Transglutaminase activity and putrescine-binding capacity in cloned cell lines with different metastatic potential

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An inverse correlation was found between cellular transglutaminase activity and metastatic potential of four cloned cell lines derived from a primary nickel-induced rat rhabdomyosarcoma. Cellular transglutaminase activity as assessed with endogenous cellular protein or exogenous methylated casein was greatest in the clone F9-4/8 which is the least metastasizing. When the putrescine-binding capacity of one cellular derived protein – fibronectin – was examined with exogenous transglutaminase, it was found that the fibronectin derived from the clone F9-4/8 showed the lowest binding capacity compared with those from the other clones. However, when the overall binding capacity of cellular proteins from each cell line was examined no differences could be detected. The results are discussed in the light of the well-known role of fibronectin in cellular adhesion.

Transglutaminase *Polyamine* *Metastasis* *Fibronectin*

1. INTRODUCTION

Transglutaminase (TGase) (EC.2.3.2.13) catalyzes the acyl-transfer reaction of a γ-carboxamide group derived from a peptide-bound glutamine residue to primary amine receptors, such as the ϵNH_2 of peptide-bound lysine or primary NH_2 of polyamines [1–2]. There are many lines of evidence in the literature showing that this enzyme may play a role in cell transformation. Indeed, it has been shown that: (i) transformed cells, in vitro, have significantly lower total TGase activity than their normal counterparts [18]; (ii) the enzymic product, ϵ-(γ-glutamyl)lysine, is present in lower amounts in transformed cells than in normal cells [3]; (iii) the TGase activity measured in rat sarcoma primary tumours decreased 4–7-fold following detection of metastases in lung [4].

In view of these observations, the question could be asked whether differences in TGase activity do exist between metastasizing and non-metastasizing cells.

To answer this question the model chosen consisted of a primary nickel-induced rat rhabdomyosarcoma cell line and clones with different metastatic potential derived from this same cell line. These cells have been well characterized with respect to their in vivo and in vitro growth and tumorigenic and metastatic capacities [5]. We report here the results of investigations on the TGase activity of these clones along with the putrescine-binding capacity of cellular proteins.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{14}C]Putrescine dihydrochloride ([1,4-^{14}C]tetramethylenediamine dihydrochloride, spec. act. 109 Ci/mol) was purchased from the Radiochemical Center (Amersham, Bucks). Putrescine dihydrochloride (PUT), phenylmethanesulfonyl fluoride (PMSF) and casein were obtained from Sigma (St. Louis, MO).

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2.2. *Methods*

2.2.1. Preparation of dimethylcasein

Dimethylcasein was prepared as described [6].

2.2.2. Purification of liver transglutaminase

TGase was purified from guinea pig liver [7]. One unit corresponds to the amount of enzyme capable of inserting 2.1 nmol PUT into 114 μg methylcasein in the presence of 67 μM PUT for 1 h at 37°C.

2.2.3. Preparation of fibronectin

Fibronectin was purified from the culture media by precipitation with spermine [8] with the modifications in [9].

2.2.4. Cells

The cells used were isolated from a primary nickel-induced rat rhabdomyosarcoma as described [5]. The parental tumor cells (9-4/0) were cloned in vitro, thus providing a series of cell lines among which we used a low metastasizing line (F9-4/8) and two high metastasizing lines (F9-4/15, F9-4/6).

The number of lung metastasizing colonies of these cells after i.v. injection into the tail vein of rats has been shown to be [5]: 7 ± 3.2 for the parental cell line (9-4/0); 1 ± 0.2 for the clone F9-4/8; 18 ± 4.2 for the clone F9-4/6; 19 ± 2.3 for the clone F9-4/14.

Growth medium (Dulbecco) supplemented with 10% fetal calf serum was used for the growth of all cells. Cells were grown in culture to subconfluence and dispersed using 0.25% trypsin, 2 mM EDTA. The suspensions were centrifuged for 10 min at 1000 rpm. The supernatants were filtered on 0.22 μm filters and PMSF was added to a final concentration of 0.4 mM. The cell pellets were washed twice with PBS at 4°C. The cell pellets and culture media were stored at −70°C until use.

2.2.5. Preparation of homogenates from cells

Cells were resuspended in 0.14 M NaCl containing PMSF (0.4 mM final concentration) then disrupted by sonication in a Branson Sonifier (model S75, Dansbury, CT) fitted with a microprobe, for 2 s at output 2.

