Cross-linking of cellular proteins by tissue transglutaminase during necrotic cell death: a mechanism for maintaining tissue integrity

Ben NICHOLAS¹, Peter SMETHURST, Elisabetta VERDERIO, Richard JONES and Martin GRIFFIN²

Department of Life Sciences, Nottingham Trent University, Clifton Campus, Clifton Lane, Clifton, Nottingham NG11 8NS, U.K.

Tissue transglutaminase (tTG) is a Ca^{2+} -dependent enzyme which cross-links proteins via $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ bridges. There is increasing evidence that tTG is involved in wound repair and tissue stabilization, as well as in physiological mechanisms leading to cell death. To investigate the role of this enzyme in tissue wounding leading to loss of Ca^{2+} homeostasis, we initially used a model involving electroporation to reproduce cell wounding under controlled conditions. Two cell models were used whereby tTG expression is regulated either by antisense silencing in ECV 304 cells or by using transfected Swiss 3T3 cells in which tTG expression is under the control of the tet regulatory system. Using these cells, loss of Ca^{2+} homeostasis following electroporation led to a tTG-dependent formation of highly cross-linked proteinaceous shells from intracellular proteins. Formation of these structures is dependent on elevated intracellular Ca^{2+} , but it is independent of intracellular proteases and is near maximal after only 20 min post-wounding. Using labelled

primary amines as an indicator of tTG activity within these 'wounded cells', we demonstrate that tTG modifies a wide range of proteins that are present in both the perinuclear and intranuclear spaces. The demonstration of entrapped DNA within these shell structures, which showed limited fragmentation, provides evidence that the high degree of transglutaminase cross-linking results in the prevention of DNA release, which may serve to dampen any subsequent inflammatory response. Comparable observations were shown when monolayers of cells were mechanically wounded by scratching. In this second model of cell wounding, redistribution of tTG activity to the extracellular matrix was also demonstrated, an effect which may serve to stabilize tissues post-trauma, and thus contribute to the maintenance of tissue integrity.

Key words: DNA capture, $\epsilon(\gamma\text{-glutamyl})\text{lysine}$, protein cross-linking, scarring.

INTRODUCTION

Transglutaminases (TGs) are a family of enzymes whose core function is to catalyse an acyl transfer reaction between peptide-bound glutamine residues and primary amines. When the primary amine comes from peptide-bound lysine, this can result in the formation of protein multimers cross-linked via $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ bridges. In mammals, eight isoenzymes have been identified at the present time, of which six have been well characterized at the protein level [1]. Examples include the plasma TG (Factor XIII) involved in clot formation, and the keratinocyte enzyme (TG1) and the epidermal enzyme (TG3) involved in the terminal differentiation of keratinocytes in the epidermis.

One form of the TG family, tissue TG (tTG, TG type II), is distributed ubiquitously throughout the body. This enzyme has been ascribed many functions as a result of its ability to cross-link a variety of intracellular and extracellular proteins in a Ca^{2+} -dependent manner, leading to effects on cell adhesion, spreading and stability of extracellular-matrix tissues [2]. Expression of tTG is found to be up-regulated in many instances of programmed cell death [3]. tTG is also a GTP-binding protein [4,5], and it is through the inhibitory effects of GTP and GDP on its Ca^{2+} -mediated cross-linking ability that the activity of the enzyme is tightly controlled within the intracellular environment [6]. Further control of enzyme activity is also thought to be induced by nitrosylation of its active-site cysteine residue [7].

The existence of such tight controls on tTG activation indicates that the cross-linking function of the enzyme may be important

in specific cellular functions; however, it is evident that the ideal conditions for activity of the enzyme occur in the extracellular space, where the Ca^{2+} concentration is high and nucleotide levels are low. Although there is evidence for a gradual externalization of the enzyme into the extracellular space via non-classical secretory pathways, there is evidence of wider-scale activity in the form of an up-regulation of tTG expression or an increase in $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ cross-links following tissue insult. Examples include tissue injury following kidney damage, leading to end-stage renal failure [8], viral infection of lymphocytes [9], liver fibrosis [10] and in neurodegenerative diseases associated with highly cross-linked polymeric aggregates such as Alzheimer's [11] and Huntington's [12] diseases.

The aim of the present paper is to explore the role of tTG in the maintenance of cell integrity following rapid loss of Ca^{2+} homeostasis. We have used two cell types, one in which the expression of tTG is constitutively high, but has been lowered by the use of antisense silencing [13], and the other in which we can regulate tTG expression using transfected cells where tTG expression is under the control of the tet regulatory system [14]. Following loss of Ca^{2+} homeostasis, we describe the rapid formation of tTG-mediated SDS-insoluble cell-like structures or 'shells', which may represent cell-sized scars, and which occur through the Ca^{2+} -induced intracellular cross-linking activity of tTG. These shells predominantly occur in cells that express high quantities of the tTG enzyme when homeostasis is lost. These stabilizing structures may be equivalent to the cornified envelope of terminally differentiated keratinocytes, and may function in

Abbreviations used: BTC, biotin cadaverine [*N*-(5-aminopentyl)biotinamide]; DAB, 3,3'-diaminobenzidine; DTT, dithiothreitol; ECM, extracellular matrix; TG, transglutaminase; tTG, tissue TG; TUNEL, terminal transferase deoxytidyl uridine end labelling.

¹ Present address: Division of Agricultural Sciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, U.K.

² To whom correspondence should be addressed (e-mail martin.griffin@ntu.ac.uk).

response to necrosis-like cell death or secondary necrosis arising from large-scale tissue damage.

MATERIALS AND METHODS

Cell culture

ECV 304 cells and the same cells transfected with a 1.1 kb antisense cDNA vector to tTG (B7 clone) were used. The B7 clone contains around 25% of the tTG activity present in the wild type cells [13]. Cells were maintained in selection medium (Dulbecco's modified Eagle's medium) containing 10% (v/v) heat-inactivated foetal calf serum and 800 units/ml G418 sulphate (Geneticin; Amersham Life Sciences, Little Chalfont, Bucks, U.K.).

Cells from the Swiss 3T3 cell line (TG3) stably transfected with tTG under the control of a tetracycline-regulated inducible promoter were cultured as previously described in [14] in selection medium containing 10% (v/v) heat-inactivated foetal calf serum. To induce tTG expression, exponentially growing cells were cultured in the absence of tetracycline for a minimum of 48 h, to give rise to a 8–10-fold increase in transamidating activity [14]. Parallel control cultures were grown under similar conditions, but with tetracycline in the medium. During the period of induction, culture medium was changed every 48 h.

