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Protein-polyamine conjugation by transglutaminase in cancer cell differentiation: Review article

A. Lentini[1], **A. Abbruzzese**[2], **M. Caraglia**[2], **M. Marra**[2], and **S. Beninati**[1]

[1] Department of Biology, University "Tor Vergata", Rome, Italy
[2] Department of Biochemistry and Biophysics, II University of Naples "Federico II", Naples, Italy

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Summary. Considerable and intense progress has been made in the understanding of the chemistry, molecular biology and cell biology of transglutaminases (TGases: EC 2.3.2.13). The knowledge that very different processes such as cell growth, reproduction and death are dependent on the presence of adequate levels of these enzymes and that the amount of both free and protein-conjugated polyamines, formed by the enzyme, are capable of modulating the differentiation and proliferative capability of several cell types, has prompted a multitude of researchers to study the role of these fascinating molecules in cancer cell differentiation.

Keywords: Transglutaminase – Polyamine – Differentiation

Introduction

TGases can catalyse the covalent incorporation of several low molecular weight amines into proteins in the form of amides of the γ-carboxyl group of a peptide-bound glutamic acid. TGase-catalyzed reactions are extremely specific for a particular glutamine residue in native protein substrates (Folk, 1980; Greenberg et al., 1991). Because of this extreme specificity, it has been suggested that TGase-catalyzed post-translational modification may be physiologically important (Davies et al., 1988). Among the naturally occurring di- and polyamines, putrescine, spermidine, and spermine are excellent substrates of TGase *in vitro* (Folk et al., 1980; Beninati et al., 1988a). The incorporation of these amines into proteins can occur through one or both of their primary amino groups. The result of these reactions is the formation of either N-mono(γ-glutamyl)- or N,N-bis(γ-glutamyl)putrescine, -spermidine or -spermine. Since the K_m of polyamines in the TGase reaction *in vitro* is in the range of concentrations that have been observed *in vivo* (Williams-Ashman and Canellakis, 1979), it has been suggested that polyamination reactions are physiologically relevant. In particular, it has been speculated that the condensation of a polyamine with a specific glutamine residue may naturally occur *in vivo* to modify the structural and/or catalytic properties of a protein (Folk et al., 1980). Additionally, the TGase-catalyzed reaction of a protein-bound polyamine with a second glutamine residue may lead to the formation of a covalent crosslink between two polypeptide chains (Schrode and Folk, 1978). In such events, the proteins would be linked by an N,N-bis(γ-glutamyl) polyamine bond. Di- and polyamines such as putrescine, spermidine and spermine are practically ubiquitous in nature. Changes in intracellular polyamine levels have been associated with many biological processes such as proliferation, embryonic development, differentiation and neoplastic growth (Heby, 1981; Erwin et al., 1983; Erwin et al., 1984). It has been reported that several animal tissues including rat liver, testis and kidney, human skin, cultured mammalian epidermis cells, contain measurable amounts of (γ-glutamyl)polyamines (Beninati et al., 1985; Beninati and Folk, 1988; Beninati et al., 1988b). Proteolytic digests of each of these tissues have been shown to contain detectable amounts of γ-glutamyl derivatives of polyamines by HPLC and enzymatic analysis (Beninati et al., 1985; Beninati and Folk, 1988). Mitogen-induced formation of (γ-glutamyl)polyamines has been observed in human lymphocytes (Folk et al., 1980). Studies on the levels of conjugated polyamines in cells and tissues have

also demonstrated a correlation between the formation of these derivatives and the activity of intracellular TGase (Davies et al., 1988).