2.2.6. Cellular transglutaminase activity with endogenous protein or dimethylcasein as acceptor proteins

To experimental tubes containing various quantities of cellular homogenate (500, 250, 125 or 62.5 μg) protein in 100 μl were added 30 μl [^{14}C]-PUT (8 μCi, 172 nmol). Additions to the mixture were 114 μg dimethylcasein dissolved in 80 μl of 0.14 M NaCl, 0.01 M Tris-HCl (pH 7.5) or 80 μl of this buffer alone. The reaction was initiated by the addition of calcium to a final concentration of 7 mM in the presence of 1 mM DTT and the volume was adjusted to 230 μl with the Tris buffer. In both cases (presence or absence of dimethylcasein) the controls contained all constituents except cellular proteins.

After incubation for 1 h at 37°C with gentle shaking, 50 μl incubation mixture were spotted on-

Table 1

Endogenous transglutaminase activity of cellular proteins (125 μg) in the presence or absence of dimethylcasein

	Clone F9-4/8	Parental cell line 9-4/0	Clone F9-4/6	Clone F9-4/14
Lung colonizing capacity (number of lung metastasis 10 weeks after i.v. injection of 10^5 cells)[a]	1 ± 0.2	7 ± 3.2	18 ± 4.2	19 ± 2.3
Endogenous transglutaminase activity (pmol PUT incorporated)				
In the absence of dimethylcasein	9.7	1.4	0.7	0.4
In the presence of 114 μg dimethylcasein	185.8	23.4	5.1	3.8

[a] From [5]

Each value represents the average of duplicate determinations carried out in two different experiments

to a Whatman 3MM filter paper disc presoaked with 100 μl of 100 mM EDTA and treated as in [10].

2.2.7. Putrescine-binding capacity of cellular proteins by action of exogenous transglutaminase

To experimental tubes containing 200 μg cellular protein were added [^{14}C]PUT (0.7 μCi) in amounts ranging from 29 to 474 μM. 60 μl of purified guinea pig liver TGase (1 U) was added to the mixture. The reaction was initiated by the addition of calcium to a final concentration of 7 mM in the presence of 1 mM DTT. The volume was adjusted to 230 μl with the Tris buffer. The control contained all the constituents except cellular proteins (which were replaced by buffer).

Incubation, sampling and washing procedures were performed as described [10].

2.2.8. Putrescine-binding capacity of fibronectin purified from culture medium by action of exogenous transglutaminase

To experimental tubes containing 30 μg fibronectin was added [^{14}C]PUT (spec. act. 1 Ci/0.2 mol) in amounts ranging from 158 to 890 μM. TGase and calcium were added to the mixture as described before. One series of control tubes contained all reagents except fibronectin which was replaced by buffer.

Incubation and further procedures were performed as stated above.

3. RESULTS

Kinetic evidence for the TGase reaction has clearly demonstrated that the amine enters the reaction sequence only after the formation of an acyl-enzyme intermediate between the acceptor protein (contributing for the acyl portion) and the enzyme [1]. The affinity for a given amine is then a function of the structure of the acyl portion of the first substrate.

3.1. *Reaction of endogenous transglutaminase with endogenous proteins (first substrate)*

In a first approach, the capacity of endogenous proteins from the different cloned cell lines to react with endogenous TGase was tested in the presence of excess PUT (67 μM) and $CaCl_2$ (6 mM). The results (table 1) show that on the basis of their TGase activity, the clones can be classed:
F9-4/8 > 9-4/0 >
F9-4/6 > F9-4/14.

The enzymic activity of the low-metastasizing clone (F9-4/8) is around 5–7-times greater than that of the parental cells (9-4/0) whose TGase activity was itself 1–3-fold greater than that of clone F9-4/6 and 4–6-fold greater than that of clone F9-4/14 both of which are highly metastasizing.

As differences in the enzyme itself and/or acceptor proteins may contribute to these results, the experiment was repeated in the presence of excess exogenous protein, dimethylcasein, as first substrate.

3.2. *Reaction of endogenous transglutaminase in the presence of an exogenous protein - dimethylcasein (first substrate)*

In the presence of dimethylcasein (114 μg), the TGase activity is greater with extracts from all 4 clones than that observed with endogenous proteins only (125 μg). As shown in table 1, PUT bound to protein in the presence of dimethylcasein (V_B) vs that in its absence (V_A) increased 7-fold for clone F9-4/6, 9.5-fold for clone F9-4/14, 17-fold for parental cells and 19-fold for clone F9-4/8. Nevertheless, in terms of their TGase activity the order of the clones remains the same: F9-4/8 > 9-4/0 > F9-4/6 > F9-4/14. Thus differences in acceptor protein are not responsible for the differences in enzyme activity. To obtain further evidence for differences in enzyme activity, increasing quantities of enzyme derived from each of the 4 clones were added to excess dimethylcasein and the amount of bound putrescine measured.