Electroporabilization of cells

Cells were electroporabilized as previously described by Smethurst and Griffin [15]. Briefly, batches of up to 10^7 cells were suspended in ice-cold poration buffer (7 mM MgSO_4 , 140 mM potassium glutamate, 1 mM EGTA, 0.5 mg/ml BSA and 10 mM Ca^{2+}) and washed three times in this buffer by centrifugation at 3000 *g* for 30 s at room temperature (20 °C) and resuspension. They were then exposed to five pulses (0.1–0.2 ms each) of an electric field of 4.0 kV/cm from a capacitance of 3 μF in a 0.4 cm-wide cuvette using a Gene-Pulser apparatus (Bio-Rad, Hemel Hempstead, U.K.).

Following electroporabilization, the cells were washed once more in poration buffer and then placed into microfuge tubes in batches of 2×10^6 cells, unless otherwise stated. The cells were pelleted as above and placed on ice.

To start the pre-incubation, the supernatant was replaced with buffer (50–100 μl) and transferred to a water bath at 37 °C, together with the addition of CaCl_2 or water and/or other inhibitors to give the final concentrations stated. Reactions were halted by the addition of stop solution (500 μl of PBS containing 100 μM iodoacetamide) and mixing thoroughly. Ca^{2+} concentrations were calculated by the method described by Yaseen et al. [16], and then checked by direct electrode measurement as described by Baudet et al. [17].

Isolation of SDS-impermeable shells

Following electroporabilization, 50 μl of lysis solution [10% (w/v) SDS, 10 mM dithiothreitol (DTT)] was added to the stop solution and the cell suspension was heated to 100 °C in a boiling water bath. The solution was allowed to cool, and then SDS-insoluble material was examined using phase-contrast light microscopy. SDS-insoluble shells were quantified using a haemocytometer, and the quantity of shells was expressed as the percentage of the initial number of electroporabilized cells. Each experiment was performed in triplicate.

For examination by scanning electron microscopy, SDS-insoluble shells were isolated as stated above and were allowed to settle to the bottom of an Eppendorf tube. The supernatant was

gently removed and the shells were rinsed three times in distilled water with a gentle resuspension each time. Finally, the shells were diluted in water to a concentration of $1 \times 10^6/\text{ml}$ and a single drop was air dried on to Number 0 glass coverslips. The coverslips were then sputter coated twice with gold, earthed with graphite onto stubs and examined using a Jeol 820I scanning electron microscope.

Detection of biotin cadaverine [BTC; *N*-(5-aminopentyl)biotinamide] incorporation into proteins in electroporabilized cells

Following electroporabilization and subsequent incubation in buffer containing either 10 nM or 1 mM Ca^{2+} , and 0.125 mM BTC (Molecular Probes, Eugene, OR, U.S.A.), cells were resuspended directly into SDS/PAGE sample buffer [125 mM Tris/HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 10 mg/ml Bromophenol Blue], sonicated for 10 s to disrupt the proteins, heated to 100 °C for 5 min and then fractionated on 10% (w/v) resolving, 2.5% (w/v) stacking polyacrylamide gels (Mini-PROTEAN II, Bio-Rad). Proteins were then transferred on to nitrocellulose membrane using an LKB semi-dry electroblotter in Towbin transfer buffer. The membrane was incubated for 1 h in 15 ml of a solution containing 5% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS with agitation. Incorporated BTC was then detected by the addition of 15 ml of a 1:5000 dilution of extravidin–peroxidase in blocking solution with agitation at room temperature for 2 h. The membrane was rinsed five times with PBS/Tween 20 with agitation, once with distilled water, and peroxidase was detected using the enhanced chemiluminescence assay (Amersham Life Sciences). Signal was captured on Kodak X-Omat film (Sigma, Poole, Dorset, U.K.).

Western blot detection of tTG in transfected clones

Cell lysates (30 μg in SDS/PAGE sample buffer) of ECV 304 and Swiss 3T3 clones (induced/non-induced) were fractionated on 10% (w/v) resolving, 2.5% (w/v) stacking polyacrylamide minigels (Mini-PROTEAN II, Bio-Rad) and transferred on to nitrocellulose membrane, as previously described in [13,14]. Membranes were blocked for 1 h in 5% (w/v) non-fat dried milk in PBS/Tween 20 blocking solution. This solution was then replaced with 15 ml of blocking solution containing a 1:4000 dilution of polyclonal goat anti-(guinea-pig liver TG) antibody (Goat 202, a gift from Dr P. J. A. Davies) and incubated for 2 h at room temperature. Primary antibody was detected using an horseradish-peroxidase-conjugated rabbit anti-goat secondary antibody and developed using the enhanced chemiluminescence assay.

Detection of apoptotic cells

Cells in suspension were air-dried on to glass slides and apoptotic cells were detected using the Apoptag® fluorescein staining kit (Oncor Inc., Gaithersburg, MD, U.S.A.). Basically, the cells were fixed on glass slides with 4% (w/v) paraformaldehyde and then treated with 20 $\mu\text{g}/\text{ml}$ proteinase K in PBS for 10 min before incubation in a buffer containing the terminal transferase with digoxigenin-labelled dUTP for TUNEL (terminal transferase deoxynucleotidyl uridine end labelling). Cells were counterstained with 5 $\mu\text{g}/\text{ml}$ (w/v) propidium iodide in PBS for 15 min and mounted in anti-fade mountant (Vectorshield, Vector Labs, Burlingame, CA, U.S.A.). Binding of an FITC-labelled anti-digoxigenin antibody to detect apoptotic cells was examined on a Zeiss-Axioskop fluorescence/UV microscope with appropriate filters, and the number of apoptotic cells was expressed as a

Figure 1 Incorporation of BTC into homogenates of electroporated ECV 304 cells

Wild-type ECV 304 cells were electroporated and then incubated in potassium glutamate buffer in the presence of 0.125 mM BTC and varying concentrations of Ca^{2+} (10 nM, 100 μM and 1 mM) for 60 min. The labelled cells were then harvested and proteins were fractionated on 10% (w/v) SDS/polyacrylamide gels before electroblotting on to nitrocellulose membrane. Incorporated biotin label was detected using extravidin–peroxidase and enhanced chemiluminescence was detected as described in the Materials and methods section. TSG and TRG indicate the tops of the stacking gel and the resolving gel respectively. MM, molecular mass.

percentage of the total number of cells in three separate fields on each slide (minimum 80 cells/field).

