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Tissue-type transglutaminase expression in the Dunning tumor

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Summary. Transglutaminases with different functions and tissue distribution patterns can be distinguished by specific antibodies and by inhibition of enzyme activity in the presence of guanosine triphosphate (GTP). The most common form is the so-called tissue-type transglutaminase that is apparently involved in membrane stabilization processes, e.g. during apoptosis, and can be inhibited by incubation with GTP at low calcium concentrations. A secretory transglutaminase that cannot be inhibited by GTP is synthesized in an androgen-dependent manner in the dorsal prostate of the rat, the site suggested to represent the origin of the Dunning tumor used as an experimental model in prostate cancer research. Here we studied the expression of transglutaminases in different Dunning tumor lines – mainly in the highly differentiated H subline – and characterized the enzyme both biochemically and immunocytochemically. A very high enzyme activity was found only in the less well differentiated HI-F tumor line. Immunohistochemical reactions and Western blot analysis showed that there is no secretory transglutaminase present in any of the Dunning tumor lines studied. Transglutaminase activity of the Dunning tumor results from the so-called tissue-type enzyme that is non-organ specific. The absence of a secretory form of transglutaminase does not suport the contention of a prostatic origin o the Dunning tumor.

Key words: Dorsal prostate – Enzyme activity – Immunohistochemistry – Mammary gland – Secretory transglutaminase – Tissue-type transglutaminase

Transglutaminases (TGases, EC 2.3.2.13) are calcium-dependent enzymes that catalyze the formation of γ-glutamyl-ε-lysyl cross-links in peptides. They are found in a variety of tissues including liver (so-called tissue-type TGase), skin (epidermal and hair follicle TGase), platelets and plasma (blood clotting factor XIII), and rodent prostate (secretory TGase, "vesiculase" [7]). In the rat, both the dorsal prostate and the coagulating gland synthesize and secrete in an androgen-dependent manner a secretory TGase that has been characterized [15] and used for antibody production. As we have shown previously [16], this protein is released from the dorsal prostate in an apocrine fashion.

The Dunning tumor system [13] is one of the most widely used experimental animal models of rat prostate cancer, as it offers the advantage of several well-established tumor lines with characteristic differences in proliferative, metastatic and clonogenic activities, hormone responsiveness, and morphology. It was first described [8] as a papillary adenocarcinoma with glandular formation similar to the dorsal prostate, detected in a retired breeder rat of the 54th inbred generation of the Copenhagen strain, where it had spontaneously developed. Romjin et al. [14] reported that TGase activity in prostate cancer appeared to be inversely correlated with the metastatic potential of the tumor, as had been found in other tumors [4, 12].

To elucidate the character of the TGase present in the Dunning tumor we carried out biochemical and immunological studies of TGase in different lines of the Dunning tumor. The findings clearly demonstrate that the TGase of the Dunning tumor is of the tissue-type. No secretory TGase is present, even in the most highly differentiated H tumor. This finding supports our previous arguments against the prostatic origin of the Dunning tumor.

Materials and methods

Materials

[1,4(n)-^{3}H]putrescine dihydrochloride (specific activity 22.9 Ci/mmol) was purchased from Amersham-Buchler (Braunschweig, FRG). Putrescine dihydrochloride, phenylmethylsulfonylfluoride (PMSF), *N,N*-dimethylcasein, Trasylol, and monodansylcadaverine were obtained from Sigma (Munich, FRG).

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Tissue

Rat tissues (ventral, dorsolateral prostate, seminal vesicle, coagulating gland and its secretion, mammary gland) were removed from intact male or lactating female Wistar rats (250–280 g body weight; Ivanovas, Kisslegg, FRG) after cervical dislocation of the animals in deep ether anesthesia and rapidly frozen in liquid nitrogen. Dunning tumor explants (line G, line H, and their established sublines HI-F, AT-2, AT-3, Mat-Lu, Mat-LyLu) were kindly provided by Dr. J. Isaacs (Brady Urological Research Laboratories, Johns Hopkins University Medical School, Baltimore, USA) and were grown in Copenhagen rats (Harlan-Orlac Ltd., Bicester, UK) to a tumor size of 0.5 cm in diameter. Tumors were removed from animals after they had been killed, rapidly frozen in liquid nitrogen, and stored at −80°C.

