Expression of Tissue Transglutaminase in Balb-C 3T3 Fibroblasts: Effects on Cellular Morphology and Adhesion

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Abstract. Tissue transglutaminase is a cytosolic enzyme whose primary function is to catalyze the covalent cross-linking of proteins. To investigate the functions of this enzyme in physiological systems, we have established lines of Balb-C 3T3 fibroblasts stably transfected with a constitutive tissue transglutaminase expression plasmid. Several cell lines expressing high levels of catalytically active tissue transglutaminase have been isolated and characterized. Transglutaminase-transfected cells showed morphologic features quite distinct from their nontransfected counterparts. Many of the cells showed an extended and very flattened morphology that reflected increased adhesion of the cells to the substratum. Other cells, particularly those showing the highest levels of intracellular transglutaminase expression, showed extensive membrane blebbing and cellular fragmentation. The results of these experiments suggest that the induction and activation of tissue transglutaminase may contribute both to changes in cellular morphology and adhesiveness.

TRANSGLUTAMINASES (TGases; EC 2.3.2.13) are a family of calcium-dependent enzymes that catalyze the cross-linking of proteins by promoting the formation of isopeptide bonds between protein-bound glutamine and lysine residues (for a review see Folk, 1980; Lorand and Conrad, 1984; Ichinose et al., 1990; Greenberg et al., 1991). These enzymes are also capable of conjugating polyamines to proteins (Davies et al., 1988; Piacentini et al., 1988). Several transglutaminases have now been isolated, cloned, and sequenced (Grundmann et al., 1986; Ichinose et al., 1986; Ikura et al., 1988; Phillips et al., 1990; Gentile et al., 1991; Kim et al., 1991), and it is clear that the transglutaminase activity appearing in different intracellular and extracellular compartments is the result of the activity of distinct enzymes encoded by distinct and unique genes.

The extracellular transglutaminases, Factor XIII, and the transglutaminase of seminal plasma, are involved in the clotting reactions of plasma proteins (for a review see Williams-Ashman, 1984; Ichinose et al., 1990; Greenberg et al., 1991). Much less is known of the function of the intracellular transglutaminases. Keratinocyte transglutaminase is a membrane-bound intracellular transglutaminase whose expression is restricted to skin and squamous epithelia (Ta et al., 1990). This enzyme is induced during terminal differentiation of keratinocytes and cross-links specific intracellular proteins contributing to the formation of the cornified cell envelope (Rice and Green, 1978; Thacher and Rice, 1985). The skin also contains a second intracellular transglutaminase, epidermal transglutaminase, whose function is not well understood (Kim et al., 1990).

Tissue transglutaminase is an intracellular transglutaminase found in many cells and tissues (Thomazy and Fesus, 1989). Tissue transglutaminase is involved in the process of apoptotic cell death (Fesus et al., 1987; Piacentini et al., 1991; Fesus et al., 1992), the enzyme is induced and activated in apoptotic cells and cross-linked proteins accumulate in the fragments (apoptotic bodies) of cells undergoing programmed cell death (Fesus et al., 1989). Tissue transglutaminase has also been implicated in the interactions between cells and the extracellular matrix (Bowness et al., 1987; Slife et al., 1986; Tyrrel et al., 1988; Kinsella and Wight, 1986 and 1990). Transglutaminase cross-linked matrix proteins have been shown to promote cellular adhesion (Fesus et al., 1986; Martinez et al., 1989; Sane et al., 1991), and the induction of tissue transglutaminase has been shown to be correlated with morphological changes (Byrd et Licthi, 1987; Nara et al., 1989) and increased adhesivity (Cai et al., 1991) in several cultured cell lines.

Conclusions on the physiological functions of tissue transglutaminase have been hampered by the lack of techniques capable of selectively modifying the activity of the enzyme in intact cells. Transglutaminase inhibitors have been used to deduce information on the function of this enzyme (Davies et al., 1984), but most of these inhibitors are not entirely specific and can inhibit other enzymes and physiological processes (Cornwell et al., 1983; Lee et al., 1985). An alternative approach has been to induce expression of the enzyme and then correlate changes in enzyme activity with biological responses. Retinoic acid and sodium butyrate have both been reported to increase tissue transglutaminase in some

cultured cells lines (Moore et al., 1984; Davies et al., 1985; Lee et al., 1987; Chiocca et al., 1989). However, both agents produce diverse effects on cellular function and it has been difficult to identify alterations in responses that are specifically attributable to the induction of the transglutaminase. An alternative approach is to express the enzyme in cells by transfection with a constitutive expression vector. We have recently isolated a full-length cDNA clone for human tissue transglutaminase (Gentile et al., 1991). In the studies reported here, we have introduced the cDNA for this enzyme into a eukaryotic expression vector and then have isolated lines of Balb-C 3T3 cells stably expressing high levels of the enzyme. Comparison of the phenotype of the transfected cells with that of their nontransfected counterparts, suggests that expression of tissue transglutaminase in these cells is associated with both a marked alteration in cellular morphology, increased cell adhesion and blebbing, and cellular fragmentation similar to that occurring during apoptotic cell death.

Materials and Methods

Human Tissue Transglutaminase Expression Vector

An expression vector containing the human tissue transglutaminase cDNA was constructed by inserting a tissue transglutaminase cDNA (Gentile et al., 1991) into the pSG5 DNA eukaryotic expression plasmid (Stratagene Inc., La Jolla, CA). This plasmid, which contains the SV-40 early gene promoter and the polyadenylation signal, was linearized with ECORI and the transglutaminase cDNA (3.3 kb) was ligated into the ECORI site of the plasmid (see Fig. 1). For cotransfection experiments, the pSV2-Neo plasmid (Clontech, Palo Alto, CA), containing the neomycin resistance gene under the control of the SV-40 early gene promoter, was used as a selectable marker.

Cell Culture and DNA Transfection

COS-1 cells (obtained from Dr. B. de Crombrugghe, M. D. Anderson Cancer Center, Houston, TX) were grown in alpha DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated FBS (Hyclone Laboratories; Logan, UT), 50 u/ml penicillin, and 50 mcq/ml streptomycin (Gibco Laboratories). Balb-C 3T3 cells (obtained from Dr. E. N. Olson, M. D. Anderson Cancer Center, Houston, TX) were grown in alpha DME supplemented with 10% (vol/vol) heat-inactivated FBS, 10% (vol/vol) Serum Plus (Hazleton Research Products Inc.), 50 u/ml penicillin, and 50 mcq/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Transfected Balb-C 3T3 cells were grown in alpha DME supplemented with 10% (vol/vol) in normal growth media containing 400 μg/ml G418 (Geneticin; Gibco Laboratories).

Transient transfection of COS-1 cells was carried out by using the DEAE-dextran procedure (Keown et al., 1990). Balb-C 3T3 cell lines were transfected by cotransfecting 5×10^5 in 10-cm dishes with 10 μg pSG5-TGase and 1 μg pSV2-Neo plasmid by calcium phosphate procedure (Cullen, B. R., 1987). Stably transfected cells were selected and cloned in medium containing 400 μg/ml G418. Confluent clonal cell lines were screened for human tissue transglutaminase expression by transglutaminase activity assay (Lorand et al., 1972), Northern blot (Ausubel et al., 1987), and Western blot (Maniatis et al., 1989) analysis.

Morphological and Immunochemical Analyses

Cells, grown on coverslips, were fixed in the culture dish for 10 min at room temperature by addition of an equal volume of 8% formaldehyde to the media. Coverslips were mounted with 50% glycerol in TBS and studied by phase-contrast microscopy.