Immunofluorescent staining of intracellular proteins in SDS-insoluble shells

SDS-insoluble shells were generated by electroporation of ECV 304 cells as described above. Shells were immobilized on glass slides and air-dried before fixation in 4% (w/v) para-formaldehyde in PBS for 30 min. The shells were then incubated in 100 μl of blocking buffer [3% (w/v) BSA in PBS] for 30 min at room temperature. The blocking solution was then discarded and replaced with primary antibodies [monoclonal anti-vinculin (V4505), anti-actin (A4700), anti-tubulin (T9028) and anti-fibronectin (F3648), purchased from Sigma], diluted 1:100 in blocking solution. The slides were incubated for 1 h at room temperature (20 °C) and then rinsed in PBS three times before the addition of FITC-conjugated goat anti-mouse antibodies for 30 min at room temperature. The slides were then washed with PBS three times before counterstaining with propidium iodide and mounting in Vectashield anti-fade mountant. Slides were examined using a fluorescence microscope as described above.

Quantification of DNA from lysis solution

Cells were electroporated as described above in buffer containing 10 nM or 2 mM Ca^{2+} at a concentration of 4×10^6 cells/ml. The cells were resuspended in 600 μl of PBS and split into two tubes. One tube was processed for the quantification of SDS-insoluble shells as described above. The cells in the other tube were lysed in 50 μl of lysis solution [10% (w/v) SDS in distilled water] and incubated with 1 unit of proteinase K overnight at 37 °C. Saturated NaCl solution (100 μl) was added and the tube was inverted gently for 30 s. The solution was

centrifuged in a microfuge at 3000 g for 10 min at 4 °C, and 2 vol. of ice-cold ethanol were added to the supernatant. This solution was incubated at –20 °C overnight and then centrifuged at 13000 g for 15 min at 4 °C to pellet the DNA. The pellet was resuspended in 70% (v/v) ethanol and centrifuged again at 13000 g for 15 min at 4 °C to collect the DNA. The supernatant was again discarded and the DNA was resuspended in 100 μl of TE buffer (50 mM Tris/HCl, pH 7.4, and 10 mM EDTA) for 2 h at room temperature. The DNA in solution was quantified by using UV absorbance at 260/280 nm. The experiment was repeated three times.

Quantification of $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ from SDS-insoluble shells

The quantification of $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ in SDS-insoluble shells was performed according to previously published methods [18]. SDS-insoluble shells were derived from ECV 304 cells by electroporation in buffer containing 2 mM Ca^{2+} for 180 min. The shells were washed in distilled water by repeated centrifugation at 3000 g for 30 s at room temperature and resuspension three times before a final resuspension of the shells in 100 μl of $(\text{NH}_4)_2\text{CO}_3$ buffer at final concentrations of 1×10^6 , 3×10^6 and 5×10^6 cells/tube. The shells were then extensively proteolysed using subtilisin (0.05 mg/tube), Pronase (0.15 mg/tube), prolidase (0.08 mg/tube) and leucine aminopeptidase (4 units/tube), and carboxypeptidase Y (0.1 mg/tube). Control samples contained proteases alone.

Separation of the dipeptide was achieved using an LKB 4151 Alpha-Plus amino acid analyser (Pharmacia) with a 46 mm $\times$ 150 mm Ultrapac 8 ion exchange column (lithium form/particle size $8 \pm 0.5 \mu\text{m}$) using a constant temperature of 25 °C and a stepwise gradient of increasing molarity with changing pH of lithium citrate buffers as described in [18]. The program of elution involved 9 min of 0.2 M lithium citrate buffer (pH 2.8), 23 min of 0.3 M lithium citrate buffer (pH 3.0) and 63.5 min of 0.6 M lithium citrate buffer (pH 2.6) at a flow rate of 25 ml/h. Detection of the dipeptide, which was eluted at approx. 88 min, was achieved using post-column derivatization with σ -phthalaldehyde/2-mercaptoethanol at a flow rate of 20 ml/h, which was analysed in a fluorescent detector (LS1, PerkinElmer Corp.) with wavelengths of 340 nm (excitation) and 450 nm (emission). Data acquisition was by means of an interface box (900 series; Nelson Analytical, PerkinElmer). Chromatograms were compared and analysed using Model 2600 version 5 software (Nelson Analytical). Some samples were 'spiked' with known quantities of commercially obtained $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ (Sigma).

Detection of fluorescein–cadaverine incorporation into electroporated cells by fluorescence microscopy

Electroporated cells were incubated in the presence of varying concentrations of Ca^{2+} (10 nM–2 mM) in potassium glutamate buffer containing 0.5 mM fluorescein–cadaverine (Molecular Probes) for the desired duration. The reaction was then stopped by the addition of 500 μl of PBS containing 100 μM iodoacetamide. The cells were then centrifuged at 2000 g for 2 min and the pellet was resuspended in 500 μl of PBS containing 25 ng/ml propidium iodide. After 2 min, the cells were washed twice in PBS by centrifugation at 3000 g for 2 min at room temperature and resuspension, and finally suspended in 500 μl of PBS buffer. A drop of each sample was then air-dried on glass slides and mounted in Vectashield anti-fade mountant prior to observation using a Leica TCSNT confocal microscope with appropriate filters for FITC.

Cell wounding

Confluent monolayers of transfected Swiss 3T3 cells (clone TG3) or wild-type ECV 304 cells were cultured in eight-well Permanox chamber slides (7×10^5 cells/well). The medium was then replaced with fresh medium containing either 0.5 mM fluorescein-cadaverine or 0.125 mM BTC and incubated for 1 h in at 37 °C and 5% CO_2 . A wound was then introduced into the monolayer using a sterile plastic pipette tip and the cells were incubated for up to 1 h. The cells were then rinsed gently with PBS three times for 1 min and fixed in methanol at -20°C for 10 min. To detect BTC incorporation, monolayers were incubated in blocking solution [3% (w/v) BSA in PBS] for 1 h, followed by incubation in streptavidin-FITC, diluted 1:100 in blocking solution, for 2 h

and then washed three times for 5 min with PBS. Nuclei were counterstained with 10 $\mu\text{g/ml}$ propidium iodide in PBS for 10 min. The slide was mounted in Vectashield anti-fade mountant and viewed in 0.1 μm stepwise sections using a Leica TCSNT confocal microscope with appropriate filters for FITC and propidium iodide.

To detect incorporation using DAB (3,3'-diaminobenzidine) stain, streptavidin-peroxidase was used instead of streptavidin-FITC. Monolayers were incubated with Sigma Fast DAB according to the manufacturer's instructions and examined using light microscopy.