Tissue preparation

Frozen samples were placed in ice-cold homogenization buffer (10 ml/g tissue) composed of 100 mM TRIS [tris(hydroxymethyl)aminomethane]/HCl buffer, pH 7.6, 5 mM dithioerythritol (DTE), and 3 mM ethylene glycol-bis(2-amino-ethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) in bidistilled water containing Trasylol (50 KIU/ml buffer) and 1 mM PMSF. Tissue samples were disrupted with an Ultraturrax and further homogenized with a Potter-Elvehjem homogenizer (ten strokes at 10 s each). Homogenates were centrifuged at 100000×*g* for 60 min at 4°C. The supernatants were used for protein determination [6], enzyme activity tests, and Western blotting.

Enzyme activity determinations

TGase activity was determined by quantitating the incorporation of [^{3}H]-putrescine into dimethylated casein. Unless otherwise indicated, 2 mM free Ca^{2+} was used in activity tests.

Substrate solution consisted of 65 µl casein solution [1% (w/v) *N,N*-dimethylcasein, 15 mM $CaCl_2$ in homogenization buffer] and 65 µl putrescine solution (310 µM n-putrescine and 22.9 µCi [1,4(n)-^{3}H]putrescine equalling 1 nmol/ml in homogenization buffer) equilibrated for 5 min at 37°C in a shaking water bath. The reaction was started by adding 65 µl ice-cold sample (2.5 mg protein/ml) to the prewarmed solution and the incubation continued at 37°C. Samples of 60 µl each were taken at 3, 6 and 9 min and transferred into 500 µl 10% trichloroacetic acid (TCA). After incubation for 30 min on ice, samples were centrifuged (2 min at 10000×*g*). The pellets were washed twice with 5% (w/v) TCA and finally with 95% ethanol, dissolved in 450 µl 0.1 M NaOH solution and mixed with 5 ml scintillation cocktail (Rotiscint; Roth, Karlsruhe, FRG). Incorporated radioactivity was counted in a Wallac scintillation counter (Pharmacia-LKB, Freiburg, FRG).

Controls were performed by measuring the TGase activity in the presence of an excess of EGTA (5 mM). The specific enzyme activity was defined as the amount of putrescine (labeled and unlabeled) that was incorporated into dimethylated casein per minute by 1 mg tissue protein present in the 100000×*g* supernatant.

As tissue-type TGase is known to be inhibited by GTP at low Ca^{2+} concentrations [1] the effect of GTP on TGase activity was tested. Enzyme activity was measured at GTP concentrations of 0.1, 0.25, 0.5, and 1.0 mM and Ca^{2+} concentrations of 2, 1, and 0.5 mM.

Western blot analysis

Western blotting was performed according to Towbin et al. [19] using the semi-dry technique. Briefly, samples were separated by SDS-PAGE (stacking gel, 4% acrylamide; separating gel, 7.5%–20% acrylamide) in a Mini-Protean II -System (BioRad, Munich,