Cells for immunocytochemistry were formaldehyde-fixed as described above or they were fixed in absolute methanol for 30 min at 4°C. Formaldehyde-fixed cells were treated with 1% $NaBH_4$ in TBS for 30 min at room temperature to quench autofluorescence. The cells were permeabilized by 0.3% Triton X-100 treatment for 10 min at room temperature. Coverslips were rinsed in TBS and blocked with a mixture of 50% normal rabbit serum-5% BSA (blocking solution) for 30 min at room temperature. Affinity-purified goat anti-guinea pig transglutaminase or IgG fraction of preimmune sera were diluted in blocking solution at 10 μg/ml and were applied to the coverslips overnight at 4°C. The coverslips were washed three times for 10 min in TBS containing 0.1% Tween 20. The secondary antibody was TRITC-conjugated rabbit anti-goat Ig (Cappel, Cochranville, PA) diluted 1:50 in blocking solution. Before use the antibody was dialyzed overnight at 4°C against PBS to remove unconjugated fluorochrome and preadsorbed with 1/20 vol of FBS for 30 min at room temperature. Incubation with secondary antibody was for 45 at room temperature followed by washings as above. Methanol-fixed cells were treated similarly except that the Triton-X 100 permeabilization step was omitted. Coverslips were mounted in 50% glycerol-TBS containing 0.2% *p*-phenylene diamine. Coverslips were studied on a Nikon Optiphot photomicroscope. Micrographs were taken on Kodak Tmax 400 black and white film.

Quantitation of Protein-conjugate Polyamines and ε-(γ-glutamyl)-lysine

Transfected Balb-C 3T3 cells were used for the measurement of the ε(γ-glutamyl)-lysine (Gln-Lys) dipeptide content 24 h after they reached confluency. The cells were washed twice with TBS and the acid-precipitated fractions were subjected to exhaustive proteolytic digestion (Fesus et al., 1985). The protein-free fraction of the digest was passed through an AG-50W-X8 column to remove lysine, then the sample was desalted on a silica HPLC column using water as eluent. The fraction collected at the position of ε(γ-glutamyl)-lysine was derivatized by phenylisothiocyanate (Bidlingmeyer et al., 1984), then analyzed by HPLC(ISCO) on a 3.9 mm × 30 cm uBondpack C_{18} column using the following elution system: eluent A was 0.14 M sodium acetate with 0.5 ml/liter triethanolamine (pH 6.35), eluent B was acetonitrile; isocratic separation at 10% B was carried out for 10 min followed by a linear gradient up to 25% within 2 min and a subsequent isocratic separation for 8 min. The isopeptide peak was used for the calculations and radiolabeled ε(γ-glutamyl)-lysine was present throughout the procedure to follow recovery.

Protein-conjugate polyamines levels in transfected Balb-C 3T3 cells (24 h after confluency was reached) were measured by addition of ^{3}H-putrescine (5 μCi) to the culture medium followed by isolation and quantitation of conjugated glutamines (Piacentini et al., 1988). Determination of radiolabeled derivatives was performed on aliquots of enzymatic digests of the acid-insoluble fraction, using the automated ion exchange chromatography procedure as described previously (Folk et al., 1980).

Protease Detachment Assay

Adhesivity analyses were carried out by following a modified version of a procedure previously described by DeLuca et al. (1990). Control and transglutaminase-transfected Balb-C 3T3 cells were grown in 35-mm Petri dishes, 5 μl of ^{3}H-labeled amino acids mixture (1 mCi/ml, Sp. Act. 204 mCi/mg; ICN) were added to each dish 12 hr before the assay. Monolayers of cells 24 h after reaching confluency were rinsed twice with alpha D-MEM medium and 1 ml of 0.125% bovine trypsin preparation (Gibco BRL) in Dulbecco's PBS was added. The dishes were then placed on a rotary shaker (American Rotator V; American Dade, Miami, FL) at 80 rpm at room temperature and 50-μl aliquots were taken each 5–10 min from the dishes. At the end of the incubation, the aliquots containing detached cells as well as the residual adherent cells (solubilized in 1% SDS) were quantitated by a scintillation spectrometry.

Results

Establishment of Transglutaminase-transfected Cell Lines

To express tissue transglutaminase in cultured cells, a 3.3-kB cDNA clone of human endothelial cell tissue transglutaminase (Clone hTG-1; Gentile et al., 1991) was inserted into the ECORI site of the eukaryotic expression vector pSG5 (Fig. 1). This clone (pSG5-TGase) included 135 bp of 5′ untranslated sequence of the tissue transglutaminase cDNA, 2,061 bp encoding the complete enzyme and 1,058 bp of 3′ untranslated sequence. To determine whether this

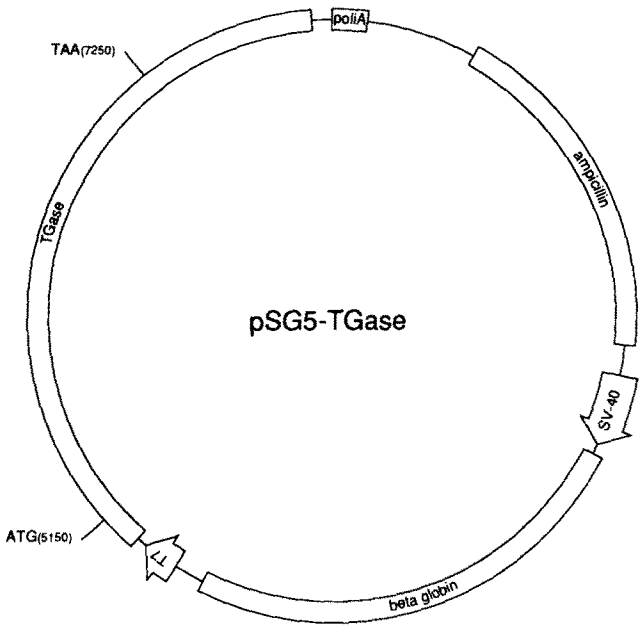

Figure 1. Schematic representation of the human tissue transglutaminase expression vector (pSG5-TGase). The vector includes the SV-40 early gene enhancer and promoter, intron II of the rabbit beta-globin gene, the T7 bacteriophage promoter, a 3.3-kb human tissue transglutaminase cDNA cloned into the ECORI site of the vector, the SV-40 polyadenylation signal sequence, and the ampicillin resistance gene.

plasmid would support the expression of active transglutaminase in mammalian cells, COS-1 cells were transiently transfected with the vector and, after 72 h, the transglutaminase activity of the cells was determined. Untransfected COS-1 cells, or COS-1 cells transfected with pSG5 alone, have very low transglutaminase activity (<0.01 pmol/min/mg of cell protein). In the transiently transfected cells the transglutaminase activity was increased >45-fold (0.270 pmol/min/mg of cell protein).

Lines of Balb-C 3T3 cells stably expressing tissue transglutaminase were established by cotransfecting Balb-C 3T3 cells with pSG5-TGase and a plasmid containing a selectable marker (pSV2-Neo). Transfected cells were selected by growth in media containing 400 μg/ml G-418 and cell lines were established by cloning cells from individual G-418 resistant colonies. The individual cell lines were then screened for the expression of tissue transglutaminase by assays of transglutaminase activity and Western blot analysis (Fig. 2). Untransfected Balb-C 3T3 cells (WT) and cells cotransfected with both the pSV2-Neo and the pSG5 (without insert) vectors show very low transglutaminase activity (<0.01 pmol/min/mg cell protein) and no tissue transglutaminase detectable by Western blot (limit of sensitivity: 1–2 ng of purified antigen) (Fig. 2, WT and clones 1 and 2). Most of the cell lines cotransfected with pSG5-TGase and pSV2-Neo that were isolated as G418-resistant colonies (clones 4, 6–8, 10, and 11) also showed very low transglutaminase activity and the absence of immunoreactive tissue transglutaminase (Fig. 2). Two lines (clones 12 and 9) showed low but detectable levels of transglutaminase activity. In both of these lines immunoreactive tissue transglutaminase was also detected by Western blot. In two other lines (clones 3 and 5) the enzyme activity was much higher (0.2 and 0.78 pmol/min/mg) than the controls and both cells gave a strong signal in the Western blot assay. These lines were selected for further characterization.