Statistical analysis

Statistical analysis of data was performed by Student's *t* test. Only *P* values < 0.05 were considered to be significant.

RESULTS

BTC incorporation into cellular proteins

Electropermeabilization of wild-type ECV 304 cells, which constitutively contain high levels of intracellular tTG, in medium containing 10 mM, 100 μM and 1 mM Ca^{2+} resulted in the incorporation of BTC into a range of intracellular proteins in a calcium-dependent manner with the greatest number of proteins labelled at 1 mM Ca^{2+} . Fractionation of proteins by SDS/PAGE, followed by Western blotting, indicated that cross-linking resulted in the accumulation of BTC-containing polymers at the stacking/resolving gel interface, and also in extremely large polymers that could not enter the stacking gel (Figure 1). Incorporation was seen into proteins of all molecular masses, with dense labelling of two proteins around 50 kDa and 55 kDa. Two smaller bands below 25 kDa were also heavily labelled. These effects were independent of apoptosis as measured by DNA fragmentation using TUNEL, which indicated only $12.1 \pm 6.54\%$ of cells showing DNA fragmentation at 20 min and $10.95 \pm 4.88\%$ of cells ($n = 3$) at 60 min when exposed to 1 mM Ca^{2+} .

Figure 2 Morphology of SDS-insoluble shells

SDS-insoluble shells were derived from electropermeabilized wild-type ECV 304 cells (1 mM Ca^{2+} for 60 min) as described in the Materials and methods section, and were then examined by (a) phase microscopy, (b) scanning electron microscopy and (c) fluorescence microscopy following staining with propidium iodide. The scale bar represents 10 μm in each panel.

Figure 3 Effect of calcium concentration on SDS-insoluble shell formation

SDS-insoluble shells were derived from electropermeabilized wild-type ECV 304 cells as described in the Materials and methods section, at varying concentrations of Ca^{2+} (0–10 mM) for 60 min. The percentage shell formation calculated from initial cell numbers at each Ca^{2+} concentration are shown. Results shown are means $\pm$ S.E.M. for three individual experiments.

Figure 4 tTG- and time-dependence of SDS-insoluble shell formation

SDS-insoluble shell formation by (a), panel (i) ECV 304 cells transfected with the antisense construct for tTG (Clone B7) and control cells (WT) and (b), panel (i) transfected Swiss 3T3 cells (clone TG3), induced (Tet —) or non-induced (Tet +) for tTG expression, following electroporation and in buffer containing 1 mM Ca^{2+} for 60 min as described in the Materials and methods section. Results shown are the means $\pm$ S.E.M. for three individual experiments performed in triplicate. Western blot analysis of the tTG content of the transfected ECV 304 cells (a), panel (ii) and Swiss 3T3 cells induced for tTG expression (b), panel (ii) analysed as described in the Materials and methods section.

Visualization of SDS-insoluble shells

Following electroporation of wild-type ECV 304 cells and incubation with 1 mM Ca^{2+} for 60 min, cells were solubilized in SDS-containing buffer and the remaining insoluble residue was visualized by phase-contrast microscopy. The shell-like insoluble residue appeared as fibrous phase-dark structures with an apparent vestigial nuclear structure (Figure 2a). Examination using scanning electron microscopy revealed greater detail of a roughly cell-sized structure with a rough surface (Figure 2b). Several SDS-insoluble shells were seen to be cross-linked together. Examination of these structures after staining with propidium iodide and visualization with a UV/fluorescence microscope revealed that DNA was trapped within the shell structure, although staining was more diffuse than that seen with a suspension of normal cells (Figure 2c). These shell structures cross-reacted with antibodies to several intracellular proteins, including vinculin, tubulin and actin, but not fibronectin (results not shown), indicating the intracellular nature of the proteins making up the shell. The SDS-insoluble structures could not be

dissolved by incubation with SDS buffer containing 25 mM DTT, thus excluding the involvement of disulphide bonds.

Calcium dependence of SDS-insoluble shell formation

SDS-insoluble shell formation was examined in wild-type ECV 304 cells at increasing concentrations (0–10 mM) of Ca^{2+} . A concentration of 1 mM Ca^{2+} was necessary for maximal shell formation after 60 min incubation (Figure 3), confirming the results obtained by BTC incorporation.

SDS-insoluble shell formation is dependent on tTG expression and occurs in different cell types

SDS-insoluble shell formation in ECV 304 and Swiss 3T3 cells indicated that large-scale shell formation took place only in cells containing high intracellular levels of tTG. This was demonstrated using the wild-type ECV 304 cells, which constitutively

Table 1 Effect of nucleotides, protease inhibitors and caspase 3 inhibitor on SDS-insoluble shell formation

ECV304 cells were electroporated and incubated in potassium glutamate buffer containing 1 mM Ca^{2+} and then incubated in the presence of TG, protease and caspase 3 inhibitors, or in varying concentrations of nucleotides as shown, as described in the Materials and methods section. The effects of these compounds on SDS-insoluble shell formation were quantified and expressed as the percentage of shells formed compared with control cells. Results are means $\pm$ S.E.M. for three individual experiments. Z-DEVD-FMK, N-benzoyloxycarbonyl-Asp-Glu-Val-Asp fluoromethyl ketone. *, $P < 0.05$ when compared with the control.

Compound	Concentration	Shell formation (%)
Cystamine	5 mM	68.3 $\pm$ 3.4*
DMSO	1% (v/v)	86.8 $\pm$ 6.2
Monodansyl-cadaverine	100 μM	75.5 $\pm$ 4.4
Pepstatin A	2.5 mM	133.5 $\pm$ 9.5
Leupeptin and PMSF	2 mM/300 mM	95.7 $\pm$ 4.2
Calpain inhibitor II	5 mM	106.6 $\pm$ 12.2
Z-DEVD-FMK	5 mM	85 $\pm$ 6.3
ATP/GTP	0.5 μM /10 μM	129.8 $\pm$ 33.3
ATP/GTP	5 μM /100 μM	102.1 $\pm$ 9.5

express high levels of tTG, and the cell line B7 in which tTG has been silenced following transfection with a 1.1 kb antisense construct [13]. The cells transfected with the antisense construct contain approx. 25% of the tTG activity of the wild-type cells (Figure 4a, ii) [13], which is clearly reflected in the difference in the numbers of shells formed by these different cells (Figure 4a, i). Similarly, using Swiss 3T3 cells transfected with tTG under the control of the tet regulatory system [14], shell formation is much more apparent in the cells induced (–tet) to express tTG. Very little shell formation is seen in the non-induced control cells (+tet) (Figure 4b, i), thus reflecting their lower endogenous tTG activity which is approx. 10–15% of the induced (–tet) cells (Figure 4b, ii) [14]. The maximum number of shells were formed following 60 min incubation, with equivalent numbers formed in both cell types expressing high levels of tTG.