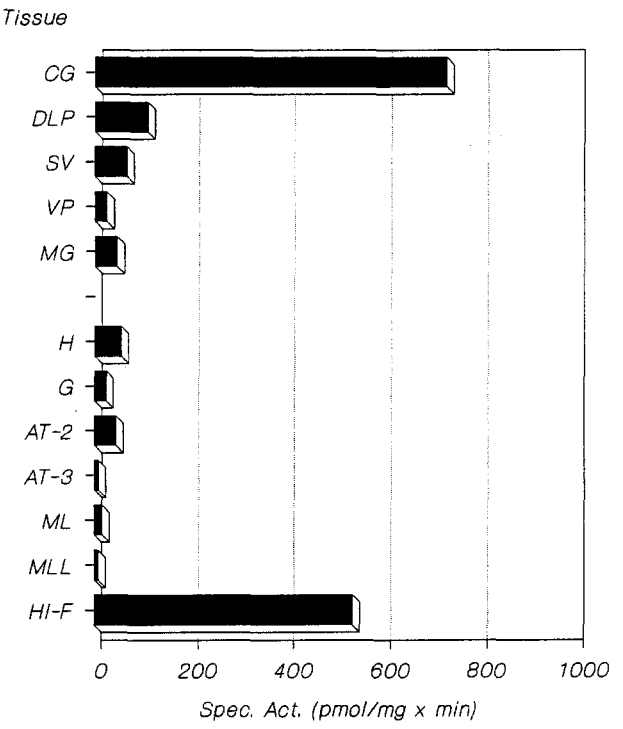

Fig. 1. TGase activity in rat accessory sex glands and Dunning tumor lines: coagulating gland *(CG)*, dorsolateral prostate *(DLP)*, seminal vesicle *SV)*, ventral prostate *(VP)*, mammary gland *(MG)*, Dunning tumor lines *(H, G, AT-2, AT-3, HI-F)*, *MLL*, (Mat-LyLu), *ML* (Mat-Lu)

FRG) and transblotted onto nitrocellulose sheets. Nitrocellulose membranes were incubated with a polyclonal rabbit antiserum against secretory TGase from rat coagulating gland [15] or a monoclonal mouse antibody against tissue-type TGase ([5]; kindly provided by Dr. P. Birckbichler, Noble Foundation, Ardmore, OK). Signal detection was performed with swine anti-rabbit Ig followed by rabbit peroxidase anti-peroxidase (PAP) complex [18] or with peroxidase-labeled anti-mouse IgG, respectively.

Visualization of peroxidase activity was performed with *p*-3,3' diaminobenzidine (DAB) [11]. In control experiments, the primary antibody was replaced by rabbit-pre immune serum or phosphate-buffered saline (PBS).

Immunohistochemistry and enzyme histochemistry

Frozen tissue and tumor samples were cut in a cryostat at 4 µm thickness, mounted on chromalum-gelatin subbed slides and briefly fixed in ice-cold acetone, followed for 10 s in ice-cold methanol, and washed for 10 min in PBS. Primary antibodies were the above-mentioned monoclonal antibody against tissue-type TGase [5] and a polyclonal antibody against secretory TGase [15]. Detection was performed using fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG or anti-rabbit IgG, respectively. Sections were covered with a drop of 80% glycerol in PBS and a cover slip.

In addition, tissue sections were processed for visualization of TGase enzyme activity using monodansylcadaverine as a substrate. Monodansylcadaverine, a substituted pentamethylenediamine, is covalently linked to the slices by TGase enzyme activity, where its localization can be visualized by fluorescence of the dansyl group. To exclude a nonspecific adsorption of monodansylcadavarine to the slices, controls were made by preincubation of the slices with an excess of EGTA (10 mM). Slices were examined in a Zeiss axiomat photomicroscope equipped with epifluorescence optics.