Based on their distinct catalytic characteristic and distribution, several forms of TGase have been identified. TGases are widely distributed in various organs, tissues, and body fluids, and they are distinguishable from each other to a large extent by their physical properties and distribution in the body. It is well known that TGases exhibit differences in specificity, which are expressed in terms of variations in susceptibility of glutamine residues to catalytic modification and appear to be dependent, at least in part, upon amino acid residues surrounding a given glutamine (Folk and Finlayson, 1977). In contrast to their limited glutamine substrate specificity, TGases possess an exceptionally wide specificity for amine substrates. Although the catalytic action of TGases and their limited specificity are known, much remains to be learned concerning tissue specificity, regulation, and structural relationships.

Biological role of TGases

Some physiological roles are unequivocally established for TGases: blood coagulation (Lewis et al., 1985), construction of a ''cornified envelope'' in epidermal keratinocytes (Rice and Green, 1977) and apoptotic bodies (Fesus et al., 1989) and formation of a postejaculatory vaginal plug by prostate TGase in rodents (Williams-Ashman, 1984). Several evidences have been found on additional functions that TGase may affect. These include irreversible membrane stiffening of erythrocytes (Siefring et al., 1978), eye lens opacification (Lorand et al., 1981), receptor-mediated endocytosis (Davies et al., 1980), regulation of cell growth and differentiation (Birckbichler et al., 1981), tumor metastasis (Beninati et al., 1993; Lentini et al., 1998) and programmed cell death (Fesus et al., 1989).

During terminal differentiation, mammalian epidermal cells acquire a deposit of protein on the intracellular surface of the plasma membrane which is termed ''cornified envelope''. This cross-linked structure is the most insoluble component of the epidermis due to disulfide as well as ε-(γ-glutamyl)lysine isopeptide and (γ-glutamyl)polyamine bonds (Rice and Green, 1977; Martinet et al., 1990). Several proteins including involucrine, keratolinin and loricrin, are components of the epidermal envelope, and it has been shown that they are cross-linked to this structure by ε-(γ-glutamyl)lysine isopeptide bonds (Zettergren et al., 1984). Recently, TGase activity was also found in higher and lower plants, following the incorporation of polyamines into plant proteins in different organs. The few reports about the localization of this enzime in plants concerns its identification in chloroplasts (Del Duca et al., 1995).

Polyamines as substrates for TGases

The history of research on TGases is intimately tied to assay based on the incorporation of isotopically labeled polyamines into trichloroacetic acid (TCA)-precipitable proteins. In spite of its many pitfalls, the Ca^{2+}-dependent incorporation of amines remains the cornerstone for detecting and measuring TGase activities in tissue homogenates. Various modifications of the original methodology are in use. A significant improvement was the blocking of the ε-lysine residues of casein by acetylation, succinylation (Lorand et al., 1971), dimethylation (Curtis and Lorand, 1976) or guanidylation (Gorman and Folk, 1980), so that no self cross-linking of the protein substrate could occur. Filter paper (Lorand et al., 1972a), chromatographic techniques (Lorand and Campbell, 1971), a recent microtiter plate TGase assay utilizing 5-(biotinamido) pentylamine as substrate (Slaughter et al., 1992) and a colorimetric assay (Jeon et al., 1989) are employed for the efficient and simultaneous handling of a large number of test samples. Dansylcadaverine opened up a new possibility for studying TGases by fluorescence methodologies. This molecule has excellent affinity for all acyl-enzymes of this kind and its incorporation into casein and into many other proteins is accompanied by a large blue-shift and by an increase in the quantum yield of dansyl group emission. These circumstances made it possible to devise continuous rate assays (Lorand et al., 1971) for measuring TGase activities and to apply histological manipulations for the staining of TGases on electrophoretic gels (Lorand et al., 1979). As such, purified guinea pig liver tTGase, which had previously been represented to comprise only a single enzyme, could be resolved by non-denaturating electrophoresis and dansylcadaverine staining into two differently charged components. Putrescine, spermidine and spermine can play a critical role in modulating TGase activity by interacting with the protein substrates. In animals, post-translational covalent binding of polyamines to various proteins have been demonstrated by isolation of (γ-glutamyl)polyamine derivatives (Folk et al., 1980). The physiological importance of polyamines has been increased, considering this new metabolic step, where the amine may be sequestered by its incorporation into a suitable protein. The covalent incorporation of a