To detect the transglutaminase transcripts in the transfected Balb-C 3T3 cell lines, total RNA from clone 1 (control Balb-C 3T3 clone transfected with pSV2-Neo and pSG5) and three clones that expressed transglutaminase activity (clones 3, 5, and 9) was subjected to Northern blot analysis with a human tissue transglutaminase cDNA probe (Fig. 3). No transcripts were detected in the control clone (lane *1*); clones 3, 5, and 9 contained multiple tissue transglutaminase transcripts (lanes *2–4*). Two transcripts (4.2 and 6.2 kB) were detected in the clone 3 cells; clone 5 cells contained an abundant 2.8-kB transcript as well as minor transcripts of 4.2 and 5.0 kB. Clone 9 cells contained a single 3.3-kB transcript. It is likely that these transcripts result from random multiple integrations of plasmid DNA into the recipient cell genome. Initiation of these transfected genes can lead to diverse transcripts accumulating in the transfected cells (Jiang and Jordan, 1992; Eghbali et al., 1990).

Induction of the Transfected Transglutaminase with Sodium Butyrate

Sodium butyrate has been reported to increase the expression of transfected DNA in both transiently and stably transfected cells (Rouault et al., 1987). Fig. 4 (*A* and *B*) shows the effect of 2 mM sodium butyrate on the expression of tissue transglutaminase in Balb-C 3T3 control cells (clone 1) and two of the transglutaminase-transfected cell lines (clones 3 and 5). Cell extracts were fractionated by SDS-PAGE and then either subject to Coomassie blue staining (Fig. 4 *A*) or Western blot analysis with an antibody to tissue transglutaminase (Fig. 4 *B*). In both untreated and butyrate-treated control cells, the level of tissue TGase is too low to be detected by protein staining (Fig. 4 *A*, lanes *1* and *2*) or short exposure (6 h) of the Western blot autoradiogram (Fig. 4 *B*, lanes *1* and *2*). Prolonged exposure (72 h) of the autoradiogram (Fig. 4 *B*, lanes *1** and *2**) demonstrated the presence of a low level (~10 ng/mg cell protein) of the endogenous mouse tissue transglutaminase in both untreated and butyrate-treated cells. As we have previously reported (Gentile et al., 1991) mouse transglutaminase has an electrophoretic mobility slightly faster than the transfected human enzyme (compare lanes 1* and 2* with lanes 3–6).

Sodium butyrate caused a large increase in the level of tissue transglutaminase in the transfected cell lines. In untreated clone 3 cells the level of transglutaminase is too low to be detected by protein staining, however, after exposure of sodium butyrate, a distinct transglutaminase band (identified by the *arrow* adjacent to lane *4* in Fig. 4 *A*) can be recognized. The level of the enzyme is even higher in the clone 5 cells. In these cells the transglutaminase is sufficiently abundant to be detected in untreated cell extracts (identified by the *arrow* adjacent to lane *5*). In the butyrate-treated cells the tissue transglutaminase is clearly visualized as a prominent 80-kD polypeptide band (lane *6*).

The level of tissue transglutaminase in the transfected cells was quantitated by Western blot autoradiograms (Fig. 4 *B*). In clone 3 cells, the basal concentration of the enzyme is 0.5

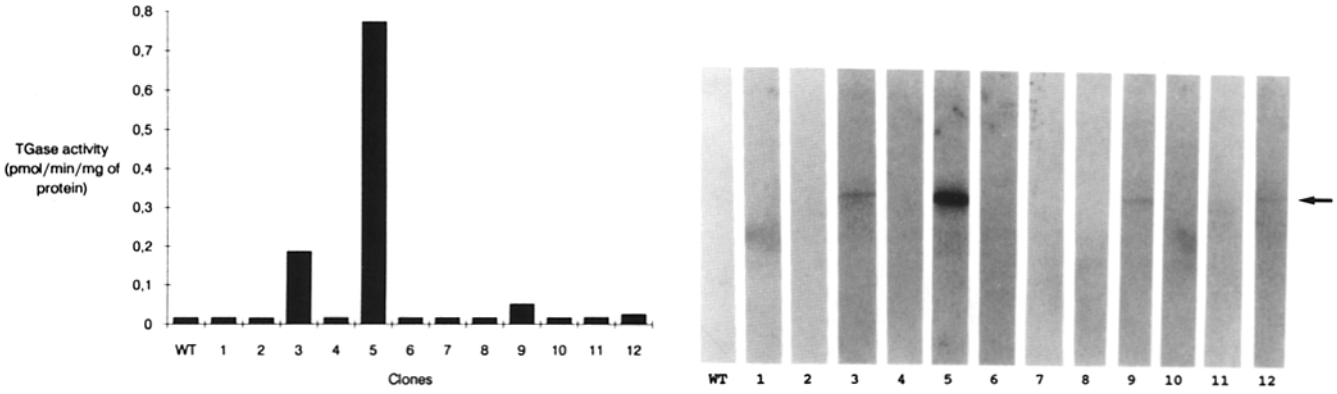

Figure 2. Transglutaminase expression in control and expression vector–transfected Balb-C 3T3 cell lines. Transglutaminase activity (*A*) was determined on total cellular homogenates (10–100 μg of protein) as described in Materials and Methods. 100 μg of total cellular homogenate protein were used for tissue transglutaminase Western blot analysis (*B*) as described in Materials and Methods. WT identifies untransfected Balb-C 3T3 cells. Clones 1 and 2 are control cell lines transfected with pSG5 vector without a transglutaminase insert and clones 3–12 are cell lines stably transfected with the pSG5-TGase expression vector. Values of enzymatic activity represent the mean of duplicate determinations.

μg/mg of cell protein (lane *3*), in the clone 5 cells it is 1.5 μg/mg of cell protein (lane *5*). After sodium butyrate treatment the level of the enzyme increases to 16 μg/mg of cell protein in clone 3 (lane *4*) and 35 μg/mg of cell protein in clone 5 cells (3.5% of total cell protein) (lane *6*).

Characterization of Transglutaminase in Transfected 3T3 Cells

Previous studies reported that tissue transglutaminase is mostly recovered in the soluble fraction of cell homogenates (Slife et al., 1986; Juprelle-Soret et al., 1988) with a small fraction of the enzyme present in the particulate components. To determine the subcellular distribution of the enzyme in the transglutaminase-transfected cells, clone 5 cells were homogenized and separated into cytosolic and particulate fractions by ultracentrifugation. Tissue transglutaminase specific activity determined in the total cellular homogenate was 3.57 pmol/min/mg of protein. The majority of this enzymatic activity (>95%) was located in the cytosolic fraction (8.43 pmol/min/mg of protein). The specific activity of the enzyme in this compartment was 40-fold higher than in the particulate fraction (0.208 pmol/min/mg of protein).

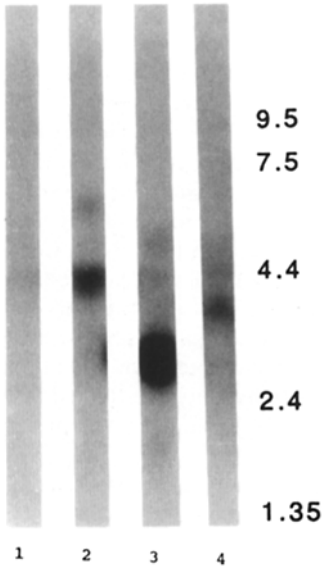

Figure 3. Tissue transglutaminase transcripts in control and expression vector–transfected Balb-C 3T3 cell lines. Total RNA from control (lane *1*) and the transglutaminase-transfected cell lines clone 3 (lane *2*), clone 5 (lane *3*), clone 9 (lane *4*) was fractionated and probed with a 3.3-kb radiolabeled human tissue transglutaminase cDNA hybridization probe. Numbers on the right side identify the mobility of RNA molecular mass standards (in kb).