SDS-insoluble shell formation is independent from proteases and the presence of nucleotides

Formation of SDS-insoluble shells could be significantly inhibited by the inclusion of the competitive primary amine substrate cystamine but was not significantly affected by a range of different protease inhibitors or by the caspase 3 inhibitor N-benzoyloxycarbonyl-Asp-Glu-Val-Asp fluoromethyl ketone. Similarly, the presence of ATP and GTP in the physiological ranges of 0.5–5 μM for ATP and 10–100 μM for GTP did not affect shell formation (Table 1).

Degree of cross-linking of the SDS-insoluble shells

SDS-insoluble shells were demonstrated to contain high levels of $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ dipeptide following exhaustive proteolysis (Table 2), thus confirming their formation by a TG-mediated mechanism. The cross-link was detected in a cell-concentration- and Ca^{2+} -dependent manner. The formation of SDS-insoluble shells was accompanied by a reduction in the concentration of free DNA in the lysis solution following electroporation (Table 3), confirming that some DNA was trapped inside the SDS-insoluble shells following their isolation.

Table 2 $\epsilon(\gamma\text{-Glutamyl})\text{lysine}$ content of SDS-insoluble shells

SDS-insoluble shells were generated from ECV304 cells as described in the Materials and methods section by incubation of electroporated cells in potassium glutamate buffer containing 1 mM Ca^{2+} for 60 min. The shells were subsequently resuspended in distilled water, quantified and then analysed for $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ content as described in the Materials and methods section. Results shown are for three experiments, each performed in triplicate, apart from that involving 5×10^6 shells, which was performed once. In the last row, 3 nmol of standard dipeptide was taken through the digestion and analysis procedure as a measure of recovery.

$10^{-6} \times$ Initial number of shells	$\epsilon(\gamma\text{-Glutamyl})\text{lysine}$ (nmol)
0	0
1	1.9 $\pm$ 0.5
3	6.5 $\pm$ 0.4
5	10.1
0 + 3 nmol standard dipeptide	2.9

Table 3 Measurement of DNA in solution surrounding SDS-insoluble shells

ECV304 cells (4×10^6 cells) were electroporated in potassium glutamate buffer as described in the Materials and methods section and incubated in the presence of either 10 nM or 1 mM Ca^{2+} in the same buffer. The cells were then suspended in 600 μl of PBS and split into two tubes, and one half was processed for SDS-insoluble shell counting and the other for measurement of DNA as described in the Materials and methods section. The cells were then lysed to obtain SDS-insoluble shells. The number of SDS-insoluble shells was determined by direct counting, and the percentage of SDS-insoluble shell formation was calculated by dividing the number of shells formed by the number of cells used, multiplied by 100. The concentration of DNA in the lysis solution surrounding the SDS-insoluble shells was determined by UV spectrophotometry. Results are means $\pm$ S.E.M. from three separate experiments. *, significant difference ($P < 0.05$) between 10 nM and 1 mM Ca^{2+} .

Electroporation conditions ...	10 nM Ca^{2+}	1 mM Ca^{2+}
SDS-insoluble shell formation (%)	0	90.5 $\pm$ 2.82*
DNA recovery in lysis buffer ($\mu\text{g}/\text{ml}$)	520.2 $\pm$ 87.6	227.7 $\pm$ 67.1*

Pattern of tTG-mediated cross-linking using fluorescein–cadaverine incorporation into electroporated wild-type ECV 304 cells

Monitoring the incorporation of fluorescein–cadaverine into cell proteins by confocal microscopy as a means of visualizing *in situ* tTG activity following loss of Ca^{2+} homeostasis indicated that, like shell formation (Figure 3), the cross-linking was Ca^{2+} -dependent, with little incorporation taking place at 10 nM and 10 μM Ca^{2+} , but with visual incorporation at 100 μM Ca^{2+} (results not shown). The incorporation increased further when the Ca^{2+} was raised to 2 mM. This incorporation was rapid with approx. 85% of the cells showing maximum labelling after 20 min in the presence of 2 mM Ca^{2+} (Figure 5B).

The subcellular pattern of incorporation when using a Ca^{2+} concentration of 2 mM indicated both perinuclear and nuclear protein cross-linking, with particular hot-spots of fluorescein–cadaverine incorporation found in the nucleus (Figure 5A).

Mechanical wounding of cell monolayers

A single scratch undertaken on adherent cell monolayers of wild-type ECV 304 cells or transfected Swiss 3T3 cells induced for the expression of tTG, in the presence of biotin or fluorescein-labelled cadaverine, was used as a means of determining whether TG-mediated intracellular protein cross-linking occurred when loss of Ca^{2+} homeostasis was introduced by mechanical wound-

Figure 5 Time course of tTG activity in electroporated ECV 304 cells

(A) The subcellular distribution of fluorescein–cadaverine incorporation (green) into ECV 304 cells electroporated in the presence of 2 mM Ca^{2+} for 0 min (panel i) and 20 min (panel ii) and counterstained with propidium iodide (red), as examined by confocal microscopy. The scale bar indicates 10 μ m. PN and IN refer to perinuclear and intracellular staining respectively. (B) Quantification of ECV 304 cells showing positive for fluorescein–cadaverine incorporation over time following electroporation. Results shown are the mean values taken from three separate experiments.

ing. Using BTC incorporation as a monitor of *in situ* TG activity, the wild-type ECV 304 cells demonstrated rapid intracellular cross-linking activity following wounding (Figure 6A), an activity that was almost completely neutralized by the addition of the competitive primary amine substrate putrescine when used at 5 mM (Figure 6A, iii). Cells that showed evidence of intracellular cross-linking remained adherent and appeared to maintain their fibroblast-like morphology. Using streptavidin–FITC to reveal BTC incorporation (Figure 6B, i, and Figure 6B, ii) indicated that, as previously observed in electroporated ECV 304 cells (Figure 5A and Figure 5B), incorporation of the labelled amine was found in both the cytoplasm and the nucleus. Maximum labelling was achieved after approx. 20 min post-wounding. When transfected Swiss 3T3 cells that were induced to express tTG were wounded in the same manner, labelled amine (fluorescein–cadaverine) was also found incorporated into the nucleus and the cytoplasmic proteins of these cells (Figure 6C, i, and Figure 6C, ii). As with ECV 304 cells, labelled amine incorporation was maximal, around 20 min post-wounding. However, unlike ECV 304 cells, labelled primary amine was also found incorporated into the extracellular matrix of the transfected Swiss 3T3 cells. The intracellular and extracellular cross-linking observed was dependent on the overexpression of tTG in the transfected Swiss 3T3 cells, since very little incorporation was observed in the non-induced transfected cells (results not shown), indicating it to be tTG-mediated. In both the ECV 304 and the Swiss 3T3 cells, DNA fragmentation could not be detected when using TUNEL staining at 20 and 60 min. post-wounding (results not shown), suggesting that at periods up to 60 min after wounding, the increase in tTG activity observed is not accompanied by classical apoptosis.