polyamine into a protein by itself is not considered sufficient for concluding that the linkage formation was catalyzed by TGase. Some strategies are available for the unequivocal identification of TGase-catalyzed formation of (γ-glutamyl)polyamine derivatives (Beninati and Folk, 1988). Several problems, similar to that cited above, were encountered in finding TGase activity in plants. Initially, TGase activity was presumed analyzing the TCA-insoluble fraction of total polyamines. Many of these data were found incorrect, because polyamines were electrostatically trapped in the TCA pellet. Since the product of TGase catalyzed reaction between protein substrates and polyamines are amides, the proteolytic digestion of the modified protein releases (γ-glutamyl)polyamines (Folk et al., 1980). Several studies performed on photosynthetically and non-photosynthetically competent cells using radiolabelled polyamines as markers, have concluded that TGase is an ubiquitous enzyme in plants and it is involved in several biological events already described for TGases found in animal cells (Serafini-Fracassini et al., 1995).

One of the most well known incorporation of polyamines into a protein in a reaction not catalyzed by TGases is the formation of protein-bound hypusine, by an unrelated sequence of events. In the effort to isolate and identify the product of TGase catalysis on polyamines, it was found that one of the chromatographic peaks, identified as (γ-glutamyl)spermidine and separated by an ion exchange column, was a component stable upon acid hydrolysis (hypusine) (Park et al., 1988). Spurious incorporation of a label originating from amines may also be encountered if the amine is oxidized in the tissue or in the culture medium to an aldehyde. Among other products, a Schiff base could ensue. In situations where interference by amine oxidases is suspected, specific inhibitors of the oxidase must be included in the test system (Beninati et al., 1988a). Another possibility for the artefactual TGase-independent incorporation of amines into proteins is due to the action of myeloperoxidase, which may convert a polyamine to R-NCl_2, which can react with proteins very rapidly (Thomas et al., 1982).

Identification of TGase substrates

Several difficulties were encountered in the finding of most suitable TGase substrates for enzymes extracted from various biological materials. One of the most widespread substrate proved to be useful for detecting TGase of animal origin has been β-casein. The water-soluble compound, benzyloxy-carbonyl-L-glutaminyl-L-glycine, has a quite poor affinity for guinea pig liver TGase, with a $K_{Mapp} = 0{,}05$ M (Lorand and Conrad, 1984). Very precious, particularly for the synthesis of (γ-glutamyl)polyamine standards, is the dipeptide benzyloxy-carbonyl-L-glutaminyl-L-leucine (Folk, 1983), also found useful for detecting TGase activities in plants (Serafini-Fracassini et al., 1995). The amounts of mono- and bis-derivatives formed vary with the time of reaction. However, the final yield of each is a function of the molar ratio of amine to benzyloxy-carbonyl-L-glutaminyl-L-leucine peptide in the starting reaction mixture. The carbonyl-terminal L-leucine is removed from the isolated derivatives by incubation with carboxypeptidase A. Finally, the free (γ-glutamyl)amines are released through chemical deblocking with HBr in acetic acid. Synthetic substrates have been most helpful from the point of view of elucidating the pathway of catalysis of TGases. Significant progress was made in recent years with regards to synthetic substrates of TGases. Folk and co-workers used nitrophenyl-esters, such as p-nitrophenylacetate or trimethylacetate (Folk, 1982), whereas Lorand and co-workers used a variety of thioesters (Lorand et al., 1972b). Amine specificity is one of the most characteristic aspects of TGase-catalyzed reactions. Affinity for the side chains of amines can be demonstrated by measuring interference with the biological formation of γ-ε cross-linked polymeric structures, or by competitively inhibiting the incorporation of a labeled amine substrate such as ^{3}H- or ^{14}C-putrescine or spermidine into β-casein (Lorand et al., 1983).

