Induction of tissue transglutaminase by dexamethasone: its correlation to receptor number and transglutaminase-mediated cell death in a series of malignant hamster fibrosarcomas

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Treatment of the hamster fibrosarcoma cell lines (Met B, D and E) and BHK-21 hamster fibroblast cells with the glucocorticoid dexamethasone led to a powerful dose-dependent mRNA-synthesis-dependent increase in transglutaminase activity, which can be correlated with dexamethasone-responsive receptor numbers in each cell line. Increasing the number of dexamethasone-responsive receptors by transfection of cells with the HG1 glucocorticoid receptor protein caused an increase in transglutaminase activity that was proportional to the level of transfected receptor. In all experiments the levels of the tissue transglutaminase-mediated detergent-insoluble bodies was found to be comparable with increases in transglutaminase activity. Despite an increase in detergent-insoluble body formation, an increase in apoptosis as measured by DNA fragmentation was not found. Incubation of cells with the non-toxic competitive transglutaminase substrate fluorescein cadaverine led to the incorporation of this fluorescent amine into cellular proteins when cells were damaged after exposure to trypsin during cell passage. These cross-linked proteins containing fluorescein cadaverine were shown to be present in the detergent-insoluble bodies, indicating that the origin of these bodies is via activation of tissue transglutaminase after cell damage by trypsinization rather than apoptosis *per se*, since Met B cells expressing the bcl-2 cDNA were not protected from detergent-insoluble body formation. We describe a novel mechanism of cell death related to tissue transglutaminase expression and cell damage.

INTRODUCTION

Transglutaminases (EC 2.3.2.13) are Ca^{2+}-dependent enzymes which catalyse the post-translational modification of proteins through an acyl-transfer reaction between the γ-carboxamide group of a peptide-bound glutamyl residue and various primary amines. Primary amine groups of polyamines and peptide-bound lysine may serve as acyl donors to form either γ-glutamyl polyamine or ϵ(γ-glutamyl) lysine bonds [1]. Covalent cross-links using ϵ(γ-glutamyl) lysine bonds are stable and resistant to enzymic, chemical and mechanical disruption. A number of transglutaminase enzymes have been characterized with distinct structures and locations, but in only two of these is the physiological function well understood. These include plasma factor XIII, involved in cross-linking fibrin during wound healing, and keratinocyte transglutaminase, involved in the formation of the cornified envelope during terminal differentiation (for reviews see refs [2,3]). A further transglutaminase, the tissue transglutaminase (tTGase), is a cytoplasmic enzyme which is present in many different cell types. Its function remains poorly understood, although it has the potential to stabilize intra- and extracellular molecules in a wide variety of physiological and pathological events.

Evidence suggests that tTGase may have a number of key roles in cells, including the cross-linking of extracellular matrix proteins [4–6], cell growth and proliferation [7,8] and as an important GTP-binding protein [8,9]. Of reference to this paper is the proposed role of the enzyme in programmed cell death (apoptosis) [10,11], in which activation of the enzyme is thought to lead to the assembly of highly cross-linked protein shells. These detergent-insoluble protein shells are thought to be comparable with cross-linked envelope formed by keratinocyte transglutaminase in the terminally differentiating keratinocyte [3]. Although the function of the tTGase-mediated envelope is not clear, it has been suggested that it prevents leakage of cellular components during the terminal stages of programmed cell death.

Given the potential importance of tTGase in cell death, a number of laboratories have been interested in the factors that regulate its expression. Previous studies have shown that retinoids [12], transforming growth factor β1 [13] and interleukin 6 [14] are capable of regulating tTGase activity in a number of cell types. Recent isolation and characterization of 1.74 kb of the human tTGase gene promoter indicated the presence of a number of potential transcription-factor-binding sites [15] including a potential glucocorticoid-response element (GRE).

In the present paper we show by using a number of different cell lines of hamster origin that their treatment with the glucocorticoid dexamethasone leads to a receptor-mediated increase in tTGase activity. We also demonstrate that an increase in tTGase activity leads to a corresponding increase in detergent-insoluble body formation, which does not appear to be linked to classical apoptosis but is instead a result of tTGase-mediated cross-linking of cellular proteins after cellular damage by trypsinization. The physiological implications are discussed.

MATERIALS AND METHODS

Contructs

The expression vector pMAMneo CAT contains the chloramphenicol acetyltransferase (CAT) gene inserted into the multiple

Abbreviations used: tTGase, tissue transglutaminase; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumour virus; HG1, human glucocorticoid receptor protein; BHK, baby hamster kidney; GRE, glucocorticoid-response element; MCS, multiple cloning site.

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cloning site (MCS) of the inducible vector pMAMneo (Clontech, Palo Alto, CA, U.S.A.). Expression of the CAT gene is controlled by the mouse mammary tumour virus (MMTV) promoter in response to either glucocorticoids or heavy metals. The CAT gene was also inserted into the MCS of the constitutive expression vector pSV_2neo (Clontech) to create pSV CAT, in which the CAT gene is permanently expressed under the simian virus 40 promoter sequence.

pSG5 HG1 contains the human glucocorticoid receptor protein HG1 cDNA (kind gift from Stephen Green, Zeneca Ltd) inserted into the pSG5 eukaryotic expression vector (Stratagene Inc., La Jolla, CA, U.S.A.), and pSV_2 bcl-2, in which the human bcl-2 cDNA was inserted into the MCS of the expression vector pSV_2neo (Clontech), was a gift from John Reed, La Jolla Cancer Research Foundation [16].

