Treatment of the human promonocytic cell line U937 with all-trans-retinoic acid (RA) commits these cells to apoptosis, which can be triggered by simply increasing intracellular calcium levels by the ionophore A23187. RA treatment of U937 cells is characterized by a decrease in Bcl-2 and marked induction of "tissue" transglutaminase (tTG) gene expression. In this study, we show that the inhibition of tTG expression in U937 cells undergoing apoptosis prevents their death. In fact, U937 cell-derived clones transfected with the human tTG gene in the antisense orientation showed a pronounced decrease in apoptosis induced by several stimuli. These findings demonstrate that the Ca\textsuperscript{2+}-dependent irreversible cross-linking of intracellular proteins catalyzed by tTG represents an important biochemical event in the gene-regulated cell death in monoblasts. In addition, our data indicate that the apoptotic program in promonocytic cells is strictly regulated by RA and that a key role is played by the free intracellular calcium concentration.

"Tissue" transglutaminase (tTG)\textsuperscript{1} catalyzes a Ca\textsuperscript{2+}-dependent reaction in which \(\gamma\)-carboxamidated groups of peptide-bound glutamine residues serve as acyl donors and primary amino groups of several compounds function as acceptor substrates (1, 2). The reaction results in post-translational modification of \(\epsilon\)-(\(\gamma\)-glutamyl)lysine and/or covalent incorporation of polyamines and histamine into proteins (2–3). Di- and polyamines may also participate in cross-linking reactions through the formation of \(\epsilon\),(\(\gamma\)-bis\(\gamma\)-glutamyl)polyamine cross-bridges (3). tTG-dependent formation of stable cross-linking determines protein polymerization, conferring resistance to mechanical breakage and chemical attack to the polypeptides involved in the linkage; in fact, these polymers can be destroyed only by proteolytic degradation of the protein.

In addition to the Ca\textsuperscript{2+}-dependent protein cross-linking activity, tTG binds guanine nucleotides and hydrolyzes GTP and ATP. Nakaoka et al. (5) have demonstrated that the 74-kDa \(\alpha\)-subunit (G\textsubscript{a}) associated with the 50-kDa \(\beta\)-subunit of the GTP-binding protein G\textsubscript{q} is tTG. This dimer acts in association with the \(\alpha\textsubscript{1}\)-adrenergic receptor in a ternary complex. Thus, tTG is a multifunctional protein that not only acts as a transglutaminase, but activates phospholipase C after receptor stimulation (5).

It has been well established in various experimental systems that tTG is one of the few genes induced during apoptosis (6–10). tTG protein is undetectable in the majority of cells, and its mRNA is transcribed as a consequence of the onset of apoptosis (6, 8). Overexpression of tTG primes cells for suicide, and the clones resistant to tTG transfections are highly susceptible to apoptosis induced by various agents (9–13). Since the intracellular tTG cross-linking activity is inhibited by GTP and nitric oxide, the accumulation of tTG protein inside the cell is not necessarily associated with the activation of its cross-linking activity (1, 14). However, the Ca\textsuperscript{2+}-dependent activation of the enzyme leads to the formation of a detergent-insoluble cross-linked protein scaffold in cells undergoing programmed cell death (4). This insoluble protein scaffold may stabilize the integrity of the dying cells before their clearance by phagocytosis, thus preventing the nonspecific release of harmful intracellular components (i.e. lysosomal enzymes, nucleic acid, etc.) and consequently inflammatory responses and scar formation in bystander tissues (9, 10). Although several laboratories have shown that tTG gene expression characterizes cells undergoing apoptosis both in physiological and experimental settings, the precise position of tTG in the cascade of events leading to establishment of the death phenotype has not yet been fully clarified (4, 6, 7, 14). Several studies indicate that induction of tTG parallels Bcl-2 down-regulation and is not sensitive to its inhibitory effect (12).

