Retinoic Acid-induced Expression of Tissue Transglutaminase in Human Promyelocytic Leukemia (HL-60) Cells*

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Addition of retinoic acid to human promyelocytic leukemia cells results in a dramatic increase in cellular transglutaminase activity. This increase is due to the induction of a specific intracellular transglutaminase, tissue transglutaminase. Retinoic acid-induced expression of tissue transglutaminase is potentiated by analogues of cyclic AMP. The introduction of the enzyme can be detected within 6 h of the addition of the retinoid to the cell and results in increases of the enzyme of at least 50-fold. The induction of HL-60 transglutaminase is a specific response of the cells to retinoic acid and is not seen with other agents that induce HL-60 differentiation. We believe that the induction of tissue transglutaminase is a useful index of the early events in retinoid-regulated gene expression in both normal and transformed cells.

Retinoic acid is a naturally occurring metabolite of vitamin A (retinol) that can modify the proliferative rate and state of differentiation of a variety of normal and transformed cells (for reviews see Refs. 1 and 2). Although retinoids can induce dramatic alterations in the phenotype of transformed cells, little is known about the molecular events involved in this process (3). Part of the difficulty in investigating this issue has been the lack of identifiable markers of retinoid action, specific proteins whose expression directly reflects the interaction of retinoids with a cell.

Recent several reports have suggested that the transglutaminase activity of cells may be directly linked to retinoid action (4–6). Transglutaminases are a group of enzymes that catalyze the covalent cross-linking of proteins (7, 8). Recently, we have reported that retinoic acid can directly regulate the rate of expression of a specific transglutaminase, tissue transglutaminase, in cultured macrophages (6). Nanomolar concentrations of retinoic acid induced a dramatic increase in the rate of synthesis of the enzyme within 90 min of addition of the retinoid to the cells (6). These observations suggested that the induction of tissue transglutaminase might be a useful probe to study retinoid action in cultured cells, particularly cells that undergo differentiation in response to retinoids. To pursue this issue, we have studied the effects of retinoic acid on tissue transglutaminase in human promyelocytic leukemia (HL-60) cells. These cells respond to retinoic acid with a marked decrease in the rate of cellular proliferation and phenotypic maturation into cells that resemble granulocytes (9). We have found that retinoic acid is indeed a specific inducer of tissue transglutaminase expression in HL-60 cells. Furthermore, this induction is greatly potentiated by analogues of cyclic AMP. The induction of tissue transglutaminase can be dissociated from the differentiation of the HL-60 cells and appears to represent an early, rapid, and specific response of these cells to retinoic acid.

**EXPERIMENTAL PROCEDURES**

Materials—Hypoxanthine, nitro blue tetrazolium (NBT), insulin, transferrin, selenium, dibutyryl cyclic AMP, dibutyryl cyclic GMP, sodium butyrate, actinomycin D, trans-retinoic acid and trans-retinol were purchased from Sigma. We also used trans-retinoic acid and cis-retinoic acid generously provided by Hofmann-La Roche, with similar results. Dimethylformamide and dimethyl sulfoxide were purchased from Eastman. Na<sup>251</sup>I was purchased from Amersham Corp. [H] Putrescine was purchased from New England Nuclear. Fresh mouse serum was obtained by cardiac puncture as described previously (10). Fresh human serum was obtained by venipuncture of a healthy volunteer. Human serum albumin was prepared from serum by ion exchange and gel filtration chromatography and was obtained as a by-product of the purification of human serum retinol binding protein. Serum retinol binding protein (SRBP) was purified from human serum using the procedure of Peterson (11). The preparation used in these studies was greater than 95% pure as judged by SDS-gel electrophoresis and spectrophotometry. Albumin and SRBP were depleted of endogenous retinoids by ethanol precipitation. The proteins were determined to be free of residual retinoids by spectrophotometry. Goat anti-guinea pig tissue transglutaminase antibodies used in these studies were described in detail previously (10). They were prepared by immunization of a goat with guinea pig liver transglutaminase, purified by affinity chromatography on a column of immobilized rat tissue transglutaminase, and iodinated by reaction with chloramine-T and Na<sup>251</sup>I as described previously (10). Rabbit anti-goat IgG immunoglobulins (Cappel) were purified on a column of immobilized goat immunoglobulin and iodinated with chloramine-T and Na<sup>251</sup>I (10).

**Cell Culture**—Continuous cultures of the HL-60 cell line were established from seed cultures provided to us by Dr. J. Fontana, Department of Medicine, University of West Virginia School of Medicine, Morgantown, WV. These cells were originally derived from a culture obtained from the laboratories of Dr. R. C. Gallo, National Cancer Institute, National Institutes of Health, Bethesda, MD. The cells were maintained in RPMI 1640 media supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), and selenium (3 nM), subsequently referred to as defined media. Cells were cultured in plastic tissue culture flasks (Falcon Plastics, Oxnard, CA) at 37 °C in a humidified atmosphere of 7.5% CO<sub>2</sub>. Cells were counted in a hemocytometer chamber and viability assessed by the exclusion of 0.1% trypan blue.

* The abbreviations used are: NBT, nitro blue tetrazolium; SRBP, serum retinol binding protein; SDS, sodium dodecyl sulfate.

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Cellular morphology was evaluated from a cytoplasm preparation stained with Wright-Giemsa stain. Maintenance cultures were maintained with cells seeded at a density of 5 × 10^6 cells/ml every 4-7 days.

Mouse peritoneal macrophages were prepared by peritoneal lavage of normal ICR strain laboratory mice as described previously (10). Treatment of Phagocytosis—Candida albicans, grown in Sabouraud and medium, were washed three times in sterile phosphate-buffered isotonic saline, opsonized by incubation with human AB serum in phosphate-buffered isotonic saline for 2 h at 37°C, and then stored in aliquots at -70°C. HL-60 cells, washed twice in RPMI 1640 media and resuspended at a concentration of 1 × 10^7/ml, were incubated with opsonized Candida at a final yeast to cell ratio of 100:1. After incubation for 20 min at 37°C, cell suspensions were fixed on slides and stained. The percentage of cells having ingested yeast was determined by direct microscopic inspection of at least 200 cells in each experimental condition.

**Measurement of NBT Reduction**—NBT reduction was determined by a modification of an assay previously described (12). Cells (1 × 10^7/ml) were incubated for 20 min at 37°C in RPMI 1640 medium with 100 ng/ml tetradecanoylphorbol acetate and 0.1% NBT. After incubation, cells were placed onto slides by cytospin, fixed, and stained. The percentage of cells that contained blue-black formazan deposits, the product of NBT reduction, was determined by direct microscopic inspection of at least 200 cells for each experimental condition.

Detection of Tissue Transglutaminase in Cell Extracts—The assay of HL-60 transglutaminase activity used conditions similar to those described for the assay of transglutaminase activity in cultured peritoneal macrophages (10). HL-60 cells (5 × 10^6 cells/ml) were cultured in suspension in 6-well tissue culture dishes (Costar, Cambridge, MA) in 5 ml of defined media. After the culture period, the cells were sedimented by low speed centrifugation, washed once with 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and then lysed in 200 μl of 20 mM Tris-HCl, pH 7.5, 1 mg/ml EDTA, and 0.5% Triton X-100. The transglutaminase activity of the lysate was assayed by measuring the time- and Ca^2+-dependent incorporation of [H]putrescine into casein (13) under conditions described in detail previously (10). To order to measure the low level of transglutaminase activity in HL-60 cells, we modified the assay by decreasing the concentration of [H]putrescine to 0.5 μM thereby increasing its specific activity to 69,000 μCi/ml. Experiments that used different concentrations of putrescine are identified in the legends to the figures and tables.

Measurement of Transglutaminase Activity in Cell Extracts—The assay of HL-60 transglutaminase activity used conditions similar to those described for the assay of transglutaminase in cultured peritoneal macrophages (10). HL-60 cells (5 × 10^6 cells/ml) were cultured in suspension in 6-well tissue culture dishes (Costar, Cambridge, MA) in 5 ml of defined media. After the culture period, the cells were sedimented by low speed centrifugation, washed once with 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and then lysed in 200 μl of 20 mM Tris-HCl, pH 7.5, 1 mg/ml EDTA, and 0.5% Triton X-100. The transglutaminase activity of the lysate was assayed by measuring the time- and Ca^2+-dependent incorporation of [H]putrescine into casein (13) under conditions described in detail previously (10). To order to measure the low level of transglutaminase activity in HL-60 cells, we modified the assay by decreasing the concentration of [H]putrescine to 0.5 μM thereby increasing its specific activity to 69,000 μCi/ml. Experiments that used different concentrations of putrescine are identified in the legends to the figures and tables.

**Results**

**Retinoic Acid-induced Expression of Tissue Transglutaminase**—Our studies on the induction of tissue transglutaminase in macrophages indicated that serum proteins can play a major role in the induction of the enzyme (6, 10). To avoid this complication, we first studied the effects of retinoic acid on the expression of transglutaminase in HL-60 cells adapted to grow in a chemically defined medium (15). Immunoblots of untreated HL-60 cells showed no detectable tissue transglutaminase (Fig. 1, lane A), indicating that the concentration of the enzyme was less than 5 ng/mg cell protein, the limit of detection of the immunoblot assay. Retinoic acid induced a large increase in the amount of tissue transglutaminase in the cells as evidenced by the appearance of a single major immunoreactive band at 82,000 daltons (Fig. 1, lane B). In three separate experiments, treatment of HL-90 cells with 1 μM retinoic acid for 48 h resulted in a level of tissue transglutaminase of 69 ± 5 ng/ml cell protein, reflecting at a minimum a 15-fold increase in the level of the enzyme. The induction of tissue transglutaminase was a specific process. Comparison of the Coomassie Blue profile of control and retinoic acid-treated cells (Fig. 1, lanes C and D) revealed no gross alterations in the number or the amounts of the major protein bands.

Retinoic acid was a potent inducer of transglutaminase in the HL-60 cells; the EC50 for induction of the enzyme was approximately 50 nM (Fig. 2). The cis- and trans-isomers of retinoic acid were equivalently effective as inducers of tissue transglutaminase whereas trans-retinol had very little inducing activity (Fig. 2). It has been reported that retinoic acid and cyclic AMP act synergistically to induce HL-60 cell differentiation (16, 17). We therefore examined the ability of a number of cyclic AMP, dibutyryl cyclic AMP, to affect retinoic acid-induced expression of tissue transglutaminase. HL-60 cells were cultured for 18 h in defined media alone (Fig. 3a, lane 2), or media containing either 1 mM dibutyryl cyclic AMP (lane 3), 1 μM trans-retinoic acid (lane 4), or the combination of retinoic acid plus dibutyryl cyclic AMP (lane 5), and the levels of tissue transglutaminase were measured by immunoblot assay. In this particular experiment, we used an indirect immunoblot procedure and long exposure times to maximize the sensitivity of the assay. The immunoblot of the standard, DNA was digested by addition of a small amount of purified DNase. The incorporation of radioactivity into protein was measured by trichloroacetic acid precipitation and liquid scintillation counting.

To pulse-label cells, HL-60 cells growing in suspension culture were pelleted and resuspended at a density of 2.5 × 10^6 cells/ml in methionine-free culture media containing 40 μCi/ml of [3H]methionine (New England Nuclear). After 30 min at 37°C, the cells were pelleted, washed twice with phosphate-buffered isotonic saline, and resuspended in 20 mM Tris-HCl, pH 7.5. The cells were then sonicated, DNA digested, and radiactivity determined as described above.

To immunoprecipitate tissue transglutaminase, equivalent aliquots of labeled cell extract were incubated at 4°C overnight with either affinity purified anti-transglutaminase antibody (6 μg) or preimmune immunoglobulin (60 μg) in 100 μl of 20 mM Tris-HCl, pH 7.5, containing 2 mg/ml bovine serum albumin. Immune complexes were sedimented by adding 300 μl of RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, and 0.1% SDS) and 50 μl of SDS-extracted, killed Staphylococcus aureus (Calbiochem-Behring) (14). The precipitated complexes were washed with RIPA and 20 mM Tris-HCl, pH 7.5, and then the proteins were solubilized in a SDS-containing electrophoresis buffer. The samples were fractionated by SDS gel electrophoresis on a 6.5% discontinuous slab gel, and protein bands were located by fluorography under conditions described in detail previously (10).
A) 48 h in media alone or media containing 1 μM retinoic acid was detected by immunoblot analysis with a 251-anti-transglutaminase antibody as described under “Experimental Procedures.” Total cellular polypeptides in untreated cell extracts (Fig. 3A, lane 1) or retinoic acid-treated (lane B) cell extracts (75 μg of cell protein) was detected by immunoblot analysis with a 251-anti-transglutaminase antibody as described under “Experimental Procedures.” Total cellular polypeptides in untreated (lane C) or retinoic acid-treated (lane D) cell extracts (100 μg of cell protein) were detected by Coomassie Blue staining. The markers at 200 kDa, 94 kDa, 68 kDa, and 44 kDa, respectively, represent the location of molecular weight standards, myosin heavy chain, phosphorylase, serum albumin, and ovalbumin.