Cell culture

The Met hamster fibrosarcoma cell lines were originally established from the lung metastases of a parental tumour (HSV-2-333-2-26) transformed by the herpes simplex virus. The cloned cell lines were given the prefix Met (metastatic variant) and a suffix of A to G. The characteristics of Met cell lines are reported by Teal and Rees [17]. The BHK-21 (baby hamster kidney) cell line is commercially available from Flow Laboratories and was used as a 'normal' control cell line to the Met lines. All cell lines were grown in a humidified atmosphere at 37 °C, 5 % (v/v) CO_2, 95 % (v/v) air in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) heat-inactivated foetal bovine serum (Sigma-Aldrich, Poole, Dorset, U.K.), 2 mM glutamine (Sigma-Aldrich), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich).

Transfections

All cell transfections were performed using Lipofectin (Life Technologies, Paisley, Scotland, U.K.).

Approx. 2×10^6 exponentially growing cells (70–80 % confluent) were transfected with 10 μg of appropriate plasmid using Lipofectin at 100 μg per 10 μg of DNA as previously described [18].

Transfection efficiency was assessed by parallel transfection of each cell line with pSVCAT and measuring the level of CAT activity after 24 h. All transfection-based calculations were corrected accordingly. Transfection efficiencies were highly reproducible, with no cell line demonstrating more than a 5 % variation between all transfections performed. For the stable transfection of Met B with bcl-2, cells were transfected with 9 μg of pSVbcl-2 and 1 μg of the selection vector pSVneo using Lipofectin. Stably transfected cell lines were selected by growth in medium containing 400 μg/ml G418 (Life Technologies) as previously described [18]. Clones expressing the bcl-2 cDNA were screened by immunoblotting using commercially obtained antibody (DAKO Ltd., High Wycombe, Bucks., U.K.). Transfected control cells were transfected with pSVneo selection vector and selected by growth in medium containing G418.

Expression of tTGase

Total RNA was extracted from a confluent 75 cm² flask using the RNA STAT-60 extraction kit (Biogenesis, Poole, Dorset, U.K.) according to the procedures devised by the manufacturer. The RNA was electrophoresed on an agarose/Mops/formaldehyde gel, stained with ethidium bromide and viewed under UV to verify loading. The RNA was capillary blotted on to Hybond N (Amersham Life Science, Bucks, U.K.) and cross-linked with 70 mJ/cm² UV radiation (Amersham UV crosslinker). Northern blots were hybridized to a random-primed specific 0.4 kb *Bam*HI deletion probe constructed from a full-length cDNA of the mouse tTGase as previously described [18].

Western blots of cell homogenates were obtained and probed with a goat anti-(guinea-pig liver tTGase) polyclonal antibody (Goat 202) as previously described [19]. The blots were probed using a horseradish peroxidase-conjugated secondary antibody (DAKO Ltd.) and revealed colorimetrically using a chloronaphthol substrate.

tTGase activity was measured as the Ca^{2+}-dependent incorporation of [1,2-^{14}C]putrescine into *N*,*N*′-dimethylcasein as described previously [19].

Isolation of envelopes formed by tTGase-mediated cell death

tTGase-mediated cell death was measured as the number of detergent-insoluble envelopes that could be isolated from a fixed number of cells (normally 10^8) in culture. Envelopes were isolated by the method of Knight et al. [11]. Cells were harvested by trypsinization and then pooled with the cells present in the culture supernatant. 50 μl of 20 % (w/v) SDS and 50 μl of 0.1 % (w/v) dithiothreitol were added to 500 μl of a single-cell suspension containing 10^8 cells, and this mixture was boiled for 5 min. After cooling, 10 units of DNase I was added for 1 h at 37 °C to prevent clumping of the envelopes. The isolated envelopes were washed in PBS containing 0.1 % (w/v) SDS and 10 units of DNase I and were counted using a haemocytometer. tTGase-mediated cell death is expressed as the number of detergent-insoluble envelopes obtained from 1×10^6 cells.

CAT assay

Cells were harvested by trypsinization, and 2×10^6 cells lysed in 200 μl of 0.25 M Tris/HCl, pH 7.5, using a freeze–thaw procedure involving liquid nitrogen. The lysis mixture was centrifuged to remove any cell debris and 20 μl was added to 2 μl of 200 μCi/ml [^{14}C]chloramphenicol, 20 μl of 4 mM acetyl-CoA, 32.5 μl of 1 M Tris/HCl, pH 7.5, and 75.5 μl of distilled water and incubated at 37 °C for 1 h. The chloramphenicol and acetylated chloramphenicol were extracted using ethyl acetate and run on TLC with a 19:1 (v/v) chloroform/methanol solvent mixture. After autoradiography, bands were localized on the TLC plate, excised and quantified by β-scintillation counting. CAT activity is expressed as percentage acetylation occurring in 1 h per 2×10^6 cells, calculated by dividing the c.p.m. of the monoacetylated product by the total c.p.m. from acetylated and non-acetylated products.