To investigate to what extent the overexpression of the tTG gene is a key biochemical event in the death program, we studied the effect on apoptosis of the all-trans-retinoic acid (RA)-dependent induction of tTG in cell lines derived from promonocytic U937 cells stably transfected with plasmids containing tTG cDNA in the antisense orientation.

EXPERIMENTAL PROCEDURES

Chemicals—[1,2,3\textsuperscript{H}]Putrescine dihydrochloride (26.3 Ci/mol) and the ECL detection system were from Amersham Pharmacia Biotech. Optifluor was from Packard Instrument Co. N,N'-Dimethylcasein, bovine serum albumin, putrescine hydrochloride, propidium iodide, staurosporine, cycloheximide, and all-trans-retinoic acid were from Sigma. Cell culture plastics were from Falcon. calphostin C, actinomycin D, and A23187 were from Calbiochem. Z-VAD and anti-CD95 mAb were

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from Alexis. RPMI 1640 medium supplemented with l-glutamine, FCS, penicillin, G418, 1,2-dimyristoyl-sn-glycero-3-phospho(1'-diacylglycerol) amonium bromide and cholesterol, Lipofectin, and streptomycin were from Life Technologies, Inc. SDS-polyacrylamide gel electrophoresis molecular mass markers, nitrocellulose membrane, and horseradish peroxidase were from Bio-Rad Laboratories. tTG mAb (clone CUB 7402) was from NeoMarkers, and anti-Bcl-2 mAb was from Dako. FITC-conjugated annexin V was from Chemicon International, Inc. Restriction and modification enzymes and DNA molecular mass markers were from Roche Molecular Biochemicals.

**Cell Culture and Treatments—**The U937 parental cell line and its derived transfected sublines (UNeo, UAS1, and UAS2) were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin and incubated at 37 °C in a humidified atmosphere of 5% CO2 in air.

In various experiments, cells (seeded at a density of 3 × 103 cells/ml) were first exposed or not to all-trans-retinoic acid (1 µM from a 5 mM stock solution dissolved in 70% ethanol) and treated as follows. (a) In calphostin C (0.25 µM from a 0.25 mM stock solution in Me2SO) studies, cells pretreated or not with 1 µM RA for 36 h were incubated for an additional 11 h in growth medium containing 0.5% serum in the presence or absence of 1 µM RA. The cells were finally treated with calphostin C for 1 h. After calphostin C addition, cells were exposed to light for 10 min, and the incubation was allowed to proceed in the dark for an additional 50 min. (b) A23187 calcium ionophore (1 µM from a 1 mM stock solution in Me2SO), apoptosis-inducing anti-CD95 mAb (100 ng/ml in culture medium), and cycloheximide (4 µg/ml) were added to cells pretreated or not with 1 µM RA for 36 h, and incubation was carried out for an additional 12 h. (c) In staurosporine experiments, cells pretreated or not with 1 µM RA for 43 h were incubated for the last 5 h in the presence of staurosporine (2 µM from a 2 mM stock solution in Me2SO). (d) Z-VAD (100 µM) was added 5 h before treatment with A23187 (1 µM) to cells pretreated or not with RA for 36 h, and the incubation was allowed to proceed for an additional 12 h in the presence of the calcium ionophore. (e) Mycobacterial infections of U937 cells were performed as described previously (15). Briefly, 1 × 105 cells were infected for 24 h with *Mycobacterium tuberculosis* strain H37Rv in RPMI 1640 medium, 10% FCS, and 2 mM l-glutamine (complete medium); washed; and maintained at 3 × 106 cells/ml in complete medium. (f) For time courses, cycloheximide (4 µg/ml) and actinomycin D (5 ng/ml) were added, and samples were analyzed after 6, 16, 24, and 48 h by flow cytometry.