Modulation of TGase activity: retinoic acid and methylxanthines

Activation of TGases at the wrong time and wrong place could have disastrous consequences. Therefore, various activators and inhibitors could exercise a regulatory role at the substrate level. The possibility of proteolytic unmasking of the amine incorporating γ-glutamine sites has already been referred to in connection with the finding that fibrin was much better substrate for Factor XIIIa than the parent protein, fibrinogen. Furthermore, in nature some forms of these enzymes are found as inactive zymogens or as latent enzymes. Elucidating the mechanisms of activation of zymogens and latent forms is essential for the understanding of biological controls. There are examples for sequestering the enzyme from the substrate, with contact occurring between them only as the need arises (Williams-Ashman et al., 1977). The conversion of vertebrate Factor XIII to Factor XIIIa is the best studied model for zymogen activation thus far. In as much as both the cross-linking enzyme and the main target substrate, called

fibrin, are generated in the fluid phase of plasma of the same tissue, the blood, the zymogen form must have represented the best evolutionary solution poised for rapid activation. An other interesting case in point is the clotting of the seminal fluid of rodents, where enzyme and substrates are secreted by two different lobes of the prostate and the two become mixed only in the ejaculate (Williams-Ashman et al., 1977). Moreover, a protease-induced activation of an intracellular TGase has been reported (Chung et al., 1988). Proteolytic enzymes play a key role in a large variety of physiological processes some of which are regulatory in nature. A protease-induced modulation of cellular TGases may be one of these regulatory processes. Apart from the proteolytic activation, there is also the possibility that substrate-level control could be exercised through the preliminary covalent modification of the target protein. Various post-translational modification of TGase substrate, might alter its reactivity towards the enzyme. In this sense, fluctuations in the intracellular concentration of polyamines might play a regulatory role by competing against the cross-linking of proteins (Beninati et al., 1994a).

While TGase synthesis and activation is normally part of a protective cellular response contributing to tissue homeostasis, the enzyme has also been implicated in a number of pathological conditions including cancer metastasis (Lentini et al., 1998), fibrosis (Grenard et al., 2001), atherosclerosis (Auld et al., 2001) and coeliac disease (Roth et al., 2003). The cross-linking activity can subserve disparate biological phenomena depending on the location of the target proteins. Intracellular activation of soluble TGase can give rise to cross-linked protein envelopes in apoptotic cells, whereas extracellular activation contributes to stabilization of the extracellular matrix and promotes cell-substrate interaction (Beninati et al., 1994b).

The first differentiation agent found to be successful in the treatment of acute promyelocytic leukemia was all-trans-RA (ATRA). ATRA induced a complete remission in about 70% of cases. Interestingly, attempts to understand the differentiation process in leukemia have been considerably by insights emerging from studies of the related process of apoptosis. For example, leukemic cells exposed to differentiation inducers ultimately die an apoptotic death, generally as a rather late event (Tallman et al., 2002). This phenomenon is accompanied by an increase in TGase expression providing additional support for a role of these enzymes in growth arrest and differentiation of cancer cells.

Addition of retinoic acid (RA) to human myeloid leukemia cells (HL-60) results in a dramatic increase in tTGase activity of at least 50-fold within 6 h of treatment, and this effect is potentiated by analogues of cyclic AMP. The enzyme induction is a specific response of the cells to RA and is not seen with other agents that induce HL-60 cells differentiation. The increase TGase activity has been proposed as a marker of the early events in retinoid-regulated gene expression in both normal and transformed cells (Martin et al., 1990). Whereas treatment of tumor cells with RA results in cellular differentiation, addition of the synthetic retinoid, N-(4-hydroxyphenyl)retinamide (HPR), induces HL60 cells to undergo apoptosis. Moreover, pretreatment of HL60 cells, as well as other cell lines (i.e. NIH3T3 cells) with RA blocks HPR-induced cell death. It has been observed that monodansyl-cadaverine (MDC), which binds to TGase, eliminated RA protection against cell death and in fact caused RA to become an apoptotic factor, suggesting that the protecting ability of RA is linked to the expression of active TGase (Antonyak et al., 2001).