Isopeptide Conjugates in Transfected 3T3 Cells

Tissue transglutaminase catalyzes cross-linking of proteins by the formation of ε-(γ-glutamyl)-lysyl isopeptide bonds (Folk, 1983). To determine the level of the transglutaminase activity in intact Balb-C 3T3 cells, the level of the ε-(γ-glutamyl)-lysyl dipeptide was measured by HPLC in enzymatic digests of extracts from control cells and one of the transglutaminase-transfected cell lines (clone 5) (Table I). The average level of isopeptide in the transglutaminase-transfected Balb-C 3T3 cells was slightly higher (70%) than in the control cells. Sodium butyrate caused a 20-fold increase in the level of isopeptide in the control cells and an even greater increase in the amount of isopeptide in the clone 5 cells.

Transglutaminase not only catalyzes the formation of isopeptide cross-links, but also the formation of bis-(γ-glutaminyl)-polyamine conjugates. The levels of these conjugates were too low to be detected in nontransfected cells, whether or not they were treated with sodium butyrate. They were detectable (16.5 pmol/mg protein) in the butyrate-treated transfected cells.

Growth Rate of Transfected 3T3 Cells

Previous studies have reported that increased transglutaminase activity often was correlated to reductions in cellular proliferation rate (Birckbichler et al., 1977). To determine whether expression of tissue transglutaminase produced a generalized effect on cellular proliferation, we measured the proliferative activity of control and transglutaminase-transfected 3T3 cell clones by ^{3}H-thymidine incorporation during log phase growth (Fig. 5). There was no significant difference between the growth rate of the control (clone 1) and transglutaminase-transfected cells. To confirm the findings we also measured the growth rate of control and transfected cell lines. The doubling time of the transfected cells

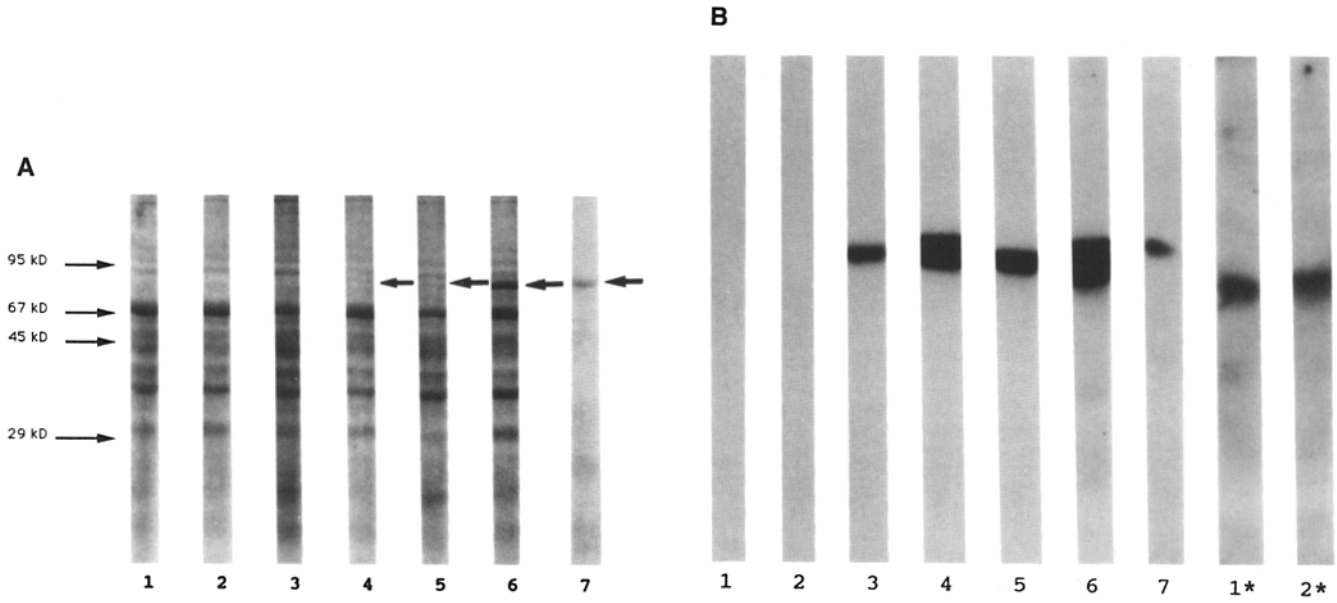

Figure 4. Effect of sodium butyrate on tissue transglutaminase expression in control and transglutaminase-transfected Balb-C 3T3 cell lines. Control (clone 1, lanes *1* and *2*) or transglutaminase-transfected (clone 3, lanes *3* and *4*; clone 5, lanes *5* and *6*) cell lines were cultured in media alone (lanes *1*, *3*, and *5*) or media plus sodium butyrate (2 mM, 48 h, lanes *2*, *4*, and *6*). Extracts of the cells were fractionated by SDS-PAGE and then either subjected to Coomassie blue staining for proteins (*A*) or Western blot analysis (*B*). *B* (lanes *1–6*) were obtained after 6 h autoradiography. Lanes 1* and 2* were obtained when the same samples shown in lanes 1 and 2 were subjected to autoradiography for 72 h. *A* (lane *7*) contains purified human tissue transglutaminase as a standard. The arrows adjacent to lanes 4–7 identify the human tissue transglutaminase band. Tissue transglutaminase amounts were calculated by interpolation with purified human tissue transglutaminase standard analyzed by laser densitometry. Purified human red blood cells tissue transglutaminase was a generous gift of Dr. P. J. Birckbickler (Noble Foundation, Ardmore, OK).

(clones 3 and 5) of 38 h was not significantly different from the doubling time of the pSV2-neo–transfected control cells ($t_{1/2}$ = 39 h).

Morphology of Tissue Transglutaminase-transfected Balb-C 3T3 Cells

We observed that increased expression of tissue transglutaminase in Balb-C 3T3 cells increased their adhesive properties. To examine this issue directly we used phase-contrast microscopy of formaldehyde-fixed control (clone 1) and transglutaminase-transfected (clone 3 and 5) cells (Fig. 6). The control cells were indistinguishable from untransfected Balb-C 3T3 cells. The majority of cells showed a typical fibroblastic morphology, bipolar compact cells with well-developed lamellipodia (Fig. 6 *A*). A few flattened cells and occasional phase-bright mitotic cells were also present. The transglutaminase-transfected cell lines showed a quite different morphologic profile. The clone 3 cells showed an extremely spread and flattened morphology (*C*). There were very few compact or bipolar cells detectable even in lower power fields. The clone 5 cells showed a more complex morphologic pattern (Fig. 6 *E*). This line included many spread and flattened cells similar to those seen in clone 3 but it also included numerous more compact, phase-bright, rounded

Table I. Quantitation of ε-(γ-glutamyl)-lysyl Dipeptide and Spermidine-conjugate Levels in Transglutaminase-transfected Clone 5 Balb-C 3T3 Cells

		Clone 1 (control)	Clone 5
ε-(γ-glutamyl)--lysyl dipeptide (nmol/mg of protein)‡	−NaB	0.26	0.44
	+NaB*	5.10	10.30
N1,N8 Bis-(γ-glytamyl)--spermidine (nmol/mg of protein)‡	−NaB	ND	ND
	+NaB*	ND	0.165

* NaB treatment, 2 mM for 48 h.
‡ Each value represents the mean of two different determinations.
ND, not detectable (<0.05 nmol/mg of protein).