**Antisense tTG Vector Construction and Expression—**Two different antisense-expressing vectors were used in this study: pSG5-AS-tTG carries the first 5′ 1.0 kilobase pair of the human tTG cDNA (human endothelial cell tTG, clone hTg-1) (12) cloned in the antisense orientation into the EcoRI site of the pSG5 vector (Stratagene) under the control of the SV40 early promoter.

For the pRC2 vector, after PCR amplification with primers carrying EcoRI restriction sites, the entire coding region for human tTG was cloned in the EcoRI site of the pEGFP vector (CLONTECH), and clones with the insert in the antisense orientation were selected. Digestion with *Heli* and EcoRV restriction endonucleases and subsequent religation of the linearized blunt-end vector gave rise to the 5′ 1.3-kilobase pair tTG cDNA in the antisense orientation under the control of the cytomegalovirus promoter. The integrity of the tTG gene was controlled by sequencing.

To assess expression of antisense transcripts, 5 µg of total RNA extracted from cells (16) were subjected to reverse transcription-PCR. Reverse transcription (Superscript II, Life Technologies, Inc.) with extracted from cells (16) were subjected to reverse transcription-PCR.

**Apoptosis Detection—**Apoptosis evaluation at different days after treatment and/or infections was carried out using flow cytometric analysis. For propidium iodide staining, aliquots of 5 × 105 cells were washed twice in PBS; placed on ice; gently resuspended with 0.5 ml of 4% paraformaldehyde and permeabilized with 0.05% saponin detergent as described under “Experimental Procedures.” To detect anti-tTG mAb as primary antibody and then stained with a FITC-conjugated goat anti-mouse antibody. A histogram profile of a relevant control antibody (dotted line) is also shown. B, Western blot analysis of total cell lysate proteins from untreated cells (−RA) and RA-treated cells (+RA) was performed on aliquots corresponding to 50 µg of total protein as described under “Experimental Procedures.” The RA-dependent induction of the tTG gene was revealed by using the anti-tTG monoclonal antibody.

**Transfections and Single Clone Selection—**Single clones were established by cotransfecting U937 cells with pSG5-AS-tTG and pSV2-Neo. DNA transfections were performed using Lipofectin according to the supplier’s instructions. In all cases, after 5 h of transfection in serum-free RPMI 1640 medium, cells were shifted to complete medium. 48 h after transfection, cells (5 × 105/well) were plated onto 96-well plates in RPMI 1640 medium and 10% FCS supplemented with G418 (500 µg/ml). After 1 month of G418 selection, resistant cells were reseeded at limiting dilution to achieve single clones.

**Western Blot Analysis—**Western blot analysis of total cell lysate proteins from untreated cells (−RA) and RA-treated cells (+RA) was performed on aliquots corresponding to 50 µg of total protein as described under “Experimental Procedures.” The RA-dependent induction of the tTG gene was revealed by using the anti-tTG monoclonal antibody.

**Apoptosis Detection—**Apoptosis evaluation at different days after treatment and/or infections was carried out using flow cytometric analysis. For propidium iodide staining, aliquots of 5 × 105 cells were washed twice in PBS; placed on ice; gently resuspended with 0.5 ml of solution containing 50 µg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate; and left at 4 °C for 20 s. STG activity was measured by detecting the incorporation of [3H]putrescine into N,N′-dimethylethacine as previously reported (6).