There are considerable evidences on the effects of RA on the growth of murine melanoma cells. Several studies on the proliferation of the murine melanoma S91 and BL6 cell lines revealed that inhibition by retinoids is time- and dose-dependent and reversible (Lotan, 1980). The inhibition does not depend on cell density and becomes apparent after 2–3 days in culture, irrespective of the initial cell density. We have found the RA is an inductor of tTGase activity in both B16-F10 and B16-F10^{Lr6} melanoma cells. The increase in the soluble enzyme activity was found inversely related with the metastatic potential of cancer cells, together with the levels of polyamine covalently incorporated into intracellular proteins. This apparent relationship suggested a role for the conjugation of polyamines to cancer cell proteins in the metastatic process. Interestingly, there was a dichotomy of response in the two cell lines with differentiative changes, only being evident in the more malignant (B16-F10) cell line (Fig. 1) (Beninati et al., 1993). cAMP-dependent mechanisms may play an important role in the establishment of experimental metastasis and highly metastatic melanoma clones possess an aberrant regulated adenylate cyclase (Lester et al., 1986). It is therefore possible that the selectivity in tTGase increase and polyamine incorporation observed for the high metastatic line is due, at least in part, to the action exerted by cAMP on RA.

Clinical studies on theophylline used in cancer therapy is limited to a case report recently published on the therapeutic efficacy of this methylxanthine in chronic lymphocytic leukemia (Makower et al., 1999). It is well known that theophylline acts as cAMP-phosphodiesterase

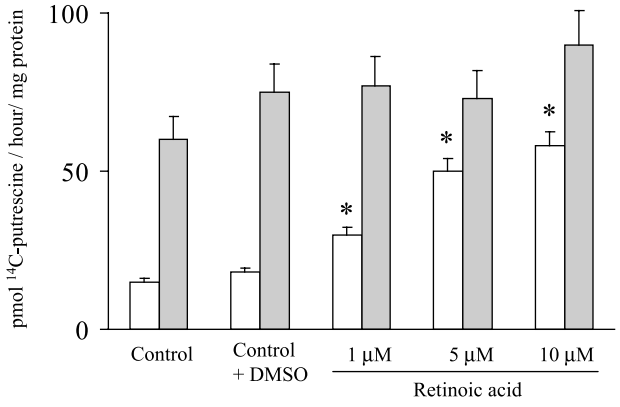

Fig. 1. Effect of retinoic acid (RA) on the activity of tTGase in B16-F10 (□) and B16-F10^{Lr6} (▩) murine melanoma cells. The data represent the mean ± SD of five experiments. * t Student's test, $p < 0{,}001$

inhibitor (Bergstrand, 1980), involving alterations in cAMP system tumor cell. Many of the biological effects of adenosine can be reverted by theophylline, suggesting a role as an adenosine antagonist (Fredholm, 1980). This was subsequently shown to be due to the fact that theophylline antagonizes the effects of adenosine. Adenosine stimulates the formation of cAMP and the adenosine-receptor-mediated actions are antagonized by this methylxanthine. There are at least two type of adenosine receptors on cells, A1 and A2. These two receptors are associated to a membrane-bound adenylate cyclase (AC) through G proteins. A1-receptors act through Gi proteins, which inhibit AC, whereas A2-receptors are linked to Gs proteins, having a stimulatory effect on AC (Fig. 2). Methylxanthines have a high affinity for the A1-receptors, leading to the increase of cAMP synthesis.