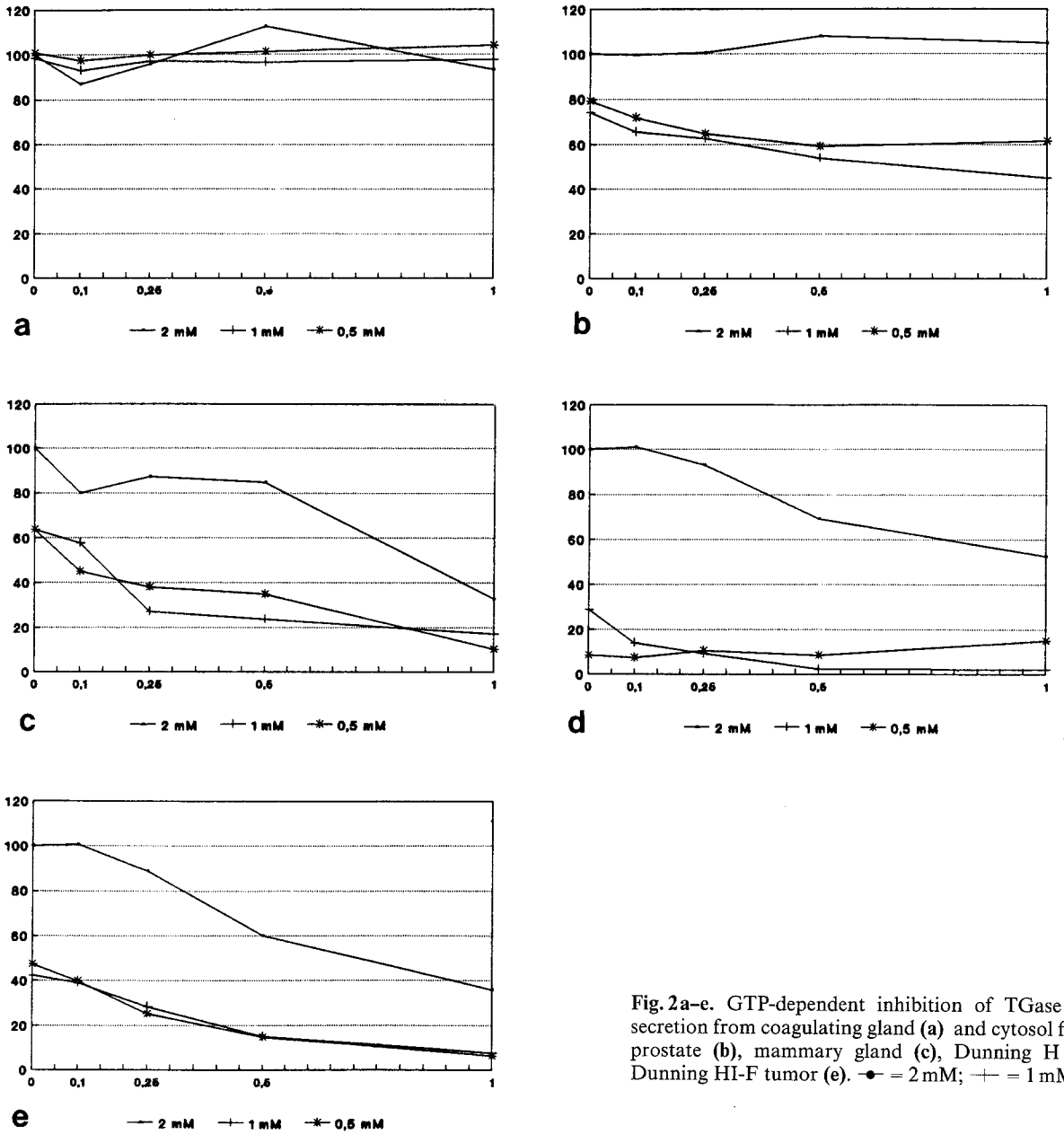

Fig. 2a–e. GTP-dependent inhibition of TGase activitiy in the secretion from coagulating gland **(a)** and cytosol from dorsolateral prostate **(b)**, mammary gland **(c)**, Dunning H tumor **(d)**, and Dunning HI-F tumor **(e)**. -●- = 2 mM; -+- = 1 mM; -*- = 0.5 mM

Results

Transglutaminase activity in rat accessory sex glands, mammary gland, and Dunning tumor lines (Fig. 1)

TGase activity was determined by measuring the amount of putrescine incorporated into TCA-precipitable material after incubation of tissue homogenate with *N,N*-dimethylated casein and labeled putrescine.

Specific enzyme activity was highest by far in coagulating gland (728 pmol putrescine per mg protein per min). It was clearly lower in dorsolateral prostate (108 pmol/mg per min), seminal vesicle (66 pmol/mg per min), lactating mammary gland (45 pmol/mg per min), and ventral prostate (24 pmol/mg per min).

The specific activity measured in the H tumor (53 pmol/mg per min) ranged between that determined in the mammary gland and that found in the seminal vesicle, and was slightly higher than that found in the AT-2 tumor (43 pmol/mg per min). Highest specific activity of all the tumor lines was that in the HI-F tumor (535 pmol/mg per min). In all other tumors activity was in the range of the sensitivity limits of the method. In the absence of free Ca^{2+} (5 mM EGTA) no TGase activity could be measured.