It is apparent (fig.1) that TGase activity is again in the order: F9-4/8 > 9-4/0 > F9-4/6 > F9-4/14. It is interesting to note that in the case of clones F9-4/6 and F9-4/14 enzyme activity increases linearly with enzyme concentrations up to 500 μg (cellular proteins) whereas, in the case of the parental cells 9-4/0 and clone F9-4/8 the slope changes from 250 μg protein onwards. If these results provided strong evidence for diminished TGase activity per se in the highly metastatic line F9-4/14, compared to that in the least metastatic F9-4/8, they provided no answer to the question raised by the results in table 1 concerning the availability of γ-carboxamide groups in glutamine residues of endogenous proteins of the different

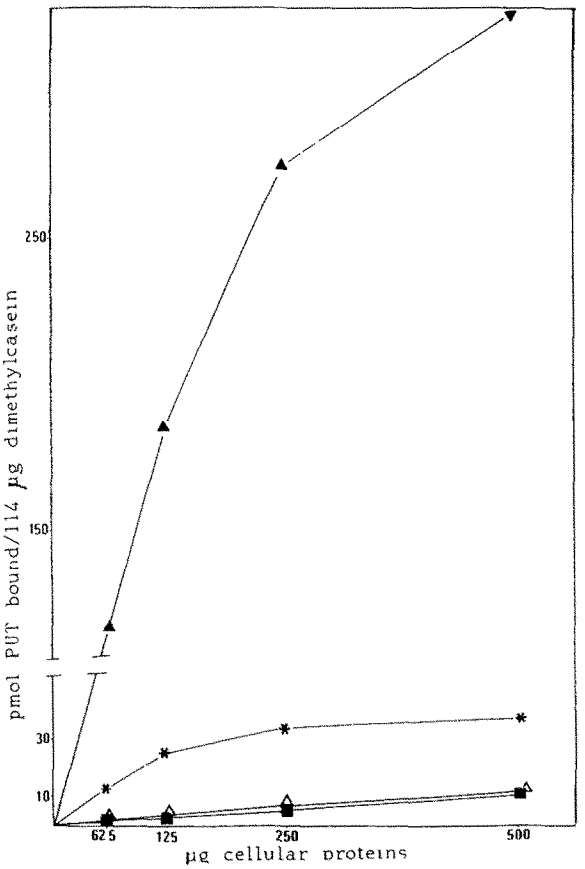

Fig.1. Endogenous transglutaminase activity (dimethylcasein) as exogenous substrate) of various quantities of cellular proteins from: (▲) clone F9-4/8, (*) parental cell line 9-4/0, (△) clone F9-4/6, (■) clone F9-4/14.

clones. To investigate this hypothesis, the PUT-binding capacity of cellular proteins was investigated in the presence of exogenous TGase purified from guinea pig liver.

3.3. *PUT binding of cellular proteins by action of exogenous transglutaminase*

The highest activity of endogenous TGase was 0.18 nmol PUT/114 µg dimethylcasein. In order to have exogenous TGase in excess, a quantity of enzyme which permits the insertion of 2.1 nmol PUT/114 µg dimethylcasein in the presence of 67 µM PUT was chosen. Under these experimental conditions, the exogenous enzyme was present in an at least 11-fold excess over endogenous enzyme. Therefore, the maximum acceptor capacity (apparant V_{max}) of the Gln residue of cellular proteins could be assessed by performing the reaction in the presence of PUT in increasing quantities (29–474 µM), cellular proteins at a fixed concentration (200 µg) and excess enzyme. As illustrated in table 2, the apparent V_{max} of the PUT-binding capacity of cellular proteins was not significantly different between clones F9-4/6, F9-4/8 and parental cells. As all cellular acceptor proteins are present simultaneously, it is not possible to determine the relative contribution of each protein to the overall reaction velocity. However, one cellular protein, fibronectin, influences cell adhesion to collagenous matrices [11] and modulates the metastatic activity of melanoma cells [12]. Furthermore, fibronectin purified from the culture medium of transformed cells compared with that of normal cells presented differences in polyamine acceptor capacity with exogenous TGase and

