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Differential Regulation of Tissue Transglutaminase in Rat Hepatoma Cell Lines McA-RH7777 and McA-RH8994: Relation to Growth Rate and Cell Death

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Abstract Close correlation between tissue transglutaminase (tTG) induction and growth regulation and/or cell death processes has been suggested in many cell lineages. In this study, the regulation of the tTG levels by various growth and differentiation factors and its relation to growth rate and cell death processes were investigated in two rat hepatoma cell lines, McA-RH7777 and McA-RH8994, using a monoclonal antibody against liver tTG. Transforming growth factor-β1 (TGF-β1) and retinoic acid (RA) each increased tTG to the level of 8- to 32-fold above that of control cultures in both cell lines after 72-h treatment. Dexamethasone (DEX) induced a 16- to 32-fold of tTG in McA-RH8994 cells while it did not change the enzyme level in McA-RH7777 cells. Simultaneous addition of DEX and RA increased the tTG level to more than 50-fold in McA-RH7777 cells as well as McA-RH8994 cells. Other factors, such as TGF-α, hepatocyte growth factor, dimethyl sulfoxide, and protein kinase C activator, did not show significant increases of the tTG levels. Although tTG induction by TGF-β1 or DEX appeared to be correlated with their growth suppressive effects, RA increased the tTG level without suppressing the growth rate of hepatoma cells. TGF-β1 was also shown to induce cell death in both cell lines. Our results demonstrate that RA and DEX are capable of modulating the TGF-β1-induced cell death processes independent of the tTG levels. We present evidence here that tTG induction by itself is not the direct cause of growth suppression and cell death in these hepatoma cells.

Key words: tissue transglutaminase, apoptosis, hepatoma, dexamethasone, retinoic acid, transforming growth factors, cell growth, cell death

Transglutaminases [EC 2.3.2.13] (TG)[4] are a family of Ca^{2+}-dependent enzymes which catalyze an acyl-transfer reaction between peptide-bound glutamine residues and primary amines including the ε-amino group of lysine residues in appropriate proteins [Folk, 1980; Lorand and Conrad, 1984; Greenberg et al., 1991]. These reactions result in the functionally significant posttranslational modification of proteins either by the specific incorporation of polyamines or the crosslinking of proteins via ε-(γ-glutamyl) lysine bridges [Folk, 1980; Folk et al., 1980; Lorand and Conrad, 1984; Greenberg et al., 1991]. The biological role of three of the four well characterized TGs in mammals, namely blood coagulation factor XIII, keratinocyte TG, and epidermal TG has previously been established [Folk, 1980; Lorand and Conrad, 1984; Greenberg et al., 1991]. However, the function of the fourth one called the tissue type is not yet completely established [Fesus and Thomazy, 1988]. Functions suggested for tissue transglutaminase (tTG) include regulation of cell proliferation, differentiation, receptor mediated endocytosis, formation of intracellular matrix, aging, and programmed cell death (apoptosis) [Fesus et al., 1987; Fesus and Thomazy, 1988; Brickbichler et al., 1981; Selkoe et al., 1981; Kannagi et al., 1982].

Recent studies revealed several cellular substrates for tTG and showed that tTG-mediated posttranslational modification plays an impor-

Abbreviations used: tTG, tissue transglutaminase; TGF-β1, transforming growth factor-β1; TGF-α, transforming growth factor-α; RA, retinoic acid; DEX, dexamethasone; HGF, hepatocyte growth factor; DMSO, dimethyl sulfoxide; ADMB, 3-(N-acetylamino)-5-(N-decyl-N-methylamino)benzyl alcohol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; MoAb, monoclonal antibody.

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tant role in various cellular functions [Fesus et al., 1985; Zatloukal et al., 1989; Hand et al., 1990; Cordella-Miele et al., 1990; Ando et al., 1991; Aeschlimann and Psulsson, 1991; Borth et al., 1991; Barsigian et al., 1991]. Especially, the involvement of tTG induction in growth regulation and/or differentiation processes has been suggested in a number of systems [Hsu and Friedman, 1983; Maccioni and Seeds, 1986; Davies et al., 1988; Suedhoff et al., 1990]. Birckbichler et al. [1981] suggested that tTG-catalyzed isopeptide crosslinks plays a central role in cell growