Differential Expression of Transglutaminase in Human Erythroleukemia Cells in Response to Retinoic Acid


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ABSTRACT

Two human erythroleukemia cell lines, HEL and K562, express transglutaminase activity. The enzyme was identified as a tissue transglutaminase following chromatographic purification. All-trans-retinoic acid (10 μM) stimulated differentiation in HEL cells as judged by a 4-fold increase in hemoglobin content and a reduction in cell proliferation. The transglutaminase activity increased 9-fold. This increase in transglutaminase was the result of a pretranslational regulation of the gene as revealed by Northern blot analysis of mRNA. These changes were not a result of cell apoptosis, since parallel DNA degradation catalyzed by a Ca2+-dependent endonuclease could not be demonstrated. The K562 cells, in contrast, showed no transglutaminase induction following exposure to retinoic acid and displayed no changes in maturation markers or cell growth.

INTRODUCTION

T-Gases1 (EC 2.3.2.13) are a family of enzymes that catalyze the formation of covalent ε-(γ-glutamyl)lysine cross-links in proteins (1). Activated Factor XIII which carries out the final stabilization of a fibrin clot during coagulation (2) and epidermal transglutaminase which produces a cornified envelope during squamous epithelial differentiation (3) are examples. A tissue or cellular form is found in nearly all tissues (4), but its biological function is still not completely understood. Recent investigations in our laboratory (5) raised the question as to whether the GTPase activity of tissue transglutaminase may be the main physiological function of the enzyme rather than protein cross-linking. Despite the lack of a definitive physiological role for tissue transglutaminase, there has been some evidence for years that this enzyme plays a role in cell differentiation. Simian virus transformation of WI-38 human lung fibroblasts resulted in a loss of transglutaminase activity and content to 2% of its original value (6). Similar observations have been made following malignant transformation of hepatocytes (7). Regulation of human transglutaminase has been extensively studied in the promyelocytic leukemia cell line, HL60 (8). It was shown that the retinoic acid-induced differentiation of these cells along the granulocytic pathway was coupled to a specific induction of the transglutaminase gene (9). In the current study, we analyzed the effect of RA on the differentiation of two human erythroleukemia cell lines, K562 and HEL, and their response in tissue transglutaminase expression.

MATERIALS AND METHODS

Cells and Cell Culture. GM06141A (HEL) and GM05372D (K562) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cells were grown in McCoy’s Medium 5a supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) to a density of 1 x 10^6 cells/ml and then split to either 1 or 2 x 10^6 cells/ml, respectively. All-trans-retinoic acid (Sigma, St. Louis, MO) was added 24 h after subculture from a 10 μM stock solution prepared in 100% ethanol, stored at -20°C. Cultures were covered with aluminum foil to protect them from light. Twenty-ml cultures were refed on days 3, 4, 5, 6, and 7 after subculture with 2, 2, 4, 4, and 8 ml medium, respectively, with or without retinoic acid. The retinoic acid concentration was 10 μM unless otherwise specified. In cases where 200-ml cultures were used, the same volume ratios were used. Cells were enumerated using a hemacytometer and cell viability was judged by trypan blue exclusion.

Transglutaminase Assay and Inhibition ELISA. Cells were pelleted by centrifugation for 10 min at 5000 g, washed once with 20 mM Tris-HCl-150 mM NaCl-1 mM EDTA, pH 7.5, and resuspended in the same solution containing 0.5 mM phenylmethylsulfonyl fluoride. Cells were disrupted by a 10-s sonication and the cytosol fraction was prepared by centrifugation at 100,000 x g for 1 h. Transglutaminase activity was measured by the incorporation of radioactive putrescine into N,N-dimethylcasein (10). One unit of enzyme activity was defined as 1 nmol of putrescine incorporated in 20 min per mg of protein at 37°C. Total cytosol protein was determined by the method of Bradford (11). The quantification of immunoreactive transglutaminase was performed by the inhibition ELISA assay as described earlier (12).

Affinity Chromatography. Purified monoclonal antibody for guinea pig liver transglutaminase was conjugated to Affi-Gel 10 (Bio-Rad, Richmond, CA) according to the manufacturer’s protocol. The cytosol fraction from 5 x 10^6 HEL cells was loaded to the column, eluted under conditions described previously (13), concentrated using Centriprep 30, and then stored at -20°C. Aliquots were subjected to electrophoresis on 10-15% polyacrylamide gradient gel with the Pharmacia Fast Gel System followed by silver staining.