**Tissue Transglutaminase Activity Assay—**Cells treated with 1 µM all-trans-retinoic acid were washed in phosphate-buffered saline (PBS) without Ca2+ and Mg2+; resuspended in 50 mM Tris-HCl, pH 8.4, and 1 mM Na3EDTA; and sonicated at 4 °C for 20 s. STG activity was measured by detecting the incorporation of [3H]putrescine into N,N′-dimethylethacine as previously reported (6).
Cytofluorometric Detection of tTG—5 × 10⁵ U937 and RA-treated cells were washed in PBS, pH 7.2, supplemented with 1% (w/v) bovine serum albumin and 0.1% NaN₃; fixed in 4% p-formaldehyde for 20 min at room temperature; washed in PBS; and then incubated for 15 min in 0.5 × NH₄Cl at room temperature. After a final wash in PBS, cells were permeabilized by incubation in PBS/bovine serum albumin/NaNO₃ containing 0.05% (w/v) saponin detergent for 15 min at room temperature. Cells were then stained intracellularly with anti-tTG mAb in saponin detergent buffer for 30 min at room temperature. After washes with saponin detergent buffer, cells were incubated with FITC-conjugated goat anti-mouse Ig for 30 min, washed and fixed with 0.05% formaldehyde in PBS, applied to a FACSCalibur flow cytometer, and analyzed as described above. Western blotting was carried out as described previously (12) on aliquots of total protein (20–50 μg) extracted from cells after different treatments.

RESULTS AND DISCUSSION

Effect of RA on Apoptosis and tTG Expression in U937 Cells—RA treatment has drastic effects on the cell cycle, blocking the U937 cells in the G₁ phase and triggering differentiation (17, 18). In parallel with these events, RA treatment induced an early and dramatic accumulation of tTG (Fig. 1), which was paralleled by a decrease in Bcl-2 protein levels (see Fig. 3D) (19).
The data reported in Fig. 2 show that the RA treatment of U937 cells is associated with their commitment to apoptosis. In fact, the RA-treated cells, which express high tTG protein levels, were particularly susceptible to apoptosis induced by different stimuli (calcium ionophore A23187, calphostin C, staurosporine, and cycloheximide), whereas untreated cells were much more resistant to death (Fig. 2A). Although U937 cells express high levels of CD95 receptor protein, which is not affected by the RA treatment (data not shown), these cells were unable to undergo apoptosis when treated with agonistic anti-CD95 IgGs (Fig. 2A). Interestingly, pretreatment of U937 cells with RA committed these cells to apoptosis, which could be triggered by increasing their intracellular calcium levels with the A23187 ionophore (Fig. 2B). The ionophore-induced apoptosis of RA-pretreated cells was mediated by caspases, as indicated by the death inhibitory effect displayed by Z-VAD (Fig. 2C) and the parallel proteolytic cleavage of poly(ADP-ribose) polymerase (data not shown). Staining with FITC-conjugated annexin V revealed that the dying U937 cells exposed to cycloheximide or actinomycin D. The U937 parental cell line and its derived clones (grown in 10% FCS) were exposed to cycloheximide (4 μg/ml; white symbols) and actinomycin D (5 ng/ml; black symbols) for 6, 16, 24, and 48 h. After treatment, cells were washed in PBS, stained with propidium iodide, and then analyzed by flow cytometry. Squares, U937 parental control cell line; diamonds, vector alone (pSV2-Neo)-transfected cells; circles, pSG5-AS-tTG (UAS1)-transfected clones; triangles, pSG5-AS-tTG (UAS2)-transfected clones. Data are the means ± S.E. of triplicate determinations from three different experiments.

The effect of the antisense construct on tTG gene expression was verified by measuring protein levels and enzyme activity.
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before and after RA treatment (Fig. 3, B and C, respectively). Whereas the U937 cell line and the clones transfected with pSV2-Neo showed the typical RA-dependent increase in tTG expression and activity, cell lines cotransfected with pSG5-AS-tTG and pSV2-Neo showed a drastic reduction in both the tTG protein levels and enzyme activity after induction by RA (Fig. 3), thus indicating the efficient inhibition achieved by the antisense strategy in the U937 cell line. To verify that the inhibitory effect on tTG expression was indeed due to the specific action of the antisense construct and not to clonal selection, we studied the effect of RA on Bcl-2 protein levels in U937 cells and its derived transfected clones. As reported in Fig. 3D, U937 cells and pSV2-Neo-transfected and pSG5-AS-tTG/pSV2-Neo-cotransfected clones showed comparable basal Bcl-2 protein levels, which were significantly decreased by RA treatment, thus confirming the specificity of tTG inhibition observed in the antisense transfectants. Similar results were obtained with the pRC2 vector (data not shown).