10 ng of pure guinea pig tissue transglutaminase, is shown in lane 1. Even under these very sensitive assay conditions, there was no detectable tissue transglutaminase in the untreated cell extracts (Fig. 3A, lane 2). The few faint bands in the autoradiogram represent the background binding of radiolabeled second antibody to cellular proteins. Treatment of cells with dibutyryl cyclic AMP alone induced a small increase in the levels of tissue transglutaminase as indicated by the appearance of a faint band at 82,000 daltons (shown by the arrow in Fig. 3A). There was a larger accumulation of the enzyme in the cells treated with retinoic acid alone (lane 4). However, the combination of dibutyryl cyclic AMP and retinoic acid was much more effective than either agent alone in inducing the enzyme (lane 5). The heavy band in the extract of cells treated with both cyclic AMP and retinoic acid (lane 4) represents at least a 150-fold increase in the levels of the enzyme compared to untreated cells (lane 2). To quantitate the effect of dibutyryl cyclic AMP on the induction of HL-60 cell transglutaminase, we measured the transglutaminase activity in cells exposed to varying concentrations of retinoic acid in the presence or absence of dibutyryl cyclic AMP, Fig. 3B. The transglutaminase activity in untreated cells was too low to be detected by enzyme assay (less than 0.017 pmol/min/mg). Retinoic acid alone induced a substantial increase in transglutaminase activity; 1 μM trans-retinoic acid increased the enzyme activity at least 5-fold. Higher concentrations of the retinoid could not be tested because they were toxic to the HL-60 cells (data not shown). Dibutylry cyclic AMP markedly potentiated the induction of enzyme activity; the transglutaminase activity in maximally stimulated cells (0.760 pmol/min/mg) was more than 50-fold higher than untreated cells. Treatment of cells with 1 mM sodium butyrate or dibutylry cyclic GMP had no effect on the levels of tissue transglutaminase either in the presence or the absence of retinoic acid (data not shown).

We next examined the time course for the induction of the enzyme in cells treated with dibutylry cyclic AMP alone, retinoic acid alone, or dibutylry cyclic AMP plus retinoic acid (Fig. 4). Dibutylry cyclic AMP alone had no effect on enzyme activity. Retinoic acid did induce an increase in enzyme activity. The earliest time at which increased transglutaminase activity could be detected was 6 h after the addition of the retinoid to the cells. If dibutylry cyclic AMP and retinoic acid were combined then there was a much larger induction of the enzyme, but still no increase was detectable until at least 6 h after the addition of both agents to the cells. Six hours was also the earliest time at which we could detect tissue transglutaminase in immunobLOTS of these cells (data not shown). Once the induction of the enzyme was initiated, the enzyme accumulated for at least 24 h and then reached a plateau which remained stable for a further 24 h, at levels that were at least 35-fold higher than untreated cells.

To examine the mechanism responsible for the induction of tissue transglutaminase in HL-60 cells, the cells were metabolically labeled by incubation in media containing radio-labeled amino acids, and the transglutaminase was then immunoprecipitated from cell lysates. In the experiment shown in Fig. 5A, cells were exposed to retinoic acid for 20 h in medium containing 14C-labeled amino acids. Lysates of control or retinoic acid-treated cells were then immunoprecip-
Retinoic Acid Induction
of Transglutaminase

A

I

1

2

3

4

5

Retinoic Acid, M

0 10^{-9} 10^{-8} 10^{-7} 10^{-6}

Tgase Activity (pmol/min/mg)

0.2 0.4 0.6 0.8

FIG. 3. Effect of dibutyryl cyclic AMP on retinoic acid-induced expression of tissue transglutaminase. A, the amount of tissue transglutaminase in untreated HL-60 cells and HL-60 cells treated for 18 h with either 1 mM dibutyryl cyclic AMP, 1 μM retinoic acid, or retinoic acid plus dibutyryl cyclic AMP was determined by indirect immunoblot analysis as described under “Experimental Procedures.” Lane 1 is the autoradiogram with 10 ng of pure guinea pig tissue transglutaminase. Lanes 2-5 show the autoradiograms obtained with extracts (75 μg of cell protein) of untreated HL-60 cells (lane 2), dibutyryl cyclic AMP-treated cells (lane 3), retinoic acid-treated cells (lane 4), and cells treated with retinoic acid plus dibutyryl cyclic AMP (lane 5). B, the transglutaminase activity (Tgase Activity) of HL-60 cells cultured in the presence of varying concentrations of retinoic acid alone (O) or retinoic acid plus 1 mM dibutyryl cyclic AMP (DBC) (△) was determined as described under “Experimental Procedures.” The values shown represent the mean and ranges of duplicate determinations.