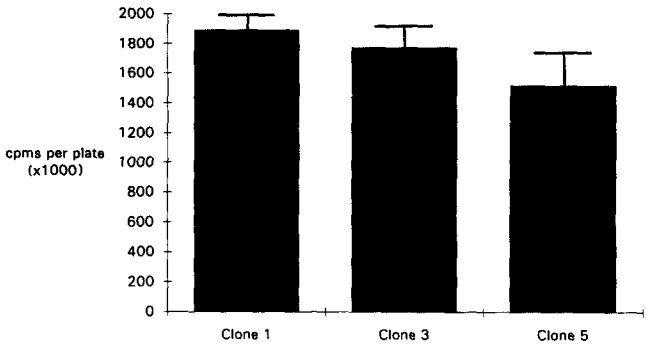

Figure 5. Growth rate of control and transglutaminase-transfected Balb-C 3T3 cell lines. Control (Clone 1) and transglutaminase-transfected Balb-C 3T3 cells (Clones 3 and 5) in log-phase growth were pulse labeled with ^{3}H-thymidine and the incorporation of thymidine into DNA was measured by TCA precipitation and scintillation spectrometry. Values presented represent the mean ± SD from three separate experiments.

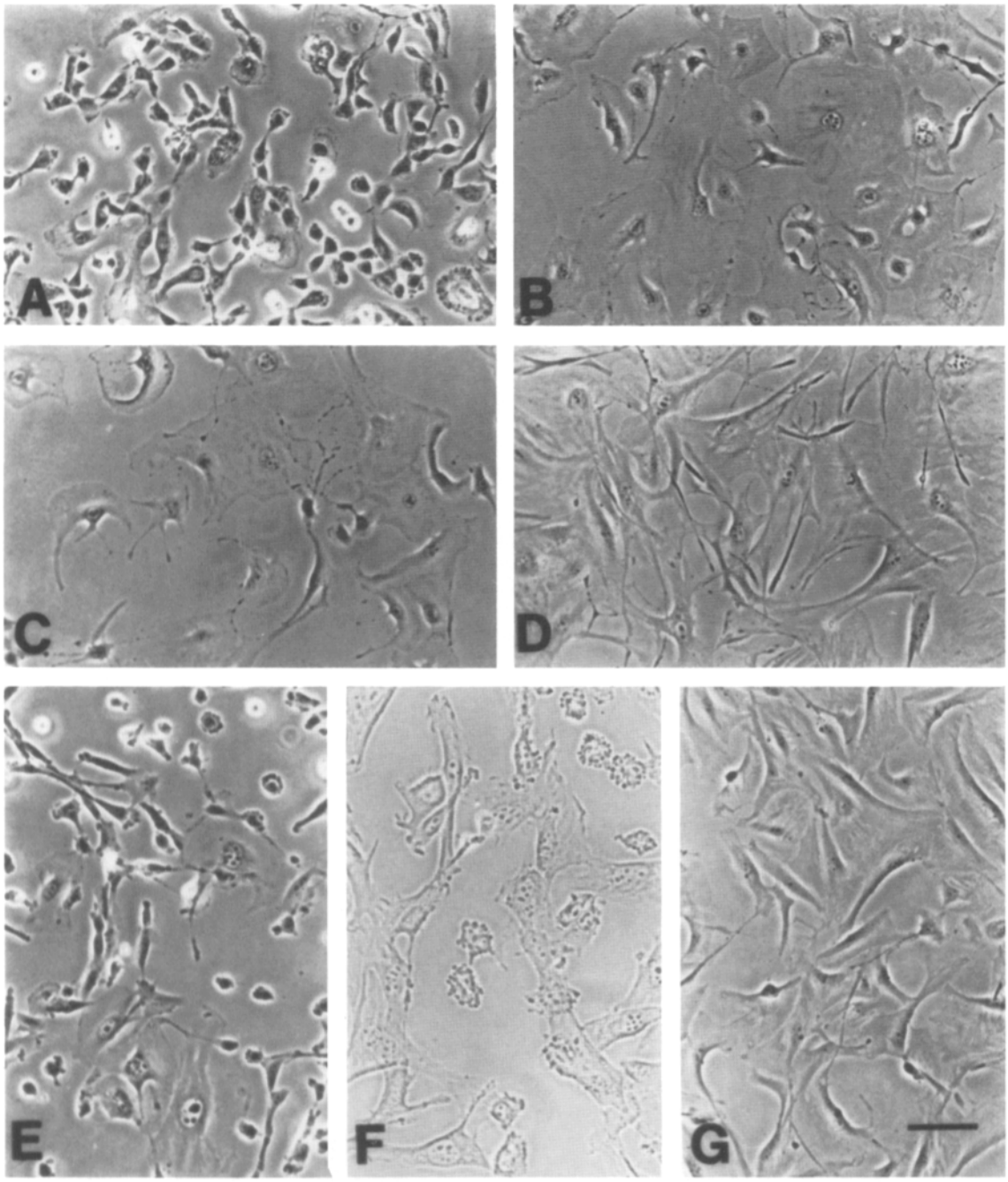

Figure 6. Phase-contrast of control and transglutaminase-transfected cells: effect of sodium butyrate. Control (clone 1; *A* and *B*) or transglutaminase-transfected (clone 3, *C* and *D*; clone *5*, *E–G*) Balb-C 3T3 cells were grown in media alone (*A*, *C*, *E*, and *F*) or media plus sodium butyrate (5 mM, 48 h; *B*, *D*, and *G*). Cells were formaldehyde fixed and photographed under phase-contrast illumination as described in Materials and Methods. *F* shows a higher magnification of clone 5 cells grown in media alone. Bar: 10 μm (*A–E*, *G*); 5 μm (*F*).

cells. Unlike the rounded cells in the control population these cells were not mitotic but were comprised of interphase cells that showed an extensive membrane blebbing. These cells, which are better visualized in a higher power field (Fig. 6 *F*), were actively shedding membrane-limited hematoxylin-negative fragments, a process that leads to the accumulation of numerous small shrunken cells with intact nuclei and an irregular blebbing outline.

Our previous studies suggested that treatment with sodium butyrate increased the level of protein cross-linking in both control and transglutaminase-transfected cells. This effect was paralleled by a marked alteration in the cell morphology of the cells (Fig. 7, *B*, *D*, and *G*). In the control 3T3 cells (clone 1), sodium butyrate treatment resulted in a spread and flattened morphology that was very similar to that seen in the transglutaminase-transfected cells (compare *B* and *C*). Butyrate treatment of the transglutaminase-transfected clones resulted in an even more uniform pattern of extremely spread and flattened cells (*D* and *G*). It is notable that butyrate treatment suppressed the expression of the pseudoapoptotic cell phenotype in the clone 5 transfected cell population (compare *E* and *G*).