Determination of the relative cell density of dexamethasone-responsive receptors

Cells were transfected with 10 μg of the CAT-inducible vector pMAMneo CAT. After 24 h, 1 μM dexamethasone was added to the culture medium and CAT activity measured 72 h after transfection. As the MMTV promoter can only be activated by the action of a dexamethasone–glucocorticoid receptor complex, it was deduced that, in the presence of a constant concentration (1 μM) of dexamethasone and corrected transfection efficiency, the CAT activity was proportional to the levels of dexamethasone-responsive receptors. When receptor number was to be determined in a co-transfection system, the pMAMneo CAT levels were reduced to 5 μg and the transfection cocktail made up to 10 μg with the vector under investigation and pSVneo. Dexamethasone-responsive receptor levels are therefore expressed as CAT activity, i.e. percentage acetylation.

Investigation of the level of apoptosis in cells

After seeding, cells were induced with either 1 μM dexamethasone or 1 μM dexamethasone and 10 μM ionomycin, a known inducer of apoptosis in Met B cells [20] for 48 h. Adherent cells were collected by trypsinization and then pooled with the cells collected from the culture supernatant. An aliquot of the cells was air-dried on to glass slides and the cells were assayed using the Apotag fluorescein kit (Oncor-Appligene, Durham, U.K.), which measures DNA fragmentation as a measure of apoptosis. The Apoalert kit (Clontech), which measures levels of CPP32, an interleukin-1β-converting enzyme protease that is an early indicator of apoptosis, was also used to verify the level of apoptosis induced by dexamethasone. Both kits were used according to the manufacturer's protocol. The percentage of cells undergoing apoptosis when measured by the Apotag fluorescein kit was analysed by manual counting and by Aequitas, an image analysis computer software package.

Determination of tTGase action after cell damage by fluorescein cadaverine incorporation into cellular proteins

Cells were induced with 1 μM dexamethasone for 48 h and then removed from culture by trypsinization. The trypsinized cells were washed in complete medium and immediately incubated in the presence of 0.5 mM fluorescein cadaverine (in complete medium) for 45 min at 37 °C. The cells were collected by centrifugation, air-dried on to glass slides and fixed in methanol (−20 °C) for 15 min. The slides were gently washed in PBS to remove any unincorporated fluorescein cadaverine, mounted and viewed by fluorescent microscopy (Zeiss Axioskop) using filters suitable for fluorescein. The photographic data were analysed by manual counting and using the Aequitas image analysis computer software program.

Envelope formation as mediated by tTGase was visualized by treating the cells exposed to fluorescein cadaverine with 2 % (w/v) SDS and 0.1 % (w/v) dithiothreitol and boiling for 5 min following the protocol of Knight et al. [11]. The isolated envelopes were placed on to a glass slide and, after mounting, observed under both fluorescent and light microscopy as described above.

Statistical analysis

For statistical analysis, paired Student's t tests were performed. P values exceeding 0.05 were considered not significant.

RESULTS

Induction of tTGase activity by dexamethasone

Dexamethasone treatment (1 μM) for 24 h causes an increase in tTGase activity of 8.5-fold in Met B, 4.4-fold in Met D, 2.2-fold in Met E and 3.6-fold in BHK-21 (Table 1). In all these cases the increase in tTGase activity was significant at $P \leqslant 0.01$. In the cell lines Met B and BHK-21, dose–response experiments were performed indicating a dose–response relationship (Table 2). Data from these experiments showed that the induction of tTGase activity plateaued at dexamethasone concentrations higher than 1 μM, and optimal induction was observed between 24 and 48 h.

To determine whether the increase in tTGase activity was paralleled by an increase in the level of tTGase antigen, immunoprobing of Western blots was undertaken using antiserum to the tTGase isolated from guinea-pig liver. The immunoprobed Western blot (Figure 1) demonstrates an increase in tTGase antigen after dexamethasone treatment in all cell lines.

Table 1 Comparison of dexamethasone-responsive receptors to dexamethasone induction of tTGase in the Met cell lines and BHK-21

For measurement of tTGase activity, 80–90% confluent cell cultures in a 75 cm^2 flask were treated for 24 h with 1 μM dexamethasone and then harvested using trypsin and counted on a haemocytometer. Aliquots (1 × 10^6 cells) were homogenized and tTGase activity determined by the incorporation of [^{14}C]putrescine into *N,N′*-dimethylcasein. Data are means ± S.E.M. from five experiments per cell line. Relative levels of dexamethasone-responsive receptors were determined by transfection of the CAT reporter gene attached to the MMTV promoter as outlined in the Materials and methods section. Receptor levels are expressed as CAT activity (percentage acetylation) adjusted for transfection efficiency and represent the mean from two experiments. The percentage increase in tTGase activity is calculated from the mean values shown in the first part of the Table.

	tTGase activity (units/10^6 cells)		Percentage increase after treatment with 1 μM dexamethasone	
Cell line	Normal medium	1 μM dexamethasone	tTGase activity	Dexamethasone receptor level (% pMAMneo CAT-mediated acetylation)
Met B	0.14 ± 0.03	1.21 ± 0.66	850	206
Met D	0.31 ± 0.07	1.35 ± 0.11	440	171
Met E	0.29 ± 0.06	0.64 ± 0.13	220	129
BHK	0.44 ± 0.06	1.57 ± 0.04	356	117

Table 2 Dose-response in Met B and BHK-21 cell lines after 48 h exposure to dexamethasone

Confluent (60–70%) cell cultures of either Met B or BHK-21 were treated for 48 h with various doses of dexamethasone and then harvested using trypsin and counted on a haemocytometer. Aliquots (1 × 10^6 cells) were homogenized and the tTGase activity was determined by the incorporation of [^{14}C]putrescine into *N,N′*-dimethylcasein. Data are means ± S.E.M. from three experiments per cell line.