FIG. 4. Time course for the induction of transglutaminase activity. HL-60 cells were cultured for varying lengths of time in 1 μM retinoic acid (RA) (△), 1 mM dibutyryl cyclic AMP (DBC) (O), or retinoic acid plus dibutyryl cyclic AMP (O) in chemically defined media, and the transglutaminase activity was measured as described under “Experimental Procedures.” Values shown represent the mean of duplicate determinations.

pared immunoprecipitates of cells pulse-labeled with [35S]methionine in the presence or absence of retinoic acid. Immunoprecipitates from untreated cells showed a few faint protein bands nonspecifically precipitated by the anti-transglutaminase antibody (Fig. 5B, lane 1). Immunoprecipitates of retinoic acid-treated cells (Fig. 5B, lane 2) showed a prominent band of tissue transglutaminase. These experiments demonstrated that the induction of tissue transglutaminase was due to an increased rate of enzyme synthesis.

The induction of tissue transglutaminase was completely blocked by actinomycin D, an inhibitor of RNA synthesis. Addition of actinomycin D (5 μg/ml) to HL-60 cells reduced retinoic acid-induced expression of the enzyme by 90% (Table I). This suggests that the induction of tissue transglutaminase depends on increased RNA synthesis and is likely to be due to increased tissue transglutaminase gene expression.

Effect of Serum Proteins on Retinoic Acid-induced Expression of Tissue Transglutaminase—The preceding studies were all carried out in cells grown in a chemically defined culture media. Because the induction of tissue transglutaminase in macrophages is modified by components present in serum (6, 10), we evaluated the effects of whole serum and specific serum proteins on retinoic acid-induced expression of tissue transglutaminase in HL-60 cells. As can be seen in Fig. 6A, addition of 10% fresh human or fresh mouse serum to HL-60 cells had no effect on transglutaminase activity. This lack of transglutaminase-inducing activity did not represent a defect in the sera themselves since the same serum samples were fully active in inducing the enzyme in cultured peritoneal macrophages (Fig. 6B). We have previously shown that the ability of serum to induce tissue transglutaminase in macrophages is due to the presence of retinoic acid in fresh mouse and human sera (6). Delipidization of serum abolishes its transglutaminase-induc-
Using anti-transglutaminase antibodies as described under "Experimental Procedures." Tissue transglutaminase was immunoprecipitated from cell extracts using anti-transglutaminase antibodies as described under "Experimental Procedures." Lane 1 is the immunoprecipitate from untreated cells and lane 2 the immunoprecipitate from cells treated with 1 μM retinoic acid and 1 mM dibutyryl cyclic AMP. B, HL-60 cells were cultured for 18 h in defined media alone (lane 1) or defined media plus retinoic acid (1 μM) and dibutyryl cyclic AMP (1 mM) (lane 2). The cells were pulse-labeled for 30 min with [35S]methionine, and the tissue transglutaminase was immunoprecipitated as described under "Experimental Procedures."

**Table I**

| Conditions* | Transglutaminase activity (pmol/min/mg) |
|-------------|---------------------------------------|
| Media alone | 0.01                                  |
| 1 μM RA     | 0.73 ± 0.08                           |
| 1 μM RA + 0.5 μg/ml Act D | 0.52 ± 0.07 |
| 1 μM RA + 5.0 μg/ml Act D | 0.06 ± 0.01 |

*HL-60 cells were cultured for 18 h in media containing 1 mM dibutyryl cyclic AMP, 1 μM retinoic acid (RA), and varying concentrations of actinomycin D (Act D). The transglutaminase activity in cell extracts was assayed as described under "Experimental Procedures." Values shown are the mean and range of duplicate determinations.

Retinoic acid induces HL-60 cells to mature into cells that have both the morphology and the functional attributes of peripheral blood granulocytes (9). It was possible that the induction of tissue transglutaminase was a component of a concerted change in gene expression due to differentiation rather than a specific response to retinoic acid. Fortunately, the maturation of HL-60 cells can be triggered by a variety of chemical agents that are unrelated to retinoic acid (20-22). We therefore investigated whether all agents that induce HL-60 differentiation are equivalent in their ability to induce expression of the enzyme and whether the induction of tissue transglutaminase is an obligatory component of HL-60 cell maturation. HL-60 cells were cultured for 24, 48, or 72 h in defined media supplemented with either retinoic acid (1 μM), hypoxanthine (50 mM), dimethylformamide (60 mM), or dimethyl sulfoxide (1.25%). Morphologic maturation was assessed by microscopic examination, and functional differentiation was determined by the incidence of cells competent to reduce NBT or to phagocytose opsonized yeast particles (Table III). Untreated cells grew as single cells in suspension and had the rounded morphology typical of agranular myeloblasts and promyelocytes. Cells grown in the presence of retinoic acid, hypoxanthine, dimethylformamide, or dimethyl sulfoxide showed marked nuclear pleomorphism, increased cytoplasmic neutrophilia, and a loss of nucleoli. This maturation was also reflected in an increase in the percentage of cells competent both to phagocytose yeast and reduce NBT (Table III). The induction of tissue transglutaminase in the cells treated with the various agents was assessed by immunoblot analysis of cell extracts (Fig. 8). In spite of the fact that all four agents induced a similar maturation of the cells, only retinoic acid induced expression of tissue transglutaminase (Fig. 8). The results shown in Fig. 8 were from cells exposed to the agents for 24 h, but similar results were obtained from cells exposed for 48 or 72 h (data not shown).

