel 2). The upper of the 2 major bands (M.W. ~70,000) was purified (Fig. 1, Gel 1) as described in "Materials and Methods." Determination of its physicochemical properties indicated a sedimentation coefficient of 5.3 s and a Stokes radius of 38 Å. A hydrodynamic M.W. of 86,800 (Table 1) was calculated as described previously (27). The purified tumor protein had a sedimentation coefficient, Stokes radius, molecular weight, frictional ratio, and amino acid composition similar to those of transferrin (Table 1). Furthermore, the purified tumor protein formed a single precipitin line with antiserum to authentic rat transferrin. Thus, transferrin is a major protein of the Dunning prostate tumor.

Transferrin was quantitated by rocket immunoelectrophoresis as shown in Fig. 2 using a commercial antiserum to authentic rat transferrin. According to this technique, transferrin represents 30 to 40% of total cyst fluid protein (Table 2), which is in good agreement with the relative concentration of transferrin revealed by the SDS:polyacrylamide gel pattern of tumor fluid (Fig. 1, Gel 3). By rocket immunoelectrophoresis, transferrin amounts to approximately 9% of total serum protein. However, considering the protein concentrations of tumor fluid (~10 mg/ml) and serum (~70 mg/ml), similar amounts of transferrin (3 to 6 mg/ml) are found in tumor fluid and serum.

A different distribution is found for albumin, the second major protein of tumor fluid (Fig. 1, Gels 3 and 6). By rocket immunoelectrophoresis, albumin amounts to ~24% of total tumor fluid protein and 46% of serum protein. Again, considering total protein concentrations, tumor fluid contains 2 to 3 mg albumin per ml, while serum contains 30 to 35 mg albumin per ml. Thus, it appears that, in comparison with albumin, transferrin is about 10-fold enriched in tumor fluid over serum. Accordingly, the presence of transferrin in tumor fluid and cytosol is likely not due to serum contamination.

The protein pattern of rat prostate Dunning tumor cytosol (Fig. 1, Gel 2) and normal rat dorsal prostate cytosol (Fig. 1, Gel 4) differ in several respects on SDS:polyacrylamide gels. Two major proteins are present in dorsal prostate (Fig. 1, Gel 4) and coagulating gland (M.W. ~62,000 and M.W. ~80,000). These proteins, referred to previously as Dorsal Proteins I and II (34), are major secretory proteins and are abundant in seminal fluid of the ejaculate (Fig. 1, Gel 5). They are not present, however, as major components in Dunning rat tumor cytosol (Fig. 1, Gel 2) or fluid (Fig. 1, Gel 3). Furthermore, Dorsal Proteins I and II cannot be detected in tumor cytosol or fluid by rocket immunoelectrophoresis or by immunocytochemistry (not shown). We have suggested previously that androgen-
Regulated gene expression appears to be altered in the Dunning tumor (35).

A low concentration of transferrin in normal prostate cytosol determined by rocket immunoelectrophoresis is another difference between normal and tumor prostate cytosols. Transferrin concentrations in all regions of the normal rat prostate, including dorsal, ventral, and lateral prostate and coagulating gland (anterior prostate) are more than 200-fold lower than in Dunning prostate tumor cytosol (Table 2). Table 2 shows in addition that the transferrin concentration of liver and kidney cytosol is low when compared with tumor prostate cytosol, providing additional evidence that the greatly increased level of transferrin in the Dunning rat prostate tumor is an unusual property of the tumor. A protein band corresponding to transferrin is also not readily detectable on SDS-polyacrylamide gels of dorsal prostate cytosol (Fig. 1, Gel 4) or in the ejaculated seminal fluid (Fig. 1, Gel 5).

In contrast to transferrin, albumin is detected as a major protein in normal prostate cytosol as well as in tumor cytosol and fluid (Fig. 1, Gels 2 to 4). Note that, in dorsal prostate cytosol (Fig. 1, Gel 4), the albumin band has been deflected slightly upward due to the abundance of the major dorsal prostate secretory protein DP I (34). Similar albumin concentrations were found in cytosols of normal and neoplastic prostate (Table 2).

**Immunocytochemical Studies.** We have used immunocytochemistry to localize transferrin within the Dunning prostate tumor and in normal rat prostate tumor. Histologically, the Dunning tumor is well differentiated in that it contains acinar glands that are characteristic of the rat prostate (Fig. 3). However, lack of a ductal system in the tumor results in the formation of cysts containing fluid of distinct protein composition (Fig. 1, Gel 3). A major component of tumor fluid has been identified as transferrin (see above). The distribution of immunoreactive transferrin also indicates that transferrin is concentrated within acinar glands of the tumor (Fig. 3A). In large tumors, transferrin is predominant in glands near the periphery (Fig. 3B), while in smaller tumors, transferrin is detected within the lumina of glands throughout the tumor (Fig. 3C). The lack of transferrin in central regions of the larger (>2 cm in diameter) tumors likely results from a low rate of metabolism due to a deficient blood supply.