Inhibition experiments

Whereas secretory TGase (present in the secretion of the coagulating gland) had an activity optimum at low Ca^{2+} concentrations (0.5 mM) [15] and hence was not inhibited after lowering of the Ca^{2+} concentration to 0.5 mM, tissue-type TGase activity in all tissues studied was clearly Ca^{2+} dependent. Lowering of the Ca^{2+} concentrations from 2 to

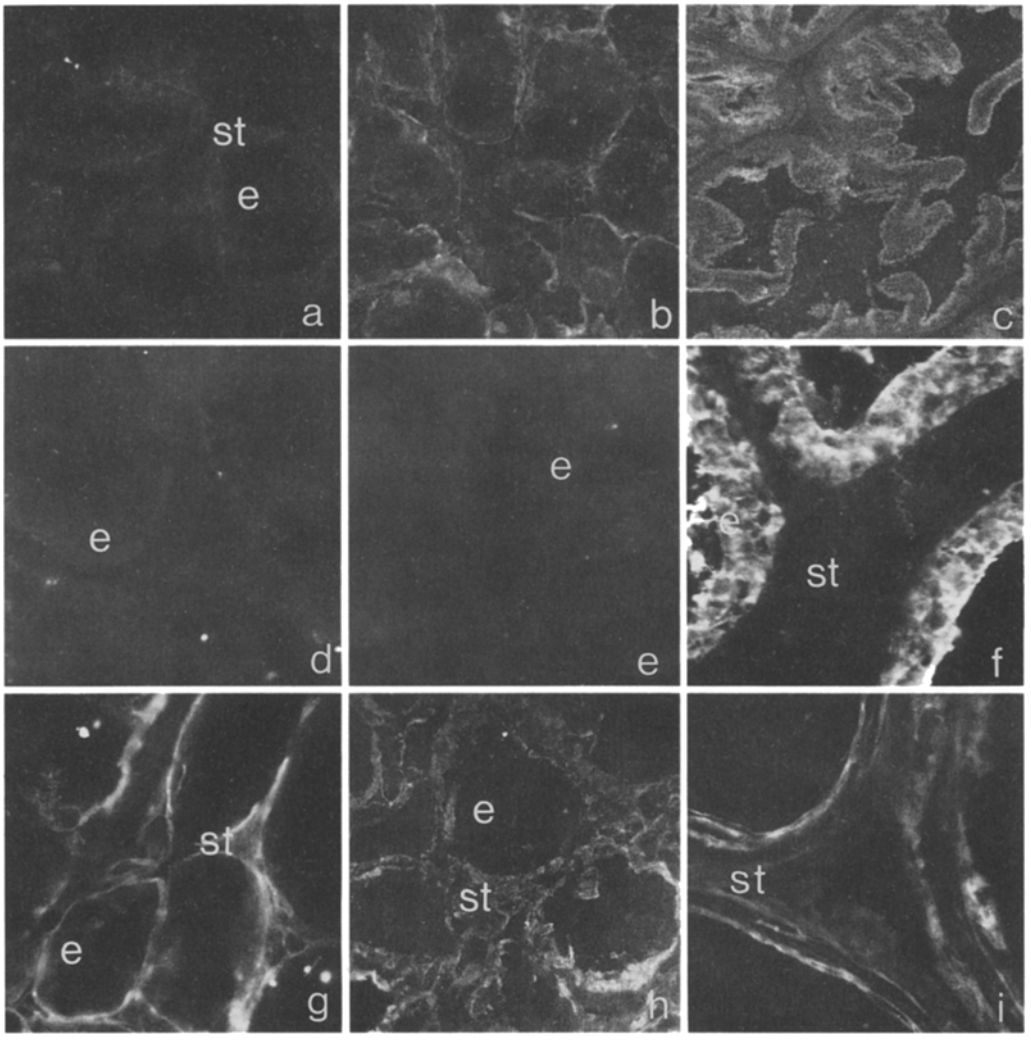

Fig. 3a–c. Enzyme histochemical demonstration of transglumatinase activity in mammary gland **(a)**, Dunning H tumor **(b)**, and dorsal prostate **(c)**. Stroma *(st)* of mammary gland and Dunning H tumor are only weakly stained. There is a strong enzyme reaction in prostatic epithelium *(e)* surrounding a lumen, but no reaction in stroma. **d–f** Immunolocalization of secretory transglutaminase in mammary gland **(d)**, Dunning H tumor **(e)**, and dorsal prostate **(f)**. Epithelium *(e)* is nonreactive in mammary gland and Dunning tumor, but strongly reactive in prostate. Some immunoreactive material is present in the prostatic lumen, while stroma *(st)* is negative. **g–i** Immunolocalization of tissue-type TGase in mammary gland **(g)**, Dunning H tumor **(h)**, and dorsal prostate **(i)**. Only stroma *(st)* is immunoreactive in all three tissues