**DISCUSSION**

We have been interested in the hormonal factors that regulate the growth and differentiation of both normal and transformed cells. Retinoids are a group of metabolites of vitamin A (trans-retinol) that have important effects on the proliferation and differentiation of many types of cells (1, 3). It has been assumed that these effects reflect the ability of retinoids to alter gene expression in susceptible cells (3), but
Transglutaminase, tissue transglutaminase (6). Synthesis of the enzyme is increased within minutes of the addition of the enzyme involved have not been elucidated. We have been interested in the regulation of transglutaminase activity in myeloid cells (6, 10, 24, 25). Retinoic acid induces dramatic increases in transglutaminase activity in both macrophages and monocytes due to the induction of a specific intracellular enzyme, tissue transglutaminase (6). Synthesis of the enzyme is increased within minutes of the addition of retinoic acid to cultured peritoneal macrophages, and retinoic acid can trigger the accumulation of the enzyme to the point that it accounts for 1-2% of the total cellular protein (6, 10). These studies suggested to us that the induction of this enzyme might be a useful tool to study retinoid control of gene expression in both normal and transformed cells. We have focused here on the effects of retinoic acid on the expression of tissue transglutaminase in cultured human promyelocytic leukemia (HL-60) cells. These cells were chosen because of their well-documented ability to undergo major changes in morphologic and functional properties following exposure to retinoic acid (9). Our first step was to determine whether retinoic acid had any effect on transglutaminase expression in HL-60 cells. Untreated HL-60 cells had no detectable tissue transglutaminase, either by enzymatic assay or immunoblot. Bierichbichler and Patterson (26) have reported that many transformed cell lines have low levels of transglutaminase activity, and HL-60 cells certainly conform to this generalization. The pulse-labeled studies we have carried out suggest that the very low levels of the enzyme in untreated HL-60 cells were cultured in defined media containing 1 mM dibutyryl cyclic AMP and varying concentrations of retinoic acid (RA) or media containing either 10% delipidized serum (DLMS), 350 µg/ml delipidized serum albumin (DLHSA), or 4 µg/ml delipidized serum retinol binding protein (SRBP). Transglutaminase activity was assayed as described under "Experimental Procedures." Values shown represent the mean and range of duplicate determinations.

Table II

| Concentration of retinoic acid | Transglutaminase activity | NBT Rdn Phagocytosis |
|------------------------------|---------------------------|----------------------|
| Media alone                  | <0.02                     | % mature cells       |
| 1 nM                         | 0.04 ± 0.01               | 2                    |
| 10 nM                        | 0.20 ± 0.02               | 63                   |
| 100 nM                       | 0.51 ± 0.03               | 83                   |
| 1000 nM                      | 0.55 ± 0.08               | 63                   |

*HL-60 cells were cultured in defined media containing 1 mM dibutyryl cyclic AMP and varying concentrations of retinoic acid in the presence or absence of 200 nM delipidized human serum retinol binding protein (SRBP). Transglutaminase activity was determined as described under "Experimental Procedures." Values shown represent the mean and range of duplicate determinations.

Table III

| Treatment | NBT Rdn | Phagocytosis |
|-----------|---------|--------------|
| None      | 2       | 10           |
| Retinoic acid (1 µM) | 63 | 67 |
| Hypoxanthine (50 mM) | 83 | 64 |
| Dimethylformamide (60 mM) | 46 | 63 |
| Dimethyl sulfoxide (1.25%) | 25 | 83 |