Table 2

Putrescine-binding capacity of cellular proteins by action of exogenous transglutaminase

Cell line	V_{max} (app.) (pmol PUT bound per 200 µg cellular proteins)
Parental cells	122 ± 20
F9-4/6	139 ± 39
F9-4/8	161 ± 44

Each value represents the average of duplicate determinations carried out in two different experiments

Table 3

Putrescine-binding capacity of fibronectin (purified from cell culture medium) by action of exogenous transglutaminase

30 µg fibronectin purified from cell medium culture of	% of PUT-binding capacity
Parental cells: 9-4/0	100 ± 5
F9-4/6	87.5 ± 2.2
F9-4/14	95.0 ± 5.0
F9-4/8	73.9 ± 1.9

Each value represents the average of duplicate determinations carried out in two different experiments. 100% = 1240 pmol PUT bound/30 µg fibronectin corresponding to the apparent V_{max}

bound polyamines which are more accessible to antipolyamine antibodies [9]. It therefore seemed reasonable to compare the PUT-binding capacity of fibronectin released from the different cell lines.

3.4. *PUT-binding capacity of fibronectin purified from cell culture medium by action of exogenous transglutaminase*

Fibronectin (30 μg) purified from the cell medium culture of each cell line was incubated with excess exogenous TGase and PUT (132–660 μM) as described in section 2. The results of these experiments (table 3) show that the protein-bound glutamine of the fibronectin released by clone F9-4/8 has the lowest binding capacity compared to the fibronectin from the other clones.

4. DISCUSSION

Our results provide evidence that cell lines with a high metastatic potential on intravenous injection into rats exhibit low transglutaminase activity. As all 4 clones were derived from the same rat rhabdomyosarcoma parental cell line, differences in enzyme activity reflect some intrinsic property of the clones and not differences in tissue specificity. Furthermore, as enzyme assays were performed on homogenates from cells which had all reached confluence prior to harvesting, fluctuations in enzyme activity with growth rate could be reasonably eliminated.

As regards enzyme activity itself, it was always in the order of greatest activity in the least metastatic to the lowest activity in the most metastatic (table 1) regardless of whether endogenous or exogenous protein (dimethylcasein) was used as acceptor protein. This observation rules out the possibility that differences in acceptor protein availability might have been responsible for differences in endogenous enzyme activity. We are therefore left with the possibilities that there are different amounts of either transglutaminase itself or of modulators of the enzyme in the clones F9-4/8. Our results do not permit us to choose between these two hypotheses but the resultant activity on combining homogenates derived from clones with high and low transglutaminase activities should allow a choice to be made.

Irrespective of the reason for the reduced activity, our results not only confirm those previously reported [4] showing a decrease in transglutaminase activity in the primary tumour with metastasizing cells compared to that without such metastasis, but they extend the validity of this finding to cell lines with different metastatic potential.

It should also be recalled that the decrease in cellular transglutaminase which takes place on cell transformation is accompanied by a change in its intracellular distribution. The enzyme becomes localised primarily on the membrane in hepatomas as compared to its cytosolic localisation in regenerating hepatocytes [13]. This redistribution could be responsible for the altered polyamine-binding capacity of at least one membrane protein – fibronectin. In accordance with this interpretation is the result that the lowest polyamine-binding capacity of fibronectin was found with that derived from clone F9-4/8 which has the highest transglutaminase activity. In contrast, when total cellular proteins were used, no differences in polyamine-binding capacity could be detected among the 4 clones. Taken together, these results do not rule out the possibility that in addition to fibronectin there may be other proteins with different binding capacities but rather suggest that the differences are masked when all the proteins are examined together.

Fibronectin is one of the proteins involved in cell adhesion [14,15] and differences similar to those reported here had previously been reported for fibronectin derived from normal rat kidney cells and from normal rat kidney cells transformed by Rous Sarcoma virus [9].

One mechanism which could explain the role of fibronectin in promoting cell adhesion is that involving the formation of isopeptide bonds between glutamine residues of fibronectin and lysine groups of collagen [11].

The accessibility of these glutamine residues could be reduced by conformational changes brought about by post-translational modifications such as phosphorylation [15], glycosylation [16] and sulphation [17]. Our results suggest that transglutamination may be another such post-translational modification which could give rise to fibronectins with γ-carboxamides already in isopeptide linkages. The determination of the number of isopeptide bonds in the fibronectins from the different clones should provide a direct answer.

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