DISCUSSION

There is an increasing number of reports to indicate that tTG-mediated cross-linking of intracellular proteins is not essential to the apoptotic process [2,19]. Perhaps the most convincing of these is the finding that tTG^{-/-} knockout mice do not display a morphological phenotype associated with perturbed apoptosis [20]. However, it still cannot be ruled out that other TG enzymes [1] are compensating in these animals. Despite these findings, there is still widespread evidence to indicate that up-regulation of tTG often occurs in cells that are more prone to or destined to undergo programmed cell death [21]. Therefore, the question arises as to what role tTG has in cell death in addition to its proposed role in the final stages of apoptosis?

In the present paper, we have tried to address this question by using two different models of cell death induced by loss of Ca^{2+} homeostasis, resulting in activation of the Ca^{2+} -dependent tTG in the wounded cell when present at different levels of expression. We have used two different cell systems in which tTG expression levels were modulated by cell transfection. In the first, we have used the wild-type ECV 304 endothelial-like bladder carcinoma cell line, which has high constitutive levels of tTG, and the same cell line in which tTG levels have been silenced by transfection with a 1.1 kb antisense construct (B7) [13]. The second cell system makes use of Swiss 3T3 fibroblasts which have been transfected with the human tTG cDNA (clone TG3) such that expression of the enzyme can be increased by the tet regulatory promoter system [14]. Both of these transfected cell lines have been well characterized previously [13,14].

We demonstrate that loss of Ca^{2+} homeostasis in both cell systems when using electroporation results in the formation of highly cross-linked shells that are detergent-insoluble

Figure 6 Time course of tTG activity in wounded cell monolayers of Swiss 3T3 cells

(A) ECV 304 cells wounded in the presence of BTC for 20 min as described in the Materials and methods section, and then detected by DAB stain and visualized using light microscopy. Control, no BTC (panel i). + BTC (panel ii). + BTC and 5 mM putrescine (panel iii). (B) ECV 304 cells wounded in the presence of BTC and incorporation revealed using streptavidin-FITC, as described in the Materials and methods section. Magnification was $10\times$ using conventional fluorescence microscopy (panel i). Magnification was $63\times$ using confocal laser microscopy, showing a $1\ \mu\text{m}$ section through the middle of the cells (panel ii). Cells are counterstained with propidium iodide as described in the Materials and methods section. (C) Swiss 3T3 cells (clone TG3) induced to overexpress tTG and wounded in the presence of fluorescein-cadaverine as described in the Materials and methods section and then examined after 20 min at $\times 10$ magnification (panel i) and $\times 40$ magnification (panel ii) using confocal laser microscopy. The micrographs shown are $1\ \mu\text{m}$ sections through the middle of these cells. Arrows indicate the direction of wounding, and scale bars represent $10\ \mu\text{m}$.

and are unable to be dissolved in high concentrations of the reducing agent DTT. These insoluble structures appear to be entirely dependent on the expression of tTG in both cell systems, thus underlying the importance of tTG and not other TGs [1], which these cells may contain, in the formation of these structures. Maximum shell formation took place regardless of the presence of ATP or GTP, which are known regulators of enzyme activity

[15], at $1\ \text{mM}\ \text{Ca}^{2+}$, although nearly half of the cells showed shell formation at $100\ \mu\text{M}\ \text{Ca}^{2+}$. Moreover, the process was relatively rapid, with the majority of shells formed at 20 min and was maximal at 60 min. We have shown previously that tTG-mediated cross-linking of intracellular proteins is independent of bcl_2 control [22]. In the present paper, we demonstrate its independence from the Ca^{2+} -activated protease calpain, from

other cellular proteases and from caspase 3. Hence, massive intracellular cross-linking of proteins by tTG following loss of Ca^{2+} homeostasis appears to be independent of the classical protease-dependent apoptotic pathway.

Using labelled cadaverine incorporation *in situ* as a means of characterizing cross-linked proteins indicated that a vast array of proteins was present in the shells when analysed by Western blotting, indicating the promiscuity of the enzyme following activation by Ca^{2+} . Importantly, these proteins seemed to be intracellular in nature, with no fibronectin found in the structures. Using immunofluorescence to localize *in situ* cross-linking activity confirmed the Ca^{2+} - and time-dependency observed when measuring insoluble shell numbers. In addition, it showed that proteins both in the cytoplasm (perinuclear) and within the nucleus are targets for tTG-mediated cross-linking.

It is well established that histones [23] are substrates for the tTG, and in some cell types, such as neuroblastoma cells [24], the levels of tTG within the nucleus can be as high as 6% of the total TG enzyme. However, in both Swiss 3T3 and ECV 304 cells, tTG is not normally found (by immunohistochemistry) [13,14] in the nucleus in high concentrations, although it cannot be ruled out that the epitopes for the enzyme are excluded by associated nuclear proteins. Clearly, measurement of *in situ* activity following loss of Ca^{2+} homeostasis confirms the presence of the TG activity in the nuclear compartment, which is not evident in the cells expressing low levels of the tTG protein. This finding is in agreement with the increased presence of tTG protein and activity in the nucleus of induced transfected Swiss 3T3 cells following their incubation with the Ca^{2+} ionophore ionomycin [14].

The cross-linking of nuclear proteins such as histones, nuclear structural proteins and possibly even DNA-fragmenting enzymes [25] may explain the limited DNA fragmentation observed within the 20–60 min time frame following cell wounding. The trapping of DNA within the cross-linked shells would prevent its release, thus modulating the inflammatory response and preventing necrosis in an alternative manner to that of apoptosis.