Immunocytochemistry of normal rat prostate with anti-rat transferrin shows intense staining for transferrin within interstitial spaces throughout the prostate glands (Fig. 4). Staining within acinar glands is also seen in dorsal and lateral prostate, although it is of variable intensity (Fig. 4, A and B). Many acini display moderate peroxidase stain, while others are faint. Immunoreactive transferrin is, however, essentially undetectable in coagulating gland and ventral prostate (Fig. 4, C and D). Seminal vesicle also shows no staining of transferrin within its glandular compartment (not shown). Thus, immunocytochemistry, a technique with high sensitivity, reveals a distribution of transferrin within the normal prostate that is not apparent by rocket immunoelectrophoresis. It is of interest that dorsal prostate shows a more intense staining reaction for transferrin within acinar glands than do other regions of the prostate, since the dorsal prostate is thought to be the site of origin of the Dunning tumor (6, 19, 28). The coagulating gland develops from a different embryonic structure than dorsal prostate (23), although it secretes the same major androgen-dependent proteins as dorsal prostate (34).

We have also examined the immunocytochemical distribution of albumin within the Dunning tumor and normal rat prostate. As noted above, albumin is a major component of tumor fluid (Fig. 1, Gel 3). Like transferrin, it is distributed within acinar glands throughout certain tumors (Fig. 3D); however, its concentration relative to transferrin is not enriched in tumor fluid (Table 2). Staining of identical tumor sections suggests that the presence of albumin within acinar glands parallels that of transferrin. The normal prostate also reveals a remarkable similarity between albumin and transferrin staining, in that dorsal prostate (Fig. 5A), and to a lesser extent, lateral prostate (Fig. 5B), show more albumin staining within acinar glands than do the coagulating gland or ventral prostate (Fig. 5, C and D). Albumin staining within the lumina of dorsal and lateral prostate glands was variable, with some regions of moderate-to-heavy staining and others with light-to-nearly-undetectable staining.

**DISCUSSION**

We have purified and characterized a major protein of the Dunning rat prostate tumor whose physicochemical and immunocytochemical properties are essentially identical to rat transferrin. The increased content of transferrin in tumor fluid (10 times higher than in serum) and its immunocytochemical staining within acinar glands suggests that transferrin may be selectively taken up by the tumor from serum. Albumin was also present in tumor fluid and revealed a staining pattern similar to transferrin, but was present at a 10-fold lower concentration than in serum. The dorsal prostate, from which the tumor is thought to be derived (6, 19, 28), appears to allow the passage of transferrin and albumin into the lumina of its acinar glands, although at low levels. Perhaps this property of the dorsal prostate has been augmented by the tumor, particularly with regard to transferrin.

High transferrin levels in the neoplastic prostate may be due to an increased uptake from blood or to the induction of
synthesis of transferrin. Our preliminary results with the in vitro translation of polyadenylic acid RNA isolated from the Dunning tumor indicate that transferrin is not produced in detectable amounts by the tumor. A more likely possibility is, therefore, that transferrin is taken up in high amounts from blood.

The function of transferrin is to bind plasma iron (Fe3+) with very high affinity (1024 M−1) (2) and deliver it to cells with high growth requirements. Iron-bound transferrin has been shown to be a primary factor required for the growth of cells in serum-free medium (24). It is taken up by cells via receptor-mediated endocytosis (12). Membrane receptors have been demonstrated on cells that have high iron requirements, such as reticulocytes (36), placental cells (26), and on various cultured cells (10). The entry of transferrin into cells is a prerequisite for the assimilation of iron bond to transferrin (11). One hypothesis concerning the fate of these molecules is that internalized iron-bound transferrin is transported to lysosomes where the low pH induces dissociation of iron. Transferrin itself appears to escape degradation in the lysosome. Rather, it is reported to be recycled into the medium intact, at least in teratocarcinoma stem cell cultures (13). In the case of the prostate tumor, our results raise the possibility that transferrin may be carried across the cell, perhaps in association with endocytotic vesicles, and released undegraded to the interior of prostate acinar glands.

The high level of transferrin in Dunning rat prostate tumor fluid correlates with that observed in human prostatic carcinoma (9). Greater than 90% of patients with prostatic carcinoma had levels of transferrin in prostatic fluid that were 4 to 5 times higher than controls (9). No statistical elevation in serum transferrin levels has been noted, however, in sera of patients with prostate cancer (9) or in rats containing Dunning tumor implants.