*HL-60 cells were cultured in defined media for 72 h in the presence of different chemical inducers of maturation and the incidence of functionally mature cells measured by the frequency of cells positive for the reduction of nitro blue tetrazolium (NBT Rdn) or the phagocytosis of opsonized yeast particles as described under "Experimental Procedures."
Transglutaminase synthesis, inducing up to a 150-fold increase in HL-60 cells. HL-60 cells were cultured for 24 h in defined media alone or the same media supplemented with retinoic acid (1 μM), hypoxanthine (50 mM), dimethylformamide (60 mM), or dimethyl sulfoxide (1.25%). Tissue transglutaminase was detected in cell extracts using the immunoblot assay described under "Experimental Procedures." Lane 1 is the immunoblot of untreated cells and lanes 2-5 the immunoblots of cells treated with retinoic acid (lane 2), hypoxanthine (lane 3), dimethylformamide (lane 4), or dimethyl sulfoxide (lane 5).

cells reflect a very low level of basal enzyme synthesis. Retinoic acid appears to have dramatic effects on the rate of transglutaminase synthesis, inducing up to a 150-fold increase in the level of the enzyme in optimally treated cells. This induction is most likely due to an increased rate of tissue transglutaminase gene expression since the effects of retinoic acid are completely blocked by inhibitors of RNA synthesis such as actinomycin D. Experiments to directly measure transglutaminase gene expression are currently underway in our laboratory. It is interesting to note that the concentration of the enzyme in fully induced cells (20 ng/mg cell protein) is comparable to the level of the enzyme in a number of normal cell lines and tissues (10) and significantly higher than in freshly isolated normal human blood monocytes (24).

We have used the induction of tissue transglutaminase as an index for characterizing retinoid action in HL-60 cells. One of the most striking features of the induction of tissue transglutaminase was its remarkable specificity. In spite of the fact that retinoid treatment induced up to a 150-fold increase in the levels of tissue transglutaminase, the levels of most other major cellular proteins, as detected by SDS-gel electrophoresis, were unaffected. Retinoic acid has been reported to increase the NAD glycohydrolase activity of HL-60 cells (27). This increase may be due to induction of the enzyme or activation. The expression of oncogenes in HL-60 cells also appears to be regulated by retinoic acid (28). We think that our results and the results of others suggest that retinoic acid induces alterations in the expression of a specific sub-set of genes in HL-60 cells and tissue transglutaminase is among these retinoid-regulated genes.

A second striking feature of the effects of retinoic acid on transglutaminase gene expression is the rapidity with which alterations in the level of the enzyme are detected. Retinoic acid has been reported to alter the expression of a diverse group of proteins in different types of cultured cells and tissues (3-6, 27, 29-36). In most instances these alterations in protein synthesis follow the retinoid-induced differentiation of the cells and can only be detected after many hours or even days of retinoid treatment (29-34). There are, however, a few examples of proteins whose expression reflects a more immediate response of cultured cells to retinoic acid. The addition of retinoic acid to cultured embryonal carcinoma cells increases the levels of the enzyme in HL-60 cells within 4-6 h (36). The response of HL-60 cells to retinoic acid is also very fast. Increased levels of tissue transglutaminase were detected within 6 h of addition of the retinoic acid to the cells and reached maximal values within 24-48 h. The actual onset of the induction may be even earlier than 6 h, but the levels of the enzyme at these early times are too low to be detected by our assay procedures. Hemmi and Breitman (27) reported that NAD glycohydrolase activity also increased 6 h after the addition of retinoic acid to HL-60 cells. The induction of tissue transglutaminase is easier to measure in macrophages than in HL-60 cells because the basal rate of enzyme synthesis can be detected in untreated cells. In these cells, increased transglutaminase synthesis occurs 60-90 min after the addition of retinoic acid (6). All these studies suggest that alterations in gene expression can be detected very rapidly after the addition of retinoic acid to cultured cells. The rapidity of the observed effects compares with the induction of proteins by steroid hormones (37) and suggests that similar types of molecular mechanisms might be involved.

Retinoic acid-induced differentiation of myeloid leukemia cells is potentiated by agents that elevate intracellular cyclic AMP (16, 17). We therefore investigated whether the same potentiation could be observed in the induction of a specific protein such as tissue transglutaminase. The results in Fig. 3 show very clearly that analogues of cyclic AMP such as dibutyryl cyclic AMP have a tremendous effect on the induction of tissue transglutaminase. The effects of retinoic acid and dibutyryl cyclic AMP are much more than additive and suggest a true synergistic interaction between the two agents. We do not yet know the site of this interaction, but these studies suggest very strongly that ability of cyclic AMP to promote retinoic acid-induced differentiation most likely reflects its ability to promote retinoic acid-induced gene expression. There are many examples of enzymes whose expression is altered by addition of cyclic AMP to cells (38). It is possible that some of these alterations may reflect the ability of cyclic AMP to potentiate the activity of retinoids present in serum. For instance, the effects of cyclic AMP on transglutaminase activity in Chinese hamster ovary cells (40) may have been due to a potentiation of the effects of retinoids present in the serum-containing culture media. We believe it is possible that many of the effects of cyclic AMP on cellular proliferation and differentiation could be due to a potentiation of retinoid action.