To further assess the specificity of our antisense construct, we treated the U937 parental cell line and its derived transfected clones with agents capable of inducing cell death by nonspecifically blocking transcription and transduction. Fig. 4 shows that treatment of cells with cycloheximide (4 µg/ml) or actinomycin D (5 ng/ml) induces a marked cell death, which was comparable in both the U937 parental cell line and its derived clones. Higher doses of cycloheximide (50 µg/ml) or actinomycin D (50 ng/ml) induce a significant level of cell death (60–80%) already after 24 h of treatment in all cells (data not shown). These data demonstrate that the antisense tTG construct did not interfere with cellular processes, including cell death, that do not require gene expression and protein synthesis.

To determine whether the priming effect shown in Fig. 2 was dependent on tTG expression, we treated the antisense tTG transfectants with the same apoptosis-inducing agents. The data indicate that the reduced tTG expression observed in the RA-pretreated antisense tTG clones is associated with a net decrease in the sensitivity to various apoptotic stimuli. In fact, the antisense transfectants were much less prone to apoptosis induction elicited by calphostin C, calcium ionophore, and cycloheximide (Fig. 5, A–B). It is interesting to note that the tTG inhibition was less effective in preventing apoptosis induced by cycloheximide than that elicited by the Ca²⁺ ionophore A23187 (Fig. 5A). This phenomenon can be explained by assuming that a continuous tTG synthesis is needed for apoptosis in U937 cells and/or that the induction of additional pro-apoptotic proteins is required to complete the death program.

M. tuberculosis replicates in cells of the M/M lineage (including U937), where, under some circumstances, it induces apoptosis and tTG expression (15). Consistent with these observations, macrophages obtained by bronchoalveolar lavage from patients with reactive pulmonary tuberculosis and from AIDS patients with disseminated pulmonary tuberculosis show increased levels of tTG protein, which is mainly expressed in cells undergoing apoptosis (7, 15). Since tTG has been shown to modulate the release of M. tuberculosis and intracellular components from the infected macrophages into the medium, apoptosis of the host cells may represent a very efficient means to prevent spreading of M. tuberculosis infection (21, 22). Based on these observations, we investigated the effect of tTG inhibition on the ability of M. tuberculosis to induce apoptosis in U937 cells. The induction of apoptosis by M. tuberculosis requires viable bacteria, is dose-dependent, and is restricted to H37Rv (15). The data reported in Fig. 5C indicate that the inhibition of tTG reduce M. tuberculosis-dependent cell death in U937 cells, thus confirming that tTG is an important effector element of apoptosis in M. tuberculosis-infected M/M cells.

The data reported in this paper indicate the tTG-mediated protein cross-linking is a critical event in the apoptotic path-
way in U937 cells. The transfection studies carried out on U937 cells clearly show that the blocking of tTG expression in these cells significantly inhibits apoptosis, thus suggesting that tTG is one of the genes responsible for the commitment of these promonocytic cells to undergo cell death by apoptosis. Furthermore, these data indicate that the apoptotic program in these mesoderm-derived cells is strictly regulated by RA and that a key role is played by the free intracellular calcium concentration. It is well known that both tTG activation and the onset of the apoptotic program require sustained high Ca\(^{2+}\) levels (1).

Thus, it is plausible to hypothesize that, at the low level of free cytosolic calcium normally present in viable RA-treated U937 cells, the accumulated tTG protein might be in its G-protein configuration, which inhibits the cross-linking activity required for apoptosis (18, 23).

In conclusion, we have presented data demonstrating that the tTG gene product is one of the effector elements of the apoptotic program in promonocytic cells. tTG activation, leading to the assembly of intracellular cross-linked protein polymers, may irreversibly modify cell organization, contributing to the determination of those ultrastructural changes typical of cells undergoing apoptosis.

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REFERENCES

1. Fesus, L., Davies, P. J. A., and Piacentini, M. (1991) *Eur. J. Cell Biol.* 56, 170–177
2. Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) *FASEB J.* 5, 3071–3077
3. Folk, J. E. (1980) *Annu. Rev. Biochem.* 49, 517–531
4. Fesus, L., Thomazy, V., Autuori, F., Ceru, M. P., Tarcsa, E., and Piacentini, M. (1989) *FEBS Lett.* 245, 150–154
5. Nakao, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M. J., and Graham, R. M. (1994) *Science* 264, 1593–1596
6. Piacentini, M., Autuori, F., Dini, L., Farrace, M. G., Gibelli, L., Piredda, L., and Fesus, L. (1991) *Cell Tissue Res.* 263, 227–235
7. Amendola, A., Gougeon, M. L., Pecia, F., Froncomand, A., Fesus, L., and Piacentini, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11057–11062
8. Nagy, L., Thomazy, V., Saydak, M., Stein, J., and Davies, P. J. A. (1997) *Cell Death Differ.* 4, 534–547
9. Piredda, L., Amendola, A., Colizzi, V., Davies, P. J. A., Farrace, M. G., Fraizano, M., Gentile, V., Urzy, L., Piacentini, M., and Fesus, L. (1997) *Cell Death Differ.* 4, 463–472
10. Knight, R. L., Hand, D., Piacentini, M., and Griffin, M. (1993) *Eur. J. Cell Biol.* 60, 210–216
11. Gentile, V., Thomazy, V., Piacentini, M., Fesus, L., and Davies, P. J. A. (1992) *J. Cell Biol.* 119, 463–474
12. Melino, G., Annichiarico-Petruzzelli, M., Piredda, L., Candi, E., Gentile, V., Davies, P. J. A., and Piacentini, M. (1994) *Mol. Cell. Biol.* 14, 6584–6596
13. Igarashi, S., Koide, R., Shimohata, T., Yamada, M., Hayashi, Y., Takano, H., Date, H., Oyake, M., Sato, T., Sato, A., Egawa, S., Ikeuchi, T., Tanaka, H., Nakano, R., Tanaka, K., Hozumi, J., Inouzuka, T., Takahashi, H., and Tsuji, S. (1998) *Nat. Genet.* 18, 111–117
14. Melino, G., and Piacentini, M. (1998) *FEBS Lett.* 430, 59–63
15. Placido, R., Mancino, G., Amendola, A., Mariani, F., Vendetti, S., Piacentini, M., Sanduzzi, A., Bocchino, M. L., Zambala, M., and Colizzi, V. (1997) *J. Pathol.* 181, 31–38
16. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* 162, 156–159
17. Harris, P., and Ralph, P. (1985) *J. Leukocyte Biol.* 37, 407–422
18. Defacque, H., Commes, T., Contet, V., Sevilla, C., and Marti, J. (1995) *Leukemia (Basingstoke)* 9, 1762–1767
19. Oliverio, S., Amendola, A., Di Sano, F., Farrace, M. G., Fesus, L., Nemes, Z., Piredda, L., Spinedi, A., and Piacentini, M. (1997) *Mol. Cell. Biol.* 17, 6040–6048
20. Zhuang, J., Ren, Y., Snowden, R. T., Zhu, H., Gogvadze, V., Savill, J. S., and Cohen, G. M. (1998) *J. Biol. Chem.* 273, 15628–15632
21. Bergamini, A., Capozzi, M., and Piacentini, M. (1994) *Immunol. Lett.* 42, 35–40
22. Fratazzi, C., Arbeit, R. D., Carini, C., and Remold, H. G. (1997) *J. Immunol.* 158, 4320–4327
23. Singh, U. S., and Cerione, R. A. (1996) *J. Biol. Chem.* 271, 27292–27298