That transferrin is selectively taken up by the Dunning prostate tumor and by human prostatic carcinoma is supported by the well-known affinity of certain soft-tissue neoplasms for 67Ga (7). Both 59Fe and 67Ga appear to be taken up through the same mechanism, i.e., the transferrin receptor (15). One could speculate that certain tumors have the capacity to assimilate iron at rates greater than in normal tissue, thereby providing the tumor with iron necessary for its rapid growth. Whether the large amount of transferrin within tumor acinar glands has a role in tumor development or maintenance remains to be investigated.

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Fig. 1. SDS-polyacrylamide gel electrophoresis of purified Dunning tumor transferrin, tissue cytosols, and fluids. An SDS-gel containing 8% acrylamide was prepared as described previously (14) with some modifications (34). Shown are protein patterns for: Gel 1, Dunning prostate tumor protein (transferrin) purified as described in 'Materials and Methods,' 6 µg; Gel 2, Dunning prostate tumor cytosol prepared as described previously (33), 45 µg protein; Gel 3, Dunning tumor fluid (15.5 mg/ml) collected from a fluid-filled cyst in the tumor, 20 µg protein; Gel 4, normal dorsal prostate cytosol, 40 µg protein; Gel 5, supernatant of a homogenate of ejaculated rat seminal fluid and sperm plug centrifuged 100,000 x g for 30 min, 75 µg protein; Gel 6, rat serum, 97 µg protein. Marker proteins are indicated for rat albumin (M.W. 64,000), ovalbumin (M.W. 45,000), and carboxypeptidase B (M.W. 35,000).

Fig. 2. Rocket immuno-electrophoresis of tumor and prostate protein fractions using anti-rat transferrin. Rockets are shown for: 1 and 2, purified tumor protein, 0.2 and 0.25 µg, respectively; 3 and 4, Dunning prostate tumor fluid collected from a cyst, 1.6 and 3.1 µg protein, respectively; 5 and 6, Dunning prostate tumor cytosol, 2.1 and 5.2 µg, respectively; 7 and 8, male rat serum, 3.7 and 7.4 µg, respectively; 9, dorsal prostate cytosol, 54 µg protein; 10, coagulating gland cytosol, 85 µg; 11, ventral prostate cytosol, 57 µg.
Fig. 3. Immunoperoxidase localization of transferrin and serum albumin in the androgen-dependent Dunning tumor (R-3327). Optimal dilution for anti-rat transferrin serum was 1:2,000 (A and C); for anti-transferrin serum raised in our laboratory against transferrin isolated from Dunning tumor, 1:1,000 (B); and for anti-rat albumin serum, 1:10,000 (D). Immunoreactive transferrin is responsible for dark heavy staining in acinar lumina of variable size and shape, and for lighter staining throughout the interstitial spaces. In larger tumors (>2 cm in diameter), staining was limited to peripheral acini (B), probably reflecting the pattern of blood supply; in smaller tumors, transferrin is stained with equal intensity in all acini (C). Rat serum albumin is uniformly distributed in all acini at an apparently lower concentration than transferrin (D). Sections were counterstained with toluidine blue. A, × 270; B, C, and D, × 35.
Fig. 4. Distribution of transferrin in the normal rat prostate as demonstrated by immunoperoxidase staining with anti-rat transferrin serum at a dilution of 1:2000. A, dorsal prostate; B, lateral prostate; C, coagulating gland; D, ventral prostate. Intense staining for transferrin in the interstitial connective tissue is visible in all regions of the normal rat prostate. Staining of variable intensity within acinar glands is seen in the dorsal (A) and lateral (B) prostate, while immunoreactive transferrin is virtually absent from the glandular compartments of the coagulating gland (C) and ventral prostate (D). Sections were counterstained with toluidine blue. A, × 108; B, × 86; C, × 270; D, × 108.
Fig. 5. Distribution of serum albumin in the normal rat prostate as demonstrated by immunoperoxidase staining with antiserum to rat serum albumin at the optimal dilution of 1:10,000. A, dorsal prostate; B, lateral prostate; C, coagulating gland; D, ventral prostate. Albumin, like transferrin, is present in the interstitial spaces in all regions of the normal rat prostate. Albumin is also present within acinar glands of the dorsal (A) and, to a lesser extent, of the lateral prostate (B). Less staining is detected within acini of the coagulating gland (C) and ventral prostate (D). Sections were counterstained with toluidine blue. × 67.
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