Endonuclease Activity. The method was based on a procedure described by Kaufmann (14) with the following modifications: (a) the release of the nuclei was monitored by Nomarski differential interference contrast microscopy; (b) the nuclei preparations were incubated for 3 h at 37°C in 350 mM sucrose-50 mM Tris-HCl, pH 7.4-5 mM MgSO4-5 mM CaCl2, with or without 10 μM retinoic acid; (c) analysis of the DNA was performed either on 1% agarose-Mini-gel, at 80 V for 3 h or on 1.2% agarose gels for 12 h at 1.5 V/cm with subsequent ethidium bromide staining.

Hemoglobin Measurement. Retinoic acid was added to exponentially growing cells which were harvested either 3 or 6 days later. Total hemoglobin was determined in the cytosol fraction after centrifugation at 100,000 x g for 1 h. The hemoglobin was determined spectrophotometrically as oxyhemoglobin following the method of Harboe (15). Essentially, 200 μl of the cytosol fraction were mixed with 1800 μl of 0.01% Na2CO3 and the absorbance was measured at 380, 415, and 450 nm. The absorbance at 415 nm representing oxyhemoglobin was corrected for impurities by subtracting the absorbance at 380 and 450 nm. A standard curve was constructed using bovine hemoglobin (Sigma). The final hemoglobin concentration was expressed as pg/cell.

RNA Preparation and Northern Blot Analysis. Polyadenylated RNA was prepared using FastTrack (Invitrogen, San Diego, CA). RNA samples (5 μg/lane) were size fractionated on an agarose formaldehyde gel and capillary blotted onto a nylon membrane following Current Protocols in Molecular Biology (16). The human tissue transglutaminase cDNA, a 3.5-kilobase insert cloned into a pBluescript SK (-) vector, was a generous gift from Dr. Peter Davies, Houston, TX. After digestion with restriction enzymes NcoI and CviI, a 2.2-kilobase fragment was obtained which contained only the coding region of the tissue.
transglutaminase sequence. A random primed labeling method was carried out according to the method of Feinberg and Vogelstein (17) using [α-32P]dATP. The nylon membrane was hybridized overnight, washed once with 5x SSC-0.5% SDS for 15 min at 42°C, once with 1x SSC-0.5% SDS for 15 min at 55°C, and twice with 1x SSC-0.5% SDS for 30 min at 60°C and then exposed to Kodak X-Omat AR films for 14 h. The bands were quantitated by scanning the filter with an image analyzer (Ambis, San Diego, CA).

RESULTS

Effect of Retinole Acid on Tissue Transglutaminase Expression. Transglutaminase activity (2 units/mg protein) was found in both human erythroleukemia cell lines, HEL and K562. More than 95% of the total activity was found in the cytosol fraction after centrifugation at 100,000 x g for 1 h and was unaffected by prior thrombin treatment (data not shown). The transglutaminase activity in a 2% deoxycholate extract from the resulting pellet never exceeded background values (data not shown).

Cytosol preparations of both erythroleukemia cell lines produced a single band with a monoclonal antibody against guinea pig liver transglutaminase in Western blots (data not shown). The same antibody was used to purify the transglutaminase from retinoic acid-treated HEL cells by affinity chromatography. Fig. 1 shows that the purified preparation gave a single band with silver staining following SDS-polyacrylamide gel electrophoresis (Fig. 1, Lane B). The molecular weight, 85,000, was identical to transglutaminase purified from mature human RBC (Fig. 1, Lane A).

In order to study transglutaminase during differentiation the cells were treated with all-trans-retinoic acid in concentrations of 0.01–10 μM. The dose-response curve (Fig. 2) showed the strongest stimulation of transglutaminase induction in HEL cells with 10 μM retinoic acid, whereas K562 cells showed no significant response over the entire concentration range. Concentrations higher than 10 μM appeared to be toxic to both cell lines as revealed by trypan blue uptake into the cells. Additional treatment with 100 μM sodium butyrate failed to show a further transglutaminase induction in HEL cells (data not shown).

The time dependency of 10 μM retinoic acid on transglutaminase activity in HEL and K562 is shown in Fig. 3. Under repetitive treatment, the enzyme activity in HEL cells increased 9-fold in a time-dependent manner, while K562 cells were unresponsive. The transglutaminase activity increase in HEL cells appeared to be the result of enzyme accumulation as revealed by inhibition ELISA assay. The level of immunoreactive transglutaminase rose 15-fold, from 20 to 300 ng/mg protein (Fig. 4). The transglutaminase activity in HEL cells after a single exposure to 10 μM retinoic acid treatment is shown in Fig. 5. The enzyme activity began to increase after 24 h and reached a maximum (8-fold) after 4 days.
RETINOIC ACID-INDUCED TRANSGLUTAMINASE

Fig. 4. Immunoreactive levels of transglutaminase in HEL cells. Conditions are the same as those described in Fig. 3, a representative experiment performed in duplicate. O, control; •, 10 μM retinoic acid.