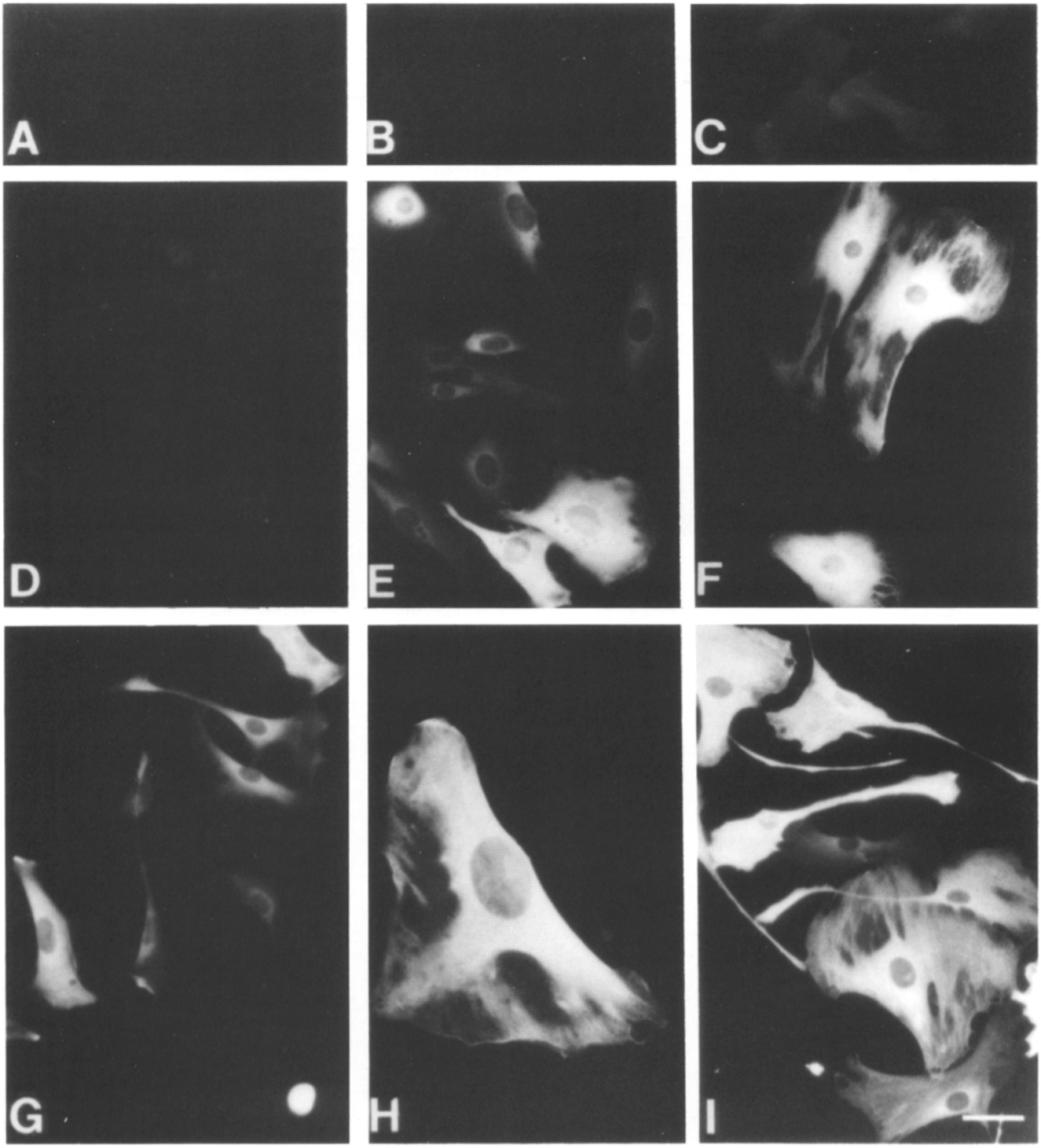

Figure 7. Tissue transglutaminase expression in control and transglutaminase-transfected Balb-C 3T3 cells. Control (clone 1, *A–C*), clone 3 (*D–F*) and clone 5 (*G–I*) Balb-C 3T3 cells were subject to paraformaldehyde fixation, detergent permeabilization, and indirect immunofluorescence microscopy with anti-tissue transglutaminase antibodies as described in Materials and Methods. *A* and *B* are from control (clone 1) cells exposed either to preimmune (*A*) or immune (*B*) IgG under standard photographic conditions. *C* shows the results when control cells treated with immune IgG were subjected to an extended exposure times (8× longer than standard). *D–F* show clone 3 cells exposed to preimmune (*D*) or immune IgG (*E* and *F*). The cells in *E* were cultured under standard conditions, those in *F* were exposed to sodium butyrate (5 mM) for 48 h. *G–I* show clone 5 cells exposed to immune IgG after culture in normal media (*G* and *H*) or sodium butyrate-containing media (*I*). *H* shows a clone 5 cell grown in normal media, exposed to immune IgG, and photographed at a higher magnification than the cells in *G*. Bar: 5 μm (*D–G* and *I*); 2.5 μm (*A–C* and *H*).

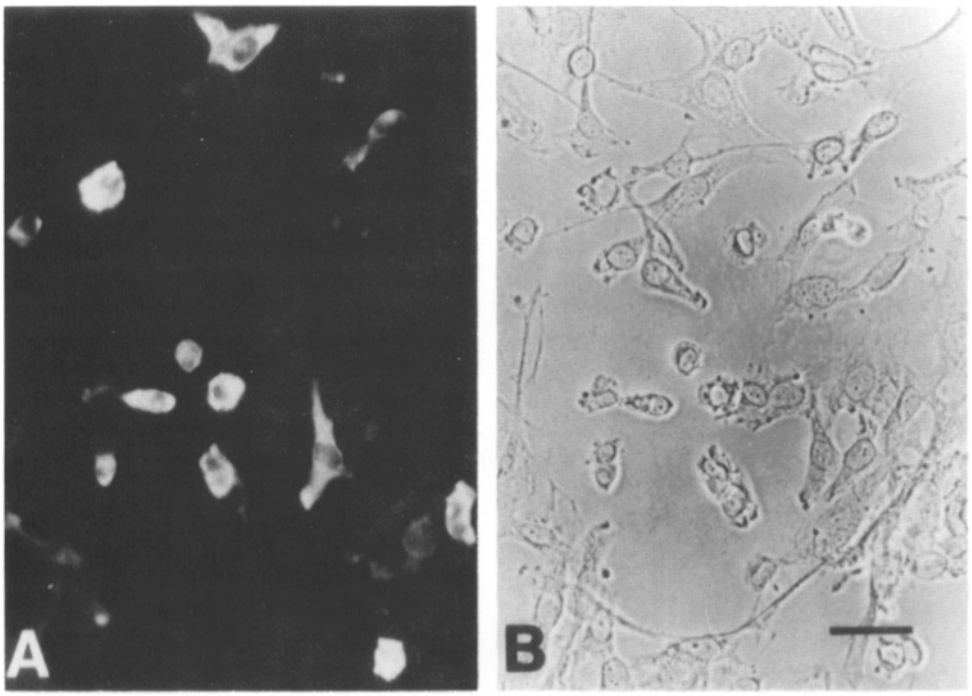

Figure 8. Detection of tissue transglutaminase in clone 5 Balb-C 3T3 cells. Clone 5 transglutaminase–transfected Balb-C 3T3 cells were fixed with methanol and subject to indirect immunofluorescence microscopy with an antitransglutaminase antibody as described in Materials and Methods. Photographs are from the same field subject to either epifluorescence illumination (*A*) or phase-contrast microscopy (*B*). Bar, 5 μm.

To evaluate the relationships between transglutaminase expression and cellular morphology, we used immunocytochemistry to visualize the level of tissue transglutaminase expression in individual control and transglutaminase-transfected 3T3 cells (Fig. 7). *A–D* show a series of control experiments. *A* and *B* show the background fluorescence signal detected in control (clone 1) cells treated with either preimmune immunoglobulin (*A*) or affinity-purified antitransglutaminase antibodies (*B*). Under photographic exposure conditions adequate to detect transglutaminase expression in the transfected cells (*E–I*) there was no background signal in control cells. Prolonged exposure (eight times longer) did result in minimally detectable immunospecific staining of the control cells (*C*) presumably due to reaction of the antibody with basal level of endogenous tissue transglutaminase in Balb-C 3T3 cells. Comparison of transglutaminase-transfected cells (clones 3 and 5) treated with either preimmune (*D*) or immune antibodies (*E*, *G*, and *H*) demonstrated diffuse immunospecific staining of the cytoplasm of the transfected cells. It was noteworthy that although all the transfected cells were immunopositive there was considerable variation in the amount of transglutaminase expressed in individual cells. These differences were largely obviated when the cells were treated with sodium butyrate (*F* and *I*), which induced an uniform strong immunospecific staining of all the transfected cells. *H* shows a higher power view of an individual transglutaminase-transfected cell. The enzyme appears to be uniformly distributed throughout the cytoplasm of these well-spread cells. The tissue transglutaminase expressed in these cells appears to be largely excluded from the nucleus and cytoplasmic vesicles.