Results comparable with that observed with cell wounding by electroporation were seen when loss of Ca^{2+} homeostasis was invoked by the mechanical wounding of an adherent cell monolayer. Using labelled cadaverine as a means of following tTG activity suggested that massive intracellular cross-linking was apparent after a 20 min period, which is in agreement with the data obtained from electroporation studies. As with electroporation, cross-linking was found both in the nucleus and in the cell cytoplasm. Intracellular cross-linking by tTG also appeared to maintain the cell morphology of the dying cells which remained adherent. Using TUNEL staining of the nucleus as a means of detecting DNA fragmentation indicated negligible staining after a 20 and 60 min period, thus confirming the data observed with cell wounding using electroporation. In Swiss 3T3 fibroblasts, cells that are known to lay down an abundant extracellular matrix [26], incorporation of labelled cadaverine, was also clearly apparent in the extracellular matrix (ECM). This incorporation increased with time and spread up to 100–150 μm beyond the initial scratch mark. It is probable that this wave of ECM-associated activity results from leakage of the enzyme from the damaged cell, since very little ECM-associated activity was observed in the Swiss 3T3 cells, in which the enzyme was not induced. However, it cannot be ruled out that the trauma induced by the initial wound led to a wave of tTG release in cells adjacent to those initially damaged. Interestingly, wound-induced intracellular free Ca^{2+} concentration waves have been described in a model of similarly wounded alveolar type II cells [27,28]. Following damage to

these cells, a calcium wave is initiated at the site of injury and is propagated throughout the monolayer. This Ca^{2+} wave could result in the activation of tTG, thus co-ordinating the healing process in response to tissue damage.

The ability of TG to kill cells independently of the apoptotic process has also been shown in hypertrophic chondrocytes. However, in this case, the enzyme involved was the active A subunit of the plasma TG [29]. Nuclei of dying cells showed no DNA fragmentation typical of apoptosis when nuclei were stained by TUNEL. However, membrane integrity was lost, leading to leakage of the enzyme into the surrounding matrix.

Further physiological evidence for the association of tTG in a form of cell death that does not fit the classical apoptotic or necrotic cell death comes from animal models of experimental renal scarring. Renal scarring is characterized by a gradual loss of tubular cells [30]. During renal scarring, these cells up-regulate tTG and, in many instances, contain high levels of ϵ -(γ -glutamyl)-lysine that are likely to be non-viable, but show no morphological signs of classical apoptosis or DNA fragmentation [8]. Moreover, increase of extracellular tTG is also found in the interstitial space of the tubular cells, which parallels increased protein cross-linking [30].

Our results define a role of tTG in cell death induced by loss of Ca^{2+} homeostasis, which protects cells from necrosis in an alternative manner to that of programmed cell death. Interestingly in a recent report, up-regulation of tTG has been implicated in the increased survival of cells by utilizing its GTP-binding activity [31].

Up-regulation of tTG expression following cell trauma and/or tissue insult is therefore likely to have a number of possible roles that could contribute to cell survival in the first instance, but, if this fails, tTG-associated cell death follows once Ca^{2+} homeostasis is lost, leading to rapid stabilization of cell proteins and the immediate surrounding matrix, thus maintaining tissue integrity by limiting massive necrosis and reducing the inflammatory response. The normal tissue-wide distribution of tTG in endothelial and epithelial tissues in particular, which are more exposed to insult, indicates that these cells contain high levels of the enzyme. This is possibly as a consequence of their increased susceptibility to tissue trauma, resulting from either mechanical damage or infection, thus providing a protective response in order to maintain tissue integrity in the event of widespread damage.

Interestingly, tTG expression has been shown to be raised during viral infection [32] and a number of viral proteins are known substrates of tTG [33,34]. Activation of tTG-mediated cross-linking under these conditions might result in the prevention of the release of virions from infected cells by cross-linking of viral proteins to the intracellular network. It is notable that, in some cells, viral infection can lead to down-regulation of tTG expression, which may be one method by which invading pathogens seek to inactivate this potentially protective enzyme.

We thank Mrs Anne Cox, Dr Claire Gaudry and Mrs Naomi Williams for their technical assistance with this work.

REFERENCES

- Grenard, P., Bates, M. K. and Aeschlimann, D. (2001) Evolution of transglutaminase genes: identification of a transglutaminase gene cluster on human chromosome 15q15. Structure of the gene encoding transglutaminase x and a novel gene family member, transglutaminase z. *J. Biol. Chem.* **276**, 33066–33078
- Griffin, M., Casadio, R. and Bergamini, C. M. (2002) Transglutaminases: nature's biological glues. *Biochem. J.* **368**, 377–396