0.5 mM led to a decrease in TGase enzyme activity (Fig. 2b–e). Addition of EGTA to TGase preparations at equimolar amounts of calcium and EGTA resulted in an inhibition of TGase enzyme activity – both the secretory type and the tissue-type (not shown).

At low calcium concentrations (0.5 mM) GTP is known as a reversible, non-competitive inhibitor of tissue-type TGase [1]. In contrast, no inhibition of secretory TGase from the secretion of coagulating gland could be achieved by addition of GTP [15]. These different susceptibilities to GTP inhibition were used to distinguish between the secretory and tissue-type forms of TGase in enzyme activity tests.

As already demonstrated by Seitz et al. [15], TGase activity in secretion from coagulating gland could not be inhibited by GTP (Fig. 2a). The TGase enzyme activity from mammary gland (Fig. 2c), H tumor (Fig. 2d), and HI-F tumor (Fig. 2e), as well as from the ventral prostate and seminal vesicle (not shown), was inhibited at low calcium concentrations (0.5 mM) by GTP concentrations above 0.2 mM, indicating the tissue-type of TGase. The dorsolateral prostate gave an intermediate result, in that its TGase activiy showed only partial inhibition by GTP. This indicates the presence of both TGases, secretory type and tissue-type (Fig. 2b), in the dorsal prostate.

Enzyme histochemistry

Using monodansylcadaverine as substrate at a calcium concentration of 2 mM, a strong enzyme activity was found in epithelium, and lumen of dorsal prostate (Fig. 3c) and coagulating gland (not shown), while in H tumor enzyme was confined to the stroma and no acinar reaction could be detected (Fig. 3b). Likewise in mammary gland (Fig. 3a), as well as in seminal vesicle and ventral prostate (not shown), enzyme activity could be visualized only in stroma.

Immunohistochemistry

Two antibodies recognizing different TGase forms were used: a polyclonal antibody raised in rabbits against secretory TGase from coagulating gland [15] and a monoclonal antibody raised against tissue-type TGase from guinea pig liver [5].