	Transglutaminase activity (units/10^6 cells)	
Concentration of dexamethasone (μM)	Met B	BHK
0	0.11 ± 0.01	0.64 ± 0.16
0.001	0.74 ± 0.12	0.91 ± 0.12
0.010	1.46 ± 0.12	1.82 ± 0.08
0.100	1.25 ± 0.16	1.65 ± 0.06
1.000	1.77 ± 0.09	2.34 ± 0.10
10.00	2.01 ± 0.14	2.48 ± 0.19

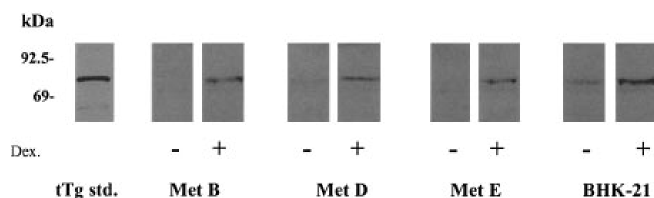

Figure 1 Immunoblot of cell homogenates from Met cell lines and BHK-21 treated for 48 h with 1 μM dexamethasone

Cells of 90% confluency were exposed to 1 μM dexamethasone (Dex; +) for 48 h, harvested by trypsinization and the cell number determined. Control cells without dexamethasone (−) were treated similarly. Cells (1 × 10^6) were homogenized and equivalent protein loaded on to a 10% (w/v) polyacrylamide gel, electrophoresed and electroblotted on to Hybond C Super Nylon. The Western blot was immunoprobed with anti-(guinea-pig liver tTGase) antibody as described in the Materials and methods section. tTg std., tTGase standard.

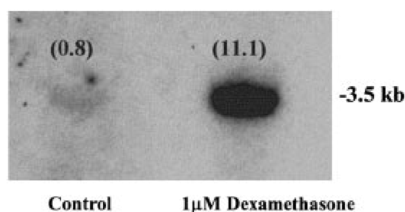

Figure 2 Northern-blot analysis of mRNA from dexamethasone-treated Met B cells

Total RNA was extracted from a 90% confluent 75 cm^2 flask of Met B cells previously treated for 48 h with 1 μM dexamethasone. The Northern blot was probed using a mouse [^{32}P]dCTP random-primed probe specific for tTGase as described in the Materials and methods section. Volume densities corrected for loading are shown in parentheses.

To determine if the increase in tTGase activity and antigen is a result of *de novo* synthesis or results from activation of an existing protein that the antibody is unable to recognize, tTGase mRNA levels were determined by Northern-blot analysis. The autoradiograph (Figure 2) shows an increase in tTGase mRNA in Met B after dexamethasone treatment, indicating that dexamethasone acts directly on tTGase transcription.

Glucocorticoid receptor involvement in tTGase expression

Experiments were undertaken to ascertain whether the increase in tTGase activity was dependent on the presence of glucocortoid receptors and if there was a correlation between the responses observed and the relative levels of glucocorticoid receptors contained in the different cell lines. Relative levels of glucocorticoid receptors were determined by transiently transfecting cells with the glucocorticoid-inducible vector pMAMneoCAT and inducing them after 24 h with 1 μM dexamethasone for 48 h, after which time CAT activity was determined. Since the MMTV promoter can only be activated by a dexamethasone–glucocorticoid receptor complex, it was deduced that, in the presence of a constant concentration of dexamethasone and corrected transfection efficiency, the CAT activity observed was proportional to the level of dexamethasone-responsive receptors. The transfection-efficiency-corrected CAT activity (expressed as percentage acetylation) representing dexamethasone-responsive receptor protein levels for each cell line is shown in Table 1. Although these data cannot be used to give an absolute dexamethasone receptor level, it does indicate the relative responsive levels of the receptor protein between different cell lines.

Comparison of the relative density of dexamethasone-responsive receptors with the increase in tTGase activity shows a direct relationship between dexamethasone receptor level and the percentage increase in tTGase activity in each cell line tested. The cell lines showing the larger increases in tTGase activity with dexamethasone treatment have a higher dexamethasone-responsive receptor level. By transfecting Met B cells with the glucocorticoid receptor HG1, it was found that the expression of tTGase could be increased further when the cells were treated with dexamethasone (Table 3), confirming the link between tTGase expression and glucocorticoid receptor level.