The extensive incubation and washings steps, involved in the standard indirect immunofluorescence protocol, resulted in the loss of most of the loosely adherent, pseudo-apoptotic cells in the clone 5 cell line. When these cells were retained they showed strong immunopositive staining with the antitransglutaminase antibodies. The brightly fluorescent cell in the lower right corner of *G* is one such cell. To better visualize the level of transglutaminase in these pseudo-apoptotic cells, monolayers of clone 5 grown on glass coverslips were subjected to a modified immunofluorescence protocol using precipitating fixatives, minimal incubation times, and very gentle washing procedures to retain the majority of these cells. Fig. 8 shows the phase-contrast and matching fluorescence micrograph from such an experiment. The phase-contrast micrograph (*B*) reveals numerous compact cells with irregular outlines due to membrane blebbing. Each of these cells is strongly immunopositive when stained with the antitransglutaminase antibodies (*A*). The strong signal in the pseudo-apoptotic cells compared to the more adherent, flattened cells suggested that these cells have very high levels of tissue transglutaminase expression.

Adhesive Properties of Transfected 3T3 Cells

It has been reported that the induction of tissue transglutaminase in cells is associated with alteration in cellular morphology (Byrd and Licthi, 1987; Nara et al., 1989) and adhesion (Cai et al., 1991). We measured the rate of detachment of both the Balb-C 3T3 control (clone 1) and two of the transglutaminase-transfected cell lines (clones 3 and 5) after exposure to 0.125% bovine trypsin preparation (Fig. 9 *A*). The transglutaminase-transfected Balb-C 3T3 were more resistant to protease detachment than the control cells. In particular, clone 5 included many cells that required prolonged exposure to the trypsin preparation before they were detached from the culture dish. Sodium butyrate treatment further increased the resistance of the transfected cells to trypsinization (Fig. 9 *B*).

Discussion

Transglutaminases are a family of enzymes that have been extensively studied, but an understanding of their physiolog-

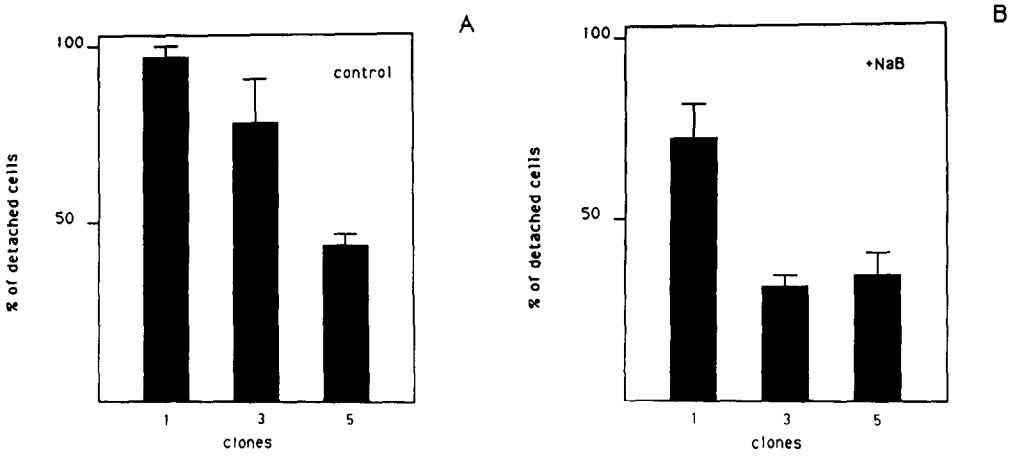

Figure 9. Trypsin-induced detachment of control and transglutaminase-transfected Balb-C 3T3 cell lines. Monolayers of control (clone 1) and transglutaminase-transfected (clones 3 and 5) Balb-C 3T3 cells were subject to trypsinization (0.125% trypsin, 30 min, 23°C with shaking) as described in Materials and Methods. *A* represents data from cells grown in normal media and *B* from cells grown in media containing sodium butyrate (2 mM, 48 h). Values, expressed as the percentage of total cells that have detached at the end of the 30 min incubation, represent the mean ± SD of triplicate determinations.

ical functions has been slow to emerge. It is clear that extracellular transglutaminases, Factor XIII and seminal plasma transglutaminase, are involved in the stabilization of clots in blood and in seminal plasma, respectively (Greenberg et al., 1991). Keratinocyte transglutaminase, an enzyme induced during the cornification of squamous epithelia, has been shown to contribute to the formation of cross-linked protein "envelopes" inside terminally differentiated keratinocytes (Rice and Green, 1978). However, the physiological role of other transglutaminases is less well understood. Tissue transglutaminase is a relatively ubiquitous intracellular transglutaminase that has been studied for many years but little clear evidence exists as to the biological function of this enzyme. This enzyme has been implicated in diverse cellular functions such as growth regulation (Birckbichler et al., 1978), endocytosis (Davies et al., 1984), secretion (Sener et al., 1985; Bungay et al., 1986), cellular morphology (Paye et al., 1986; Byrd and Licthi, 1987; Nara et al., 1989), cellular adhesion (Cai et al., 1991), and most recently in apoptotic cell death (Fesus et al., 1992), but unambiguous experimental evidence for an essential role for the enzyme in these processes has been hard to obtain. The goal of the studies reported here has been to develop a novel approach to investigating the biological activity of tissue transglutaminase. We have selectively increased the level of expression of tissue transglutaminase (by expression vector transfection) in a cell type that normally expresses very low levels of the enzyme and then we have searched for the biological effects attributable to the over-expression of the enzyme.

One of our major concerns in initiating these studies was the feasibility of developing cell lines capable of tolerating high levels of intracellular transglutaminase activity. Transglutaminase induces irreversible protein cross-linking and one might anticipate that the expression of high levels of tissue transglutaminase inside a cell might be associated with a profound inhibition of proliferative activity. Several cell types, such as endothelial cells (Greenberg et al., 1987; Korner et al., 1989; Nara et al., 1989) and activated or retinoid-stimulated macrophages (Murtaugh et al., 1983; Moore et al., 1984), have been shown to contain high levels of intracellular transglutaminase activity (as much as 1–2% of total cellular proteins), but these cells are usually terminally differentiated and show little proliferative activity in vitro. Birckbichler et al. (1978) have shown that transglutaminase activity tends to be very low in undifferentiated or rapidly proliferating cell types and to rise when the cells are induced to differentiate or to undergo growth arrest. Transglutaminase levels are high in cells undergoing apoptotic cell death (Fesus et al., 1987), again suggesting that expression of the enzyme is associated with terminally differentiated or senescent cells. In spite of these observations, the data reported here show that it is possible to isolate viable cell lines expressing a high level of tissue transglutaminase. Moreover, in spite of increased levels of enzyme expression (0.2% of total cellular protein and ~3.5% after butyrate treatment in clone 5) both clone 3 and 5 were stable in long-term tissue culture and showed a proliferative activity that was comparable to nontransfected or control cells.

At least two factors may contribute to the viability of cells expressing high levels of transglutaminase activity. The first of these is that the enzymatic activity expressed in the cells appears to be largely cryptic in Balb-C 3T3 cells under normal growth conditions. Although the transfected cells express high levels of the enzyme, there is only a modest increase in the level of cross-linked proteins or protein-conjugated polyamines in the transfected cell lines. The actual level of isopeptide in both the nontransfected and transfected cell lines (260–440 pmols/mg) is similar to that detected in other cultured cells and in tissues (Fesus and Tarcsa, 1989; Hand et al., 1990). Tissue transglutaminase is a Ca^{2+}-dependent enzyme and it is likely that the levels of Ca^{2+} sufficient to activate the enzyme are not normally achieved in intact and viable cells (Hand et al., 1985). Furthermore, guanine nucleotides have been reported to inhibit tissue transglutaminase activity (Achyuthan et al., 1987) and some cells have been reported to contain specific intracellular transglutaminase inhibitors (Korner et al., 1989). The low level of cross-linking activity in the intact transfected cells suggests that these factors can serve to limit the extent of protein cross-linking in cells expressing even very high levels of the enzyme. A less likely possibility, but one that

needs to be considered, is that the transfected human tissue transglutaminase is unable to utilize the endogenous substrate proteins present in mouse cells. Such strict species specificity has not been reported in the literature of transglutaminase biochemistry (Greenberg et al., 1991).