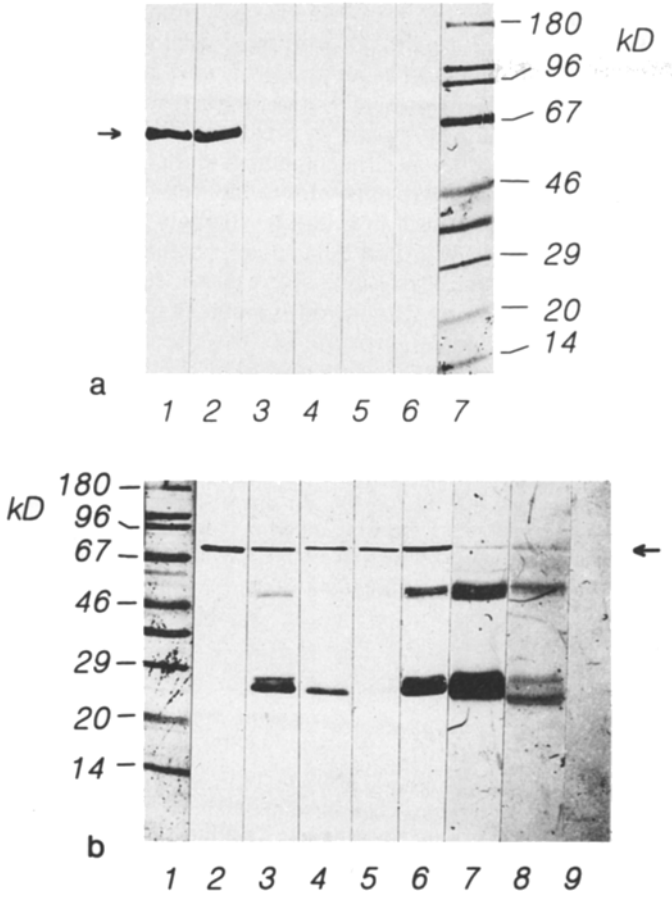

Fig. 4a. Western blot of homogenates of dorsal prostate *(1)*, coagulating gland *(2)*, ventral prostate *(3)*, mammary gland *(4)*, H tumor *(5)*, and HI-F tumor *(6)* using an antibody against secretory TGase *(arrow)*. Each lane contains 10 µg protein with the exception of lanes 1 and 2, which contain 0.5 µg protein. *Lane 7*, molecular weight standard proteins. **b** Western blot of homogenates (30 µg protein each) of dorsal prostate *(3)*, ventral prostate *(4)*, coagulating gland *(5)*, HI-F tumor *(6)*, H tumor *(7)*, and mammary gland *(8)* with a monoclonal antibody against tissue-type TGase *(arrow)*. *Lane 2*, 0.5 µg guinea pig liver TGase stained as positive control. In contrast to the tissue homogenate, secretion from coagulating gland contains no tissue-type TGase (*lane 9*, 5 µg protein). *Lane 1*, molecular weight standard proteins

Using the antibody against secretory TGase, only dorsal prostate (Fig. 3f) and coagulating gland (not shown) displayed an immunoreaction of epithelium and intraluminal secretion. No immunofluorescence was seen in the mammary gland (Fig. 3d) and the Dunning H tumor (Fig. 3e), or in the other tumor lines (not shown).

Using the antibody against tissue-type TGase, immunofluorescence was observed in stroma of dorsal prostate (Fig. 3i), mammary gland (Fig. 3g), and the H tumor (Fig. 3h). In coagulating gland, ventral prostate, seminal vesicles, and in the AT-2 and HI-F tumor also only the stroma was stained. A faint immunoreaction was found in G, AT-3, Mat-Lu, and Mat-LyLu tumors (results not shown).

Western blotting

In Western blot analyses using the antibody against secretory TGase, only homogenates of dorsal prostate and coagulating gland showed an immunoreactive band at 65 kDa (Fig. 4a). In ventral prostate, mammary gland, and Dunning H and HI-F tumor lines no immunoreaction was found with the antibody against secretory TGase.

In homogenates of the rat tissues and Dunning tumor lines tested (e.g. dorsal and ventral prostate, coagulating and mammary gland, and H and HI-F tumors) a protein band was stained at 75 kDa that could represent tissue-type TGase (Fig. 4b). In H tumor and mammary gland, however, the staining was very weak. No staining was achieved in secretion of coagulating gland (Fig. 4b). It remains unclear whether protein bands stained with the monoclonal antibody represent proteolytic products of TGase. Since the staining was rather diffuse and present in almost all rat tissues, it may represent a cross-reaction of the antibody with other proteins.

Discussion

The Dunning tumor, first described by Dunning [8] as a papillary adenocarcinoma derived from the dorsal prostate, is one of the most important model systems for prostate cancer research. One of the characteristic proteins of the dorsal prostate is a secretory TGase [16]. To elucidate the nature of the TGase present in the Dunning tumor we have carried out biochemical and immunological studies of TGase in the well-differentiated H line and other less differentiated lines of the Dunning tumor and compared them with TGases in different rat accessory sex glands and mammary gland. This last tissue was used as we have previously shown that there are more similarities between the H tumor and mammary gland than between H tumor and dorsal prostate [3, 10].

In rat prostatic tissue there are two isoforms of TGase, the secretory and the tissue-type, that can be distinguished using GTP. This reagent is know to be a non-competitive inhibitor of tissue-type TGase at low Ca^{2+} concentrations [1]. According to Seitz et al. [15] this is not the case for the secretory TGase from dorsal prostate and coagulating gland. In dorsal prostate GTP-dependent inhibition was only partial, since some TGase activity was due to secretory TGase that cannot be inhibited by GTP. In ventral prostate, seminal vesicles, and mammary gland, as well as in the Dunning H and HI-F tumors, TGase activity was significantly inhibited by GTP at low Ca^{2+} concentrations. TGase activity in these tissues must therefore be attributed to tissue-type TGase. The reason for the differences in calcium-dependence of this enzyme in different organs is unknown.