The transfected control (Met B − pMAMneoCAT) just receiving the control vector pSVneo and not exposed to dexamethasone showed no alteration in tTGase levels and CAT activity from those previously recorded. The normal dexamethasone response group (Met B + pMAMneoCAT) behaved as expected, with levels of tTGase activity within the statistical range normally observed (1–1.4 units/10^6 cells). In the HG1-transfected groups, tTGase activity increased from 0.96 unit in the normal dexamethasone response group to 3.4 units in the group transfected with 5 μg of HG1. The increase was observed to be steady and relatively linear, confirming the link of dexamethasone receptor level to tTGase response. An approximate measure of the level of HG1 receptor protein transfected into the cell population was undertaken by co-transfection of the inducible reporter gene pMAMneo CAT. This showed that 0.5 μg of HG1 transfected into Met B cells produced a 1.6-fold increase in CAT activity ($P \leqslant 0.05$), 1 μg of HG1 produced a 2.3-fold increase ($P \leqslant 0.05$), 2 μg a 24-fold increase ($P \leqslant 0.01$) and 5 μg a 25-fold increase ($P \leqslant 0.001$). This result was surprising in that there was such a large increase between 1 and 2 μg of transfected HG1 in the CAT activity that was not mirrored by an increase in the tTGase activity, which suggests that a critical concentration ratio of the two plasmids must be present before receptor-mediated transcription can occur from the pMAMneoCAT vector. Regardless, there is still a good correlation between the percentage increase in tTGase activity and dexamethasone-responsive receptors ($r = 0.72$, $P < 0.05$). Interestingly, co-transfection of cells with the HG1 protein and pMAMneoCAT vector in the absence of added dexamethasone also caused an increase in both CAT and tTGase activity, probably as a result of the endogenous glucocorticoids present in the serum-containing medium.

Table 3 Effect on tTGase activity of increasing the glucocorticoid receptor protein in Met B

Various amounts of glucocorticoid receptor protein HG1 expressed in the mammalian expression vector pSG5 were co-transfected into Met B cells with 5 μg of the dexamethasone-inducible vector pMAMneo CAT. Transfection of increasing levels of the HG1 receptor were monitored by measuring CAT induction resulting from the activation of the pMAMneo CAT vector when exposed to 1 μM dexamethasone for 48 h. The relative levels of dexamethasone-responsive receptor protein are expressed as CAT activity (percentage acetylation). For control Met B cells, the cells were not transfected with HG1 and were assayed after transfection with or without pMAMneoCAT. tTGase activity was measured in cell homogenates as described in the Materials and methods section. Data represents mean values ± S.E.M. from three experiments.

Cell line	Addition of 1 μM dexamethasone	tTGase activity (units/10^6 cells)	Level of dexamethasone-responsive receptor protein (% of pMAMneo CAT-mediated acetylation)
5.0 μg HG1	−	0.50 ± 0.13	10.5 ± 5.1
5.0 μg HG1	+	3.41 ± 0.15	797.0 ± 10.1
2.0 μg HG1	+	2.58 ± 0.04	775.8 ± 9.2
1.0 μg HG1	+	2.26 ± 0.05	71.6 ± 4.3
0.5 μg HG1	+	1.95 ± 0.19	50.5 ± 5.2
Control Met B + pMAMneo CAT	+	0.96 ± 0.07	33.4 ± 3.1
Control Met B − pMAMneo CAT	−	0.14 ± 0.06	1.5 ± 0.1

Dexamethasone-induced cell death

Dexamethasone has previously been reported as an inducer of apoptosis in lymphoid tissue [20]. Met B cells treated with this agent were therefore evaluated for the level of apoptosis present by two different criteria: the appearance of DNA fragmentation and the activation of CPP32, an ICE protease that is an early indicator of apoptosis. As a positive control, Met B cells were induced to undergo apoptosis by treatment with 10 μM ionomycin for 48 h, achieving approx. 35–50 % apoptosis, which is in agreement with previously published data [21].

Table 4 Number of detergent-insoluble bodies in the Met cell lines, BHK and HG1-receptor-protein-transfected Met B treated with 1 μM dexamethasone for 24 h and comparison of the calculated percentage increases in tTGase activity and envelope formation

Cells were treated for 24 h with 1 μM dexamethasone. Adherent cells were recovered from the plate using trypsin and pooled with those present in the supernatant. Total cell number was determined using a haemocytometer and detergent-insoluble bodies isolated by boiling the cells in the presence of reducing SDS as described in the Materials and methods section. Detergent-insoluble bodies were counted using a haemocytometer. Counting was performed by at least three workers who were unaware of the experimental groups. Data represent means ± S.E.M. from three experiments. Percentage increases in detergent-insoluble body formation were calculated from the mean values shown. NA, data not available.

	Number of detergent insoluble bodies/10^6 cells		Percentage increase after treatment with 1 μM dexamethasone	
Cell line	Normal medium	1 μM dexamethasone	tTGase activity (from Table 1)	Detergent-insoluble bodies
Met B	157 ± 8	518 ± 8	850	329
Met D	689 ± 9	2182 ± 27	440	316
Met E	339 ± 9	896 ± 14	220	264
BHK	530 ± 8	1201 ± 10	356	226
Met B + 5 μg HG1	157 ± 8	703 ± 10	NA	448

In both experiments, the extent of DNA fragmentation as an indicator of apoptosis was verified by the level of activation of CPP32 (results not shown). Met B cells treated with dexamethasone for 24 h did not show any significant increase in apoptosis over uninduced cells when measured by the number of cells undergoing DNA fragmentation (no cells showed DNA fragmentation after treatment with dexamethasone). In fact, treatment of the wild-type Met B cells with dexamethasone seemed to reduce the amount of spontaneous apoptosis occurring in these cells (13.0 ± 0.5 % of Met B cells showed spontaneous levels of apoptosis; data expressed as mean ± S.E.M. from four experiments). Since these results clearly indicated that dexamethasone did not induce Met B cells to undergo apoptosis, as measured by DNA fragmentation and activation of CPP32, it was essential to see if dexamethasone could affect the production of tTGase-mediated detergent-insoluble bodies, a method previously used to determine the level of apoptosis in cell lines.