- 3 Fesus, L., Madi, A., Balajthy, Z., Nemes, Z. and Szondy, Z. (1996) Transglutaminase induction by various cell death and apoptosis pathways. *Experientia* **52**, 942–949
- 4 Liu, S. P., Cerione, R. A. and Clardy, J. (2002) Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 2743–2747
- 5 Park, H., Park, E.-S., Lee, H. S., Yun, H.-Y., Kwon, N. S. and Baek, K. J. (2001) Distinct characteristic of $G\alpha_q$ (transglutaminase II) by compartment: GTPase and transglutaminase activities. *Biochem. Biophys. Res. Commun.* **284**, 496–500
- 6 Zhang, J. W., Lesort, M., Guttman, R. P. and Johnson, G. V. W. (1998) Modulation of the *in situ* activity of tissue transglutaminase by calcium and GTP. *J. Biol. Chem.* **273**, 2288–2295
- 7 Lai, T. S., Hausladen, A., Slaughter, T. F., Eu, J. P., Stamler, J. S. and Greenberg, C. S. (2001) Calcium regulates S-nitrosylation, dinitrosylation, and activity of tissue transglutaminase. *Biochemistry* **40**, 4904–4910
- 8 Johnson, T. S., Griffin, M., Thomas, G. L., Yang, B., Skill, J., Cox, A., Nicholas, B., Kubara, C. and El Nahas, A. M. (1997) Transglutaminase intracellularly crosslinks tubule cells and stabilizes the ECM in kidney fibrosis. *Kidney Int.* **52**, 279–279
- 9 Amendola, A., Fesus, L., Piacentini, M. and Szondy, Z. (2002) "Tissue" transglutaminase in AIDS. *J. Immunol. Meth.* **265**, 145–159
- 10 Grenard, P., Bresson-Hadni, S., El Alaoui, S., Chevallier, M., Vuitton, D. A. and Ricard-Blum, S. (2001) Transglutaminase-mediated cross-linking is involved in the stabilization of extracellular matrix in human liver fibrosis. *J. Hepatol.* **35**, 367–375
- 11 Cooper, A. J. L., Jeitner, T. M. and Blass, J. P. (2002) The role of transglutaminases in neurodegenerative diseases: overview. *Neurochem. Int.* **40**, 1–5
- 12 Chun, W. J., Lesort, M., Tucholski, J., Faber, P. W., MacDonald, M. E., Ross, C. A. and Johnson, G. V. W. (2001) Tissue transglutaminase selectively modifies proteins associated with truncated mutant huntingtin in intact cells. *Neurobiol. Dis.* **8**, 391–404
- 13 Jones, R. A., Nicholas, B., Mian, S., Davies, P. J. A. and Griffin, M. (1997) Reduced expression of tissue transglutaminase in a human endothelial cell line leads to changes in cell spreading, cell adhesion and reduced polymerisation of fibronectin. *J. Cell. Sci.* **110**, 2461–2472
- 14 Verderio, E., Nicholas, B., Gross, S. and Griffin, M. (1998) Regulated expression of tissue transglutaminase in Swiss 3T3 fibroblasts: effects on the processing of fibronectin, cell attachment, and cell death. *Exp. Cell. Res.* **239**, 119–138
- 15 Smethurst, P. A. and Griffin, M. (1996) Measurement of tissue transglutaminase activity in a permeabilized cell system: its regulation by Ca^{2+} and nucleotides. *Biochem. J.* **313**, 803–808
- 16 Yaseen, M. A., Pedley, K. C. and Howell, S. L. (1982) Regulation of insulin secretion from islets of Langerhans rendered permeable by electric discharge. *Biochem. J.* **206**, 81–87
- 17 Baudet, S., Hove-Madsen, L. and Bers, D. M. (1994) How to make and use calcium-specific minielectrodes and microelectrodes. *Methods Cell Biol.* **40**, 93–113
- 18 Griffin, M. and Wilson, J. (1984) Detection of $\epsilon(\gamma\text{-glutamyl})\text{lysine}$. *Mol. Cell. Biochem.* **58**, 37–49
- 19 Boehm, J. E., Singh, U., Combs, C., Antonyak, M. A. and Cerione, R. A. (2002) Tissue transglutaminase protects against apoptosis by modifying the tumor suppressor protein p110 Rb. *J. Biol. Chem.* **277**, 20127–20130
- 20 De Laurenzi, V. and Melino, G. (2001) Gene disruption of tissue transglutaminase. *Mol. Cell. Biol.* **21**, 148–155
- 21 Piacentini, M., Farrace, M. G., Piredda, L., Matarrese, P., Ciccocanti, F., Falasca, L., Rodolfo, C., Giammarino, A. M., Verderio, E., Griffin, M. and Malorni, W. (2002) Transglutaminase overexpression sensitises neuronal cell lines to apoptosis by increasing mitochondrial membrane potential and cellular oxidative stress. *J. Neurochem.* **81**, 1061–1072
- 22 Johnson, T. S., Scholfield, C. I., Parry, J. and Griffin, M. (1998) Induction of tissue transglutaminase by dexamethasone: its correlation to receptor number and transglutaminase-mediated cell death in a series of malignant hamster fibrosarcomas. *Biochem. J.* **331**, 105–112
- 23 Kim, J. H., Nam, K. H., Kwon, O. S., Kim, I. G., Bustin, M., Choy, H. E. and Park, S. C. (2002) Histone cross-linking by transglutaminase. *Biochem. Biophys. Res. Commun.* **293**, 1453–1457
- 24 Lesort, M., Attanavanich, K., Zhang, J. W. and Johnson, G. V. W. (1998) Distinct nuclear localization and activity of tissue transglutaminase. *J. Biol. Chem.* **273**, 11991–11994
- 25 Ballestar, E. and Franco, L. (1997) Use of the transglutaminase reaction to study the dissociation of histone N-terminal tails from DNA in nucleosome core particles. *Biochemistry* **36**, 5963–5969
- 26 Balklava, Z., Verderio, E., Collighan, R., Gross, S., Adams, J. and Griffin, M. (2002) Analysis of tissue transglutaminase function in the migration of Swiss 3T3 fibroblasts: the active-state conformation of the enzyme does not affect cell motility but is important for its secretion. *J. Biol. Chem.* **277**, 16567–16575
- 27 Hinman, L. E., Beilman, G. J., Groehler, K. E. and Sammak, P. J. (1997) Wound-induced calcium waves in alveolar type II cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **273**, L1242–L1248
- 28 Griffin, M. and Verderio, E. (2000) Tissue transglutaminase in cell death. In *Programmed Cell Death in Animals and Plants* (Bryant, J. A., Hughes, S. G. and Garland, J. M., eds.), pp. 223–240, BIOS Scientific Publishers, Oxford
- 29 Nurminkaya, M., Magee, C., Nurminsky, D. and Linsenmayer, T. F. (1998) Plasma transglutaminase in hypertrophic chondrocytes: expression and cell-specific intracellular activation produce cell death and externalization. *J. Cell. Biol.* **142**, 1135–1144
- 30 Johnson, T. S., Skill, N. J., El Nahas, A. M., Oldroyd, S. D., Thomas, G. L., Douthwaite, J. A., Haylor, J. L. and Griffin, M. (1999) Transglutaminase transcription and antigen translocation in experimental renal scarring. *J. Am. Soc. Nephrol.* **10**, 2146–2157
- 31 Antonyak, M. A., Singh, U. S., Lee, D. A., Boehm, J. E., Combs, C., Zgola, M. M., Page, R. L. and Cerione, R. A. (2001) Effects of tissue transglutaminase on retinoic acid-induced cellular differentiation and protection against apoptosis. *J. Biol. Chem.* **276**, 33582–33587
- 32 Amendola, A., Gougeon, M. L., Poccia, F., Bondurand, A., Fesus, L. and Piacentini, M. (1996) Induction of "tissue" transglutaminase in HIV pathogenesis: evidence for high rate of apoptosis of $CD4^+$ T lymphocytes and accessory cells in lymphoid tissues. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 11057–11062
- 33 Mariniello, L., Esposito, C., DiPierro, P., Cozzolino, A., Pucci, P. and Porta, R. (1993) Human immunodeficiency-virus transmembrane glycoprotein gp41 is an amino acceptor and donor substrate for transglutaminase *in vitro*. *Eur. J. Biochem.* **215**, 99–104
- 34 Mariniello, L., Esposito, C., Gentile, V. and Porta, R. (1993) Transglutaminase covalently incorporates amines into human immunodeficiency virus envelope glycoprotein gp120 *in vitro*. *Int. J. Pept. Protein Res.* **42**, 204–206

Received 17 December 2002/15 January 2003; accepted 17 January 2003

Published as BJ Immediate Publication 17 January 2003, DOI 10.1042/BJ20021949