Fig. 5. Transglutaminase activity in HEL cells following a single exposure to retinoic acid. O, control, •, 10 μM retinoic acid. Data show the mean from two different experiments performed in duplicate.

decreased at later time points but was still significantly higher than control values at day 7.

Hemoglobin as a Differentiation Marker. Both erythroleukemia cell lines offer the opportunity to investigate hemoglobin as a marker of erythroid differentiation. K562 and HEL cells produce an embryonic hemoglobin with predominating γ chains (19). Total cytosolic hemoglobin was determined after 3 and 6 days exposure to RA. As seen in Table 1, control and retinoic acid-treated K562 cells contain similar amounts of hemoglobin on days 3 and 6. In HEL cells, the parent cells and one derived clone were tested. Both of these cell populations showed the same range of transglutaminase induction (data not shown). The hemoglobin content was 2- and 4-fold higher in the retinoic acid-treated group after 3 and 6 days, respectively.

Inhibition of Cell Growth. There was also a differential effect of RA on the growth rate of the two cell lines. Whereas cell growth was unaffected in K562 cells (data not shown), there was a reversible inhibition of growth in HEL cells (Fig. 6). Cells exposed to retinoic acid showed a biphasic growth rate. The treated cultures grew somewhat slower over the first 48 h and reached a plateau with about 50% fewer cells than the control cultures at day 3. This inhibitory effect persisted up to day 5 after which time the cell number started to rise again in the retinoic acid-treated cultures.

Endonuclease Activity. We were unable to detect any evidence of a Ca2+-dependent endonuclease activity either in HEL or in K562 cells during 48-h exposure to retinoic acid (Fig. 7). All

Table 1 Hemoglobin content of erythroleukemia cells

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* ND, not determined.

Fig. 6. Cell growth in HEL cells following a single exposure to retinoic acid. Cell numbers were determined at indicated times with the use of a hemacytometer. Cell viability was judged by trypan blue exclusion. O, control; •, 10 μM retinoic acid. Data show the mean from two different experiments performed in duplicate.

Fig. 7. DNA integrity in HEL cells following exposure to retinoic acid. Sample lanes were loaded with DNA from approximately 8 x 10⁶ cells. A, K562. Lane 1, molecular weight markers; Lane 2, 0 h, no retinoic acid; Lane 3, 5 h, retinoic acid; Lane 4, 24 h, retinoic acid. B, HEL. Lane 1, molecular weight markers; Lane 2, 0 h, no retinoic acid; Lane 3, 5 h, retinoic acid; Lane 4, 24 h, retinoic acid; Lane 5, 48 h, retinoic acid. Retinoic acid concentration equals 10 μM. Gels were run as described in “Materials and Methods.” Gel A, 1.2% agarose; Gel B, 1% agarose. Molecular weight markers, 0X 174 RF DNA/HaeIII fragments supplied by Bethesda Research Labs.
DNA preparations showed an intact DNA complex with a size close to 23 kilobases. Additional exposure of the nuclei to 10 μM retinoic acid in the presence of 5 mM CaCl₂ failed to show evidence of chromatin change indicative of the activation of the Ca²⁺-dependent endonuclease.

**Molecular Aspects in Transglutaminase Induction.** The regulation of transglutaminase by retinoic acid occurred at the gene level. Northern blots of polyadenylated RNA isolated from HEL cells hybridized with a tissue transglutaminase-specific probe revealed two distinct mRNAs of about 4 and 7.4 kilobases. Within the first 12 h of retinoic acid exposure, the transglutaminase message accumulated to 3-fold control values (Fig. 8, Lane 3). This elevation persisted after 24 and 48 h with 3.3- and 2.5-fold, respectively. Treatment with 20 μM cycloheximide alone led to a 5.5-fold increase (Fig. 8, Lane 2) as compared to an 8-fold increase when cells were additionally exposed to retinoic acid (Fig. 8, Lane 4).