The effect of dexamethasone on tTGase-mediated detergent-insoluble body formation in cells was determined by counting these bodies present in cell cultures in all cell lines and in Met B cells transfected with 5 μg of the HG1 glucocorticoid receptor protein (Table 4). In all the cell lines examined, the application of dexamethasone gave rise to a statistically significant ($P \leqslant 0.05$) rise in detergent-insoluble bodies, which was further increased in the cells transfected with HG1. Table 4 demonstrates the relationship between the increase in tTGase activity and the production of detergent-insoluble bodies, which shows that cells with the highest percentage increase in tTGase activity also have the highest percentage increase in detergent-insoluble bodies, but this increase in the body formation appears to plateau when a 3-fold increase is reached.

Since the level of classical apoptosis found in these cells measured by DNA fragmentation and ICE-like protease activation did not appear to agree with the number of detergent-insoluble bodies counted, it was possible that the formation of the detergent-insoluble bodies could be an alternative form of cell death mediated by tTGase during the treatment of cells for the determination of body formation. One possibility was that cells carrying high tTGase activity form these cross-linked shells after damage by trypsin during the harvesting process before treatment with detergent. This was tested by incubating cells after trypsinization with a fluorescently labelled substrate (fluorescein cadaverine) for tTGase. Incubation of adherent growing Met B cells with fluorescein cadaverine for 45 min indicated that this amine substrate is not toxic to the cells when used at 0.5 mM and tested by their ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, nor is the fluorescent amine incorporated into intracellular proteins of fixed adherent cells when used in this manner (results not shown). However, when trypsinized cells were observed by fluorescent microscopy (Figures 3A and 3B), an approx. threefold increase in the number

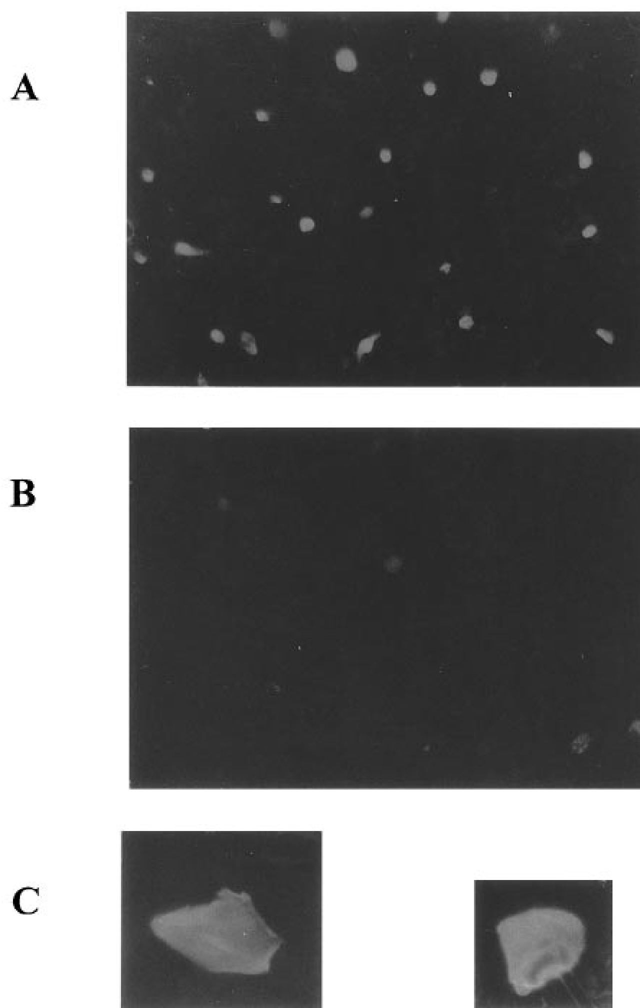

Figure 3 Cellular damage by trypsinization in induced Met B cells (A) and non-induced cells (B) as indicated by the incorporation of fluorescein cadaverine (× 20 magnification) and (C) SDS-insoluble bodies isolated from induced cells showing incorporation of fluorescein cadaverine (× 40 magnification)

Cells were incubated in complete medium in (**A**) the presence or (**B**) the absence of 1 μM dexamethasone for 48 h. Cells were trypsinized using the standard protocol, pelleted and incubated in the presence of 0.5 mM fluorescein cadaverine for 45 min at 37 °C. An aliquot of cells was air-dried on to a glass slide, fixed in methanol (−20 °C), washed and viewed by fluorescent microscopy. SDS-insoluble bodies were isolated from dexamethasone-induced cells as described in the Materials and methods section.

of Met B cells showing incorporation of the fluorescent substrate was seen in the cells induced with dexamethasone (22.5 ± 3.0 % in dexamethasone-associated cells, compared with 7.2 ± 0.8 % in the non-induced Met B cell line; data expressed as mean ± S.E.M. from four experiments). Our data therefore suggest that cells showing incorporation of the fluorescent substrate have been damaged during the trypsinization process (the initial step in measuring apoptotic bodies) resulting in Ca^{2+} influx and activation of tTGase, since a higher proportion of cellular damage was indicated by the increased extent of incorporation of fluorescein cadaverine into the cellular proteins of the induced cell culture.