There is considerable evidence that the induction of tissue transglutaminase may be linked to cellular maturation and differentiation. Treatment of SV-40-transformed human lung fibroblasts with sodium butyrate causes alterations in cellular morphology and growth rate and a concomitant increase in transglutaminase activity (41). The differentiation of murine
myeloblastic leukemia cells into macrophages is associated with a very large increase in cellular transglutaminase activity (42). The maturation of monocytes into macrophages also appears to be associated with induction of tissue transglutaminase (24). We therefore evaluated the role of differentiation in the induction of tissue transglutaminase in HL-60 cells. We took advantage of the fact that the differentiation of HL-60 cells is induced by a diverse group of chemical agents whose mechanisms of action are likely to be distinct from the effects of retinoic acid (20–22). Compounds such as dimethyl sulfoxide, dimethylformamide, and hypoxanthine induce the maturation of HL-60 cells into cells that are functionally equivalent to retinoic acid-treated cells (20–22). However, as the results in Fig. 8 clearly demonstrate, only retinoic acid-treated cells show elevated levels of tissue transglutaminase. This suggests that the induction of tissue transglutaminase is a specific response of HL-60 cells to retinoids rather than a general response of the cells to differentiation. A similar specificity has also been reported for the induction of NAD glycohydrolase (27). Interestingly, the suppression of oncogene expression does not share this same specificity in that both retinoic acid and dimethyl sulfoxide suppress myc expression in HL-60 cells (28). It would appear that the induction of some enzymes such as tissue transglutaminase and NAD glycohydrolase may be a very selective response to retinoids whereas alterations in the expression of oncogenes may represent a more generalized consequence of retinoid-induced differentiation.

We were able to investigate the effects of serum proteins on the ability of retinoic acid to induce expression of tissue transglutaminase in the HL-60 cells because these cells can be cultured in a chemically defined medium. Free retinoic acid is fully capable of inducing gene expression in the complete absence of serum proteins. Retinoids are very lipid-soluble compounds and presumably free retinoic acid is able to diffuse directly into the HL-60 cell and trigger the expression of transglutaminase. Retinoic acid can bind to serum albumin (18), and exogenous retinoic acid administered to either rats or humans is transported in plasma bound to albumin (43). We found that the addition of albumin to HL-60 cells had no effect on the induction of tissue transglutaminase. Presumably, retinoic acid is bound to albumin in a freely dissociable form and still can diffuse into the cells and induce gene expression. Plasma also contains a specific retinoid binding protein whose primary function appears to be the transport of retinol in plasma (44, 45). Some cells appear to have specific surface receptors for SRBP (46, 47) which are thought to facilitate the delivery of retinol to specific cells and tissues (44–47). SRBP also binds retinoic acid (19), and this binding has been shown to facilitate the delivery of retinoic acid to the pigmented epithelium of the retina (48). We have found that SRBP potentiates retinoic acid-induced expression of tissue transglutaminase in cultured macrophages, suggesting that this protein also may play a role in the delivery of retinoic acid to macrophages (6). SRBP did not potentiate the effects of retinoic acid in HL-60 cells, in fact it actually reduced it. Retinoic acid is known to bind to SRBP very tightly (19), and presumably the complex prevents the diffusion of retinoic acid into the HL-60 cells. We think that cells lacking cell-surface SRBP receptors, and this may be particularly true of transformed cells, are only able to respond to free retinoic acid or retinoic acid bound to albumin. Normal cells may be able to respond preferentially to retinoic acid complexed to a specific transport protein such as SRBP. Unfortunately, little is known of the transport of endogenously produced retinoic acid present in normal plasma (39), so it is not possible yet to determine whether the retinoic acid found in normal plasma is bound to albumin, SRBP, or some other transport protein. It is possible that the proteins that transport retinoic acid in plasma may have a large effect on the delivery of retinoids to specific susceptible cells and tissues. Defects in this targeting process, such as defects in the expression of cell surface receptors for the transport proteins, would render cells oblivious to the effects of plasma retinoic acid and thus capable of escaping the antiproliferative and differentiation-inducing effects of this hormone. We speculate that such defects may make an important contribution to the phenotype of some transformed and leukemic cells.

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