**DISCUSSION**

Retinoic acid, the main physiological mediator of vitamin A activity, modulates normal and leukemic myeloid proliferation and differentiation (18, 19). In our current study, we report a differential response of HEL and K562 cells toward retinoic acid. Growth and differentiation in K562 were undisturbed, while the HEL cells stopped proliferation and increased production of hemoglobin as a marker of erythroid phenotype induction. The growth inhibition was found to be reversible. Considering that the half-life of retinoic acid under these conditions to be 2 h (20), it is evident that the cells demonstrated a commitment because the growth-inhibitory effect reached a maximum after 72 h, a time where the calculated amount of active RA has dropped far below effective levels. In agreement with studies of Papayannopoulou et al. (21), the cells failed to give rise to a well coordinated terminal maturation program, as has been found in murine erythroleukemia cells. This may be the result from a heterogenic response of HEL cells to cellular commitment. However, the combination of increased expression of a differentiated gene product (hemoglobin) with reduced growth rate suggests that cell differentiation is occurring.

This differentiation in HEL cells by retinoic acid was accompanied by an increase in tissue transglutaminase. The TGase mRNA increased 3-fold within the first 12 h after retinoic acid exposure, most probably by an increased transcription rate since it has been shown that RA has no influence on message stability (9). The superinduction of the transglutaminase mRNA in the presence of cycloheximide might be the effect of a prolonged half-life of transglutaminase mRNA in the presence of cycloheximide. Stabilization of certain mRNAs such as histone H3 and H4 by protein synthesis inhibitors has been reported (22). Additionally, Almendral et al. (23) demonstrated that exposure of NIH 3T3-fibroblasts to cycloheximide at a comparable concentration prolonged the transcription periods for certain genes. Another explanation could be the presence of a repressor protein, which when its synthesis is inhibited by cycloheximide gives rise to an increased transcription of the TGase gene.

Northern blot analysis with a TGase-specific probe showed two distinct bands of 7.4 and 4 kilobases. The smaller form is similar in size to the TGase mRNA previously reported in mouse peritoneal macrophages (9). Even though our specific probe contained only the TGase coding sequence and high stringency washing conditions were used, it is still an open question if the production of the 7.4-kilobase nucleotide transcript is the result of an altered expression of the TGase gene or a closely related gene. In order to answer this question, the molecular structure of the mRNAs and their genes must be determined.

The differential response of HEL and K562 cells to retinoic acid supports the idea of a close relationship between retinoic acid-induced differentiation and transglutaminase expression in myeloid leukemic cells. The regulatory effect of retinoic acid on leukemic cell differentiation could be mediated by a direct influence on growth-promoting proteins, which has been reported for c-myc and ornithine decarboxylase (24, 25). It would appear that transglutaminase can be used to predict the response of human myeloid leukemia cells to retinoic acid.

Recently, the activation of tissue transglutaminase in apoptotic cells has been documented by Fesus et al. (26). Furthermore, acute leukemic cells were shown to undergo spontaneous apoptosis (27) and retinoic acid was found to induce programmed cell death during mouse embryogenesis (28). These reports led us to determine whether transglutaminase increase and growth inhibition in HEL cells was related to cell apoptosis. Since we could find no indication of DNA degradation, the main characteristic feature in apoptosis, during the first 48 h following retinoic acid treatment, we concluded that transglutaminase induction in HEL cells by retinoic acid was not related to cell apoptosis.

The reason for the differential response of the two human erythroleukemia cell lines to retinoic acid still remains to be answered. With the discovery of two distinct nuclear retinoic acid receptors, RARα and RARβ, the understanding of retinoic acid action reached new dimensions (29–32). A third receptor, RARγ, seems to exert its action predominantly in the skin (33). Gallagher et al. (34) found no close correlation between responsiveness to retinoic acid and steady state levels of RARα RNA in HL60 and K562 cells. Furthermore, a similar RARα gene expression has been described in HEL and K562 after treatment with RA (35). Thus, it would be interesting to know whether the current findings could be explained by a differential RARβ gene expression.

While evidence is accumulating that there is a close correlation between transglutaminase activity and cell differentiation, the physiological function of this enzyme is still in question. Because the biochemical approach alone could obtain only a slow progress, the additional use of molecular biological tools, now available with the recent cloning of the human tissue...
transglutaminase cDNA by Davies, promises to aid in this endeavor in the near future.

ACKNOWLEDGMENTS

The authors thank Dr. Peter J. A. Davies, University of Texas Health Science Center at Houston, for the human transglutaminase cDNA probe; Dr. Davies and Dr. Laszlo Fesus, Debrecen University School of Medicine, for invaluable suggestions and discussions; and Don Darling, Memorial Hospital of Southern Oklahoma, for making the hemoglobin assay available to us. We also thank Laura Smith for preparing the manuscript.

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