Enzyme distribution was also confirmed histochemically at the tissue level using monodansylcadaverine as a substrate. Strong enzyme reactions in intraluminal secretion and epithelium were observed only in coagulating gland and dorsal prostate, while all the other tissues studied showed a moderate stromal enzyme activity that must be attributed to tissue-type TGase.

Evidence for the distribution pattern of tissue-type and secretory TGase was provided by immunocytochemistry. Secretory TGase could be stained exclusively in epithelium and lumen of dorsal prostate and coagulating gland, whereas tissue-type TGase was present in stroma of all rat tissues examined and in Dunning tumor lines. In Western blotting studies only tissue-type TGase and no secretory TGase could be detected in Dunning tumor lines (H and HI-F tumor).

Our results are important with regard to the histogenesis of the Dunning tumor. The absence of one of the most characteristic proteins of dorsal prostate, secretory TGase, in the well-differentiated Dunning H tumor is in line with the absence of the dorsal-prostate-specific proteins DPI and DPII in this tumor [21] and argues against the prostatic origin of the Dunning tumor.

We have recently pointed to the fact that the cellular composition of the Dunning tumor, namely epithelial secretory and myoepithelial as well as stromal nonmuscular cells, is difficult to reconcile with the contention of a prostatic origin of the H tumor [3]. Since many similarities were observed between the H tumor and the mammary gland, we studied gene expression of different prostatic and mammary proteins in the Dunning tumor grown in male (normal and castrated) and female (lactating and non-lactating) rats and compared it with that in dorsal prostate and mammary gland, respectively [10]. Mammary proteins, such as milk fat globule membrane proteins and transferrin, were expressed both in mammary gland and in the H tumor, and some of them were increased in amount when exposed to lactogenic signals. These proteins, however, were lacking in dorsal prostate.

Here we have demonstrated that one of the most characteristic proteins of dorsal prostate, secretory TGase, is lacking in the Dunning tumor. The TGase activity measured in the Dunning tumor is clearly of so-called tissue-type TGase. This enzymic form is present in different rat tissues including mammary gland, although there are differences in the calcium dependence of the enzyme activity in different tissues.

Romijn et al. [14] have argued that in prostate cancer, including the Dunning tumor, TGase activity is inversely correlated with the metastatic potential of the tumor, i.e., that strongly metastasizing tumors contain low TGase activity whereas highly differentiated, nonmetastasizing tumors contain considerably higher TGase activity. Similar statements have been made previously for other tumor systems [4, 12]. In the present study the highest enzyme activity was found in the relatively well-differentiated HI-F tumor, whereas the highly differentiated H tumor exhibited considerably lower enzyme activity in the range found in the anaplastic AT-2 tumor. Contrary to these tumors, which display a low metastatic ability [13], in all other tumor lines, displaying a high metastatic ability, enzyme activity was in the range of the sensitivity limits of the method. Our results are difficult to interpret as regards the G and HI-F tumors. The G tumor, which has a low metastatic potential, contained an enzyme activity in the range found in the metastasizing tumors, whereas the HI-F tumor, which is less well differentiated than the H tumor, showed a significantly higher enzyme activity. It is not clear at present whether TGase functions as a metastasis-prohibiting agent in tumors or is involved in programmed cell death as an apoptotic enzyme.

In summary, our present work supports our view that the Dunning tumor is not a prostate-derived tumor. Absence of prostate-specific proteins and the presence of mammary-gland-specific proteins in the well-differentiated Dunning H tumor [10] argue strongly against its prostatic origin. Additional evidence is provided by the observed difference in steroid hormone receptor expression in Dunning tumor and mammary gland on the one hand and dorsal prostate on the other [20]. The Dunning tumor must therefore be regarded as an inappropriate model for prostate cancer research.

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