To confirm that the cells showing incorporation of the fluorescein cadaverine are giving rise to the detergent-insoluble bodies, the induced and non-induced Met B cells that had been incubated with fluorescein cadaverine after trypsinization were treated with 2 % (w/v) SDS and 0.1 % (w/v) dithiothreitol, and detergent-insoluble bodies then isolated. These bodies show extensive incorporation of fluorescein cadaverine (Figure 3C), indicating a high level of protein cross-linking as mediated by tTGase. The observed threefold increase in the number of detergent-insoluble bodies in cells treated with dexamethasone (Table 4) correlates well with the threefold increase in the number of cells incorporating fluorescein cadaverine after trypsinization, and would appear to substantiate the claim that the large majority of these bodies are the result of tTGase cross-linking cellular proteins after protease-mediated cellular damage. The finding that the number of cells showing fluorescein cadaverine incorporation is much higher than the resulting detergent-insoluble bodies is not surprising, since not all cells are likely to have the same order of cross-linking and only those shells showing extensive cross-linking are likely to survive boiling in reducing SDS.

As a means of demonstrating that the detergent-insoluble bodies resulting from cell damage are not a result of classical programmed cell death, Met B cells stably transfected with either the anti-apoptotic gene bcl-2 in the vector $pSVbcl_2$ or the control vector pSVneo were also subjected to the same analysis. The presence of bcl-2 in these cells was confirmed by Western blotting (results not shown). The active nature of the bcl-2 protein present in the Met B cells was demonstrated by its ability to protect against the induction of apoptosis after exposure of cells to 10 μM ionomycin for 48 h. No apoptosis was observed in bcl_2-transfected cells, which was not the case for the pSVneo-transfected control cells, where 38.6 ± 15.0 % of the cells were judged to be undergoing apoptosis compared with 49.8 ± 5.7 % of ionomycin-induced apoptosis seen in the wild-type Met B cells (data expressed as means ± S.E.M. from four experiments). These same transfected cells when treated with dexamethasone gave increases in tTGase expression comparable with that shown for the wild-type cells (results not shown). However, when the bcl-2-transfected cells were analysed for fluorescein cadaverine incorporation after trypsinization, the number of cells showing the incorporation of the fluorescent amine was found to be comparable with the dexamethasone-treated pSVneo-transfected controls and wild-type Met B cells (results not shown).

DISCUSSION

Given the proposed roles of tTGase in cell adhesion [4,22,23], stabilization of the extracellular matrix [4,5,6,24], terminal differentiation [25,26] and programmed cell death [10,11,27], it is not surprising that perturbations in tTGase expression have been associated with a number of disease states. For example, reduced tTGase expression has been associated with the malignant phenotype [18,28,29], whereas raised tTGase activity has been linked to fibrotic disorders [5,30], cataract formation [31], Hb-Kolns disease and Alzheimer's disease [23]. It is therefore important to gain a better understanding of the regulatory mechanisms that govern expression of this enzyme.

Dexamethasone, a synthetic glucocorticoid with a long half-life, was initially reported to be an inducer of tTGase in myeloid leukaemia cells of human and murine origin [32,33]. More recently, characterization of 1.7 kb of human tTGase promoter [15] has indicated a potential GRE. In this report, tTGase expression in response to dexamethasone has been examined in three hamster fibrosarcoma cell lines and a 'normal' hamster fibroblast cell line. In all cell lines examined, dexamethasone increased tTGase activity in a dose-dependent manner. This increase in tTGase expression is accompanied by an increase in tTGase antigen and a corresponding increase in the level of transcription.