A second factor that may favor the viability of cell lines transfected with the transglutaminase expression vector is the variation in the level of enzyme expressed in individual cells. Immunocytochemical analysis of both clone 3 and clone 5 cell lines indicated that there were marked differences in the level of tissue transglutaminase that accumulated in individual cells. Since all the cells became immunopositive after treatment with sodium butyrate, a drug that has been reported to increase the transcriptional activity of transfected DNA (Rouault et al., 1987), and since each of these lines was cloned from an individual colony, these differences are not likely to be due to genetic heterogeneity. We believe they reflect epigenetic variability in the degree to which the transfected transglutaminase construct is expressed in individual cells. It is possible that individual cells with the lowest accumulations of the enzyme may be capable of undergoing mitosis, whereas those cells with very high accumulations preferentially undergo fragmentation and cell death. The heterogeneity of expression of the transfected DNA in these clones may have contributed to their viability as stable cell lines.

Transglutaminase and Cellular Adhesion

Several recent reports have linked the induction of tissue transglutaminase activity in cells with alterations in cellular morphology (Byrd and Licthi, 1987; Nara et al., 1989) and adhesive properties (Cai et al., 1991). Each of these studies have used pharmacologic agents (sodium butyrate or retinoic acid) to increase endogenous tissue transglutaminase gene expression and it has been uncertain whether the morphologic changes seen were due to altered transglutaminase activity or independent effects of the drugs. The observations made with the transglutaminase-transfected cells strongly support the idea that increased expression of tissue transglutaminase can be associated with dramatic alterations in the pattern of interactions between cells and their substratum. There have also been reports that transglutaminases can cross-link cytoskeletal proteins in vitro, although the physiological significance of these observations has not been established (Selkoe et al., 1982). Unlike the control Balb-C 3T3 cells, the transglutaminase-transfected cells showed a marked flattening of their morphology indicating increased cell–substratum interactions. This morphologic effect was also reflected in an increased resistance of the cells to detachment with protease (trypsin). Therefore, several independent lines of experimental evidence suggest that transglutaminase expression affects cellular adhesiveness.

The association between transglutaminase expression and increased cellular adhesion poses something of an enigma. The enzyme has classically been considered to be an intracellular enzyme; the majority of its catalytic activity is found in the cytoplasm of cells and yet its biologic effects seemed to be linked to alterations in the interactions between the cell and extracellular proteins. It is possible that tissue transglutaminase is involved in the modification of specific intracellular proteins important for stabilization of cell–substrate interactions. These might be structural proteins within the cell or the intracellular domains of receptors involved in adhesion. However, the studies carried out with the transglutaminase-transfected cells suggested that most of the intracellular transglutaminase activity is cryptic and the levels of both isopeptide cross-linked proteins and polyamine conjugates inside cells is very low. An alternative possibility is that the physiological site of tissue transglutaminase activity is actually in the extracellular compartment. Several proteins associated with the extracellular matrix, such as collagen III (Bowness and Tarr, 1990), fibronectin (Fesus et al., 1986), vitronectin (Sane et al., 1988), and nidogen (Aeschlimann and Paulsson, 1991) are specific substrates for tissue transglutaminase activity. Cell-associated transglutaminase activity has been shown to promote covalent cross-linking of extracellular matrix proteins (Tyrrel et al., 1988; Sane et al., 1991). The enzyme has been shown to bind to both collagen (Juprelle-Soret et al., 1988) and fibronectin (Turner and Lorand, 1989) and to be readily deposited in the extracellular matrix associated with cultured cells and tissues (Upchurch et al., 1987; Thomazy and Fesus, 1989; Aeschlimann and Paulsson, 1991). We speculate that the high levels of tissue transglutaminase associated with the transglutaminase-transfected Balb-C 3T3 cells is associated with the cross-linking of unidentified matrix proteins and alterations in cellular adhesiveness. Further studies on the association between the transfected cells and specific matrix proteins will be necessary to resolve this issue unambiguously.

Tissue Transglutaminase and Apoptotic Cell Death

Recently Fesus and his associates have reported on a striking association between transglutaminase activity and the induction of apoptotic cell death (Fesus et al., 1987). Tissue transglutaminase expression is induced and the enzyme accumulates to high levels in a number of in vitro (Piacentini et al., 1991) and in vivo (Fesus et al., 1987; Fesus and Thomazy, 1988; Davies et al., 1992) models of apoptosis. Activation of the enzyme is apparently involved in the process of fragmentation since the membrane-limited fragments ("apoptotic bodies") shed by apoptotic cells have been shown to contain high levels of isopeptide bonds and extensively cross-linked proteins (Fesus et al., 1989). The results obtained with transglutaminase-transfected cells suggest that this association is not incidental. The Balb-C 3T3 cells that expressed highest levels of intracellular transglutaminase activity (detected by immunocytochemistry) appear to be undergoing active fragmentation in vitro, their membranes are blebbing and multiple small hematoxylin-negative membrane-limited fragments accumulate in the culture media. This process is strikingly similar to some of the processes that contribute to apoptotic cell death (Arends and Wyllie, 1991).

Apoptotic cells became dissociated from neighboring cells, their cytoplasm becomes condensed, and they fragment into multiple small membrane-limited fragments (apoptotic bodies). These remnants are in turn ingested by neighboring phagocytic cells. A striking difference between the morphology of apoptotic cells and that of cells transfected with tissue transglutaminase is that the chromatin of apoptotic cells is characteristically condensed and the nucleus itself frequently fragments. We did not detect such changes in

the morphology of the nuclei of the transglutaminase-transfected cells. Thus, expression of high levels of the enzyme in Balb-C 3T3 cells seems to reproduce some, but not all, of the changes characteristic of cells undergoing apoptotic cell death. Our results suggest that the induction and activation of tissue transglutaminase in apoptotic cells may contribute to the active fragmentation of these cells and to the subsequent stability of apoptotic bodies.

Biological Significance of Transglutaminase Expression

We inaugurated these studies to gain some insight into the biological roles of the enzyme tissue transglutaminase. At least two seemingly distinct processes, alterations in cellular adhesion and apoptotic cell death, have emerged from these studies as candidates for the physiological functions of this enzyme. It is interesting to note that each of these processes represents tissue transglutaminase expressed in a different cellular compartment. There is considerable evidence available to suggest that the link between transglutaminase activity and adhesion involves enzymatic reactions occurring outside the cell. In this environment the Ca^{2+} concentration is high enough to support transglutaminase activity and the enzyme may be freed from inhibitory factors such as GTP or proteins that restrict its activity inside cells. An involvement in the processing of extracellular matrix proteins may explain the accumulation of tissue transglutaminase in basement membrane structures in tissues, as well as its involvement in the process of wound healing and tissue repair. An unanswered issue is how the enzyme gains access to the extracellular space. Like Factor XIII and several other extracellular proteins tissue transglutaminase does not contain a leader peptide or obvious secretory signal and it remains to be determined how the enzyme becomes deposited in the extracellular compartment.

While the effects of tissue transglutaminase on cellular adhesion appear to be linked to activation of the enzyme outside the cell, the effects on cellular fragmentation and apoptotic cell death may well reflect the consequences of the activation of the enzyme inside the cell. Apoptotic cells contain levels of intracellular tissue transglutaminase comparable to those achieved in the transglutaminase-transfected fibroblasts. During apoptosis this enzyme appears to be activated by increased levels of intracellular Ca^{2+}. Orrenius and associates have shown that Ca^{2+} levels rise to levels sufficient to activate intracellular transglutaminase activity (Hand et al., 1989; Orrenius et al., 1989). The fact that apoptotic cells accumulate both isopeptide cross-linked and protein-conjugated polyamines indicates that the process involves activation of the enzyme inside the apoptotic cells. Thus, it seems likely that biological functions of tissue transglutaminase may vary depending on the cellular compartment in which the enzyme is both expressed and activated.

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