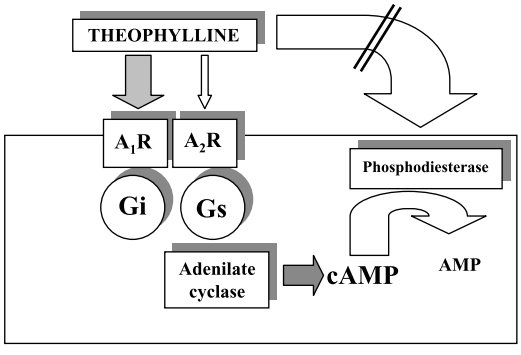

Fig. 2. Mechanism of action of theophyllline. The intracellular concentration of cAMP is mainly increased by phosphodiesterase inhibition but also by adenosine antagonism. In the latter case, theophylline exerts a higher affinity for A1 (inhibitory) adenosine receptors versus the A2 (stimulatory) ones, thus increasing the production of cAMP by adenilate cyclase

The correlation between metastatic power of cancer cells and the post-translational modification of protein catalyzed by TGases, appears supported by our recent findings on methylxanthines. We reported that theophylline is able to increase tTGase activity of cancer cells inducing melanoma differentiation markers. The *in vivo* capacity of this methylxanthine to influence B16-F10 melanoma cells metastatic behaviour was investigated by injecting methylxanthine-treated melanoma cells into the bloodstream of syngeneic mice (C57BL6/N). This produced an impaired ability of treated cells to invade the target organ, with a frequency of metastatic foci lowered by about 70%, as well as a significant increase of the lifespan of the treated animals as compared to controls. In order to discriminate between antiproliferative and anti-invasive properties of theophylline *in vivo*, we recently published a new procedure based on integrated image analysis performed on histological section of the target organ of syngeneic animals (Lentini et al., 2000). The data obtained by this procedure suggest that theophylline and partially caffeine, possess two dominant effects on B16 melanoma cells. One exerted in the inhibition of invasion, which affects the frequency of metastasis in the target organ, and the second concerned the reduction of the growth of melanoma cells in the colonized organ, likely through the induction of differentiation.

Conclusive remarks

Differentiation therapy of cancer was conceived from the observation that tumor cells can regain control of growth and differentiation in response to a number of natural and synthetic compounds. Although, several markers have been identified on tumor cells, their casual relationship to neoplastic competence has not been characterized in sufficient detail to warrant their evaluation as novel pharmacological targets for the design of new differentiative agents. The current rationale for targeting cAMP and tTGase reactions as contributing factors in cancer competence is based on several interrelated correlations (Prasad et al., 2001).

In recognition of this information, and in search of a novel approach to the investigation of the function of tTGase and polyamines in neoplastic growth, we have contributed examining the post-translational modification of protein in which structural elements of polyamines are involved. Although such reaction may affect marginally the cellular metabolism of polyamines, the function of the polyamine-modified protein is specifically perturbed (Cordella-Miele et al., 1993). It is still unclear, however,

whether this protein modification is casually associated with the acquisition and expression of high metastatic capacity, or alternatively is merely a secondary property that is not essential for the pathogenesis of the metastatic disease. We have followed these criteria in analyzing RA and methylxanthines responsiveness of two B16 melanoma lines with different metastatic potential. Results indicate that either RA or theophylline induce change in the rate of post-translational modification of proteins, likely because these drugs increase the activity of tTGase and affect the intracellular levels of cAMP (Murtaugh et al., 1986). While this result is based on one histological class of tumor, the most important implication of this observation is that it establishes a new way in the screening systems for detecting new differentiative agents. Furthermore, the absence of a chemotherapeutic strategy fully selective for tumor cell justifies the evaluation of this new approach inherent to cAMP, TGase, polyamines, and their biosynthetic pathway.

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Authors' address: Prof. Simone Beninati, Department of Biology, Room 350, University of Rome "Tor Vergata", Via della Ricerca Scientifica, 00133 Rome, Italy,
Fax: +390672594227, E-mail: beninati@bio.uniroma2.it