The induction of tTGase by dexamethasone in Met B can be compared with tTGase induction in Met B by retinoids [34], since both compounds combine with members of the group of proteins that constitute the zinc-finger superfamily of nuclear receptors. Like dexamethasone, treatment of Met B cells with all-*trans*-retinoic acid causes an mRNA-dependent increase in tTGase activity in these cells. Investigations conducted into the potential regulation of tTGase activity by post-translational modification by tyrosine phosphorylation and the effect of dexamethasone on this mechanism showed no obvious increase, eliminating this as a possible regulatory mechanism in this process (results not shown). Studies on mononuclear phagocytes and macrophage-like tumour cell lines indicated that dexamethasone always enhanced tTGase activity, whereas retinoic acid either induced or suppressed activity depending on the cell type [33]. Work by Fukuda et al. [35] investigating the relationship of tTGase expression to growth suppression in rat hepatoma cell lines found that, whereas both dexamethasone and retinoic acid increased tTGase activity, dexamethasone-induced tTGase correlated well with growth suppression, but retinoic acid-induced tTGase did not. These studies illustrate the level of complexity in tTGase regulation between cells and demonstrate that induction of tTGase by different stimuli may be related to a number of physiological effects. It is not surprising that regulation of the enzyme is highly complex when it is considered how many other agents have been shown to cause changes in tTGase expression, including transforming growth factor β1 [15], interleukin 6 [16], sodium butyrate [36,37], DMSO [38] and NF$\kappa\beta$ [39]. Enzyme activity is also further controlled at the protein level by GTP binding [40,41], Ca^{2+} binding [42], by endogenous inhibitors [43] and the possible existence of inactive forms of tTGase in cells [19], which adds further to the complexity of enzyme regulation. Studies on both the guinea-pig liver tTGase promoter [44] and human tTGase promoter [15] indicate the presence of strong constitutive promoter elements, suggesting that important negative or tissue-specific regulatory elements must control the expression of tTGase in many cells and tissues, and characterization of 1.74 kb of the human tTGase promoter [15] has indicated a potential GRE. This element has a similar sequence to the potential GRE sites reported in the PIT/GHF1 gene silencer region in human placental cells [45] and the rat hepatic lipase gene [46], but not that reported in mouse glutamine synthetase gene isolated from preadipocyte cells [47]. Moreover there has been no evidence to suggest the presence of a potential GRE on the 3.8 kb promoter region of the mouse tTGase gene [48]. The mouse tTGase promoter region has been reported to contain a novel retinoid-response element consisting of three rather than two repeat sequences [48], and suggestions have been made that the induction of tTGase requires the binding of three

receptors in a tripartite retinoic acid-response element [48], one of which must be the retinoid x receptor. This retinoic acid-response element has yet to be elucidated on the promoter of the human tTGase gene. The lack of a potential GRE on the mouse tTGase promoter may indicate an evolutionary divergence of this gene when compared with the sequences of other rodent and human tTGase promoter regions.

Analysis of dexamethasone-responsive receptors in the cell lines studied in this report indicate a good correlation between the relative density of the receptor and the induction of tTGase, confirming the dexamethasone induction of tTGase in these cells is a receptor-mediated process. These data confirm that, in the hamster, the potential GRE observed in the human tTGase promoter is indeed an active transcriptional component. The relationship between receptor number and tTGase expression was further reinforced by the transfection of the glucocorticoid receptor protein HG1 into Met B cells, where an increase in the transfected level of HG1 was mirrored by an increase in the level of tTGase activity. These data suggest that the level of glucocorticoid receptor in a cell could influence the level of tTGase expression. One could therefore speculate that some of the disease states involving changes in tTGase expression may be linked to the expression of the glucocorticoid receptor; however, the complexity surrounding the regulation of the enzyme has already been eluded to.

tTGase has been implicated in the formation of detergent-insoluble bodies, previously reported as apoptotic envelopes, found in cells undergoing programmed cell death [10,11], which have now been isolated from a number of cells [26]. It has been shown in this study that cells treated with dexamethasone do not appear to undergo apoptosis, as measured by the presence of DNA fragmentation or the activation of CPP32, an ICE protease. The data presented do, however, show good correlation between levels of tTGase induction and increases in the number of detergent-insoluble bodies. It was therefore important in this study to investigate other events that may lead to increased numbers of detergent-insoluble bodies when tTGase expression is increased in cells. One obvious candidate is that, when cells are passaged by incubation with trypsin, they are damaged, which could lead to an influx of extracellular Ca^{2+} resulting in activation of the tTGase present. Met B cells incubated with a fluorescently labelled substrate for tTGase, which on activation of the enzyme would be incorporated into proteins with available γ-glutamyl groups, showed a number of cells damaged by the trypsinization process, since they demonstrated the ability to incorporate the fluorescent amine. The number of cells was approx. 3–5-fold higher in the cells induced with dexamethasone, demonstrating an increased ability of these cells to incorporate fluorescein cadaverine into cellular proteins when damaged. The detergent-insoluble bodies subsequently isolated from these cells showed extensive incorporation of fluorescein cadaverine, confirming that tTGase is involved in the formation of these envelopes. The ability of tTGase to kill cells by a mechanism that does not appear to be related to either apoptosis or necrosis is intriguing. The absence of increased apoptosis in dexamethasone-induced cells and the inability of bcl-2 to block the tTGase-mediated cross-linking suggests that this cell death mechanism has no similarity to apoptosis, but may have some similarity to the type of cell death seen in the terminally differentiating keratinocyte [5], in which both the epidermal and keratinocyte transglutaminase play a key role. Furthermore, if tTGase is pivotal to the cell death process, then this may account for its overly complex regulatory pathways and its ubiquitous but varied presence in cells and tissues [49].

The ability of tTGase to cross-link cellular proteins into detergent-insoluble aggregates after cellular damage, such as that resulting from exposure to proteases, physical wounding or perhaps even anoxia when intracellular Ca^{2+} levels are likely to rise, would serve as an immediate cellular wounding response leading to structural stabilization of the dying cell and maintenance of tissue integrity. We have recently observed such a death process during renal tubulointerstitial scarring in rats submitted to subtotal nephrectomy [29], indicating the possible physiological importance of this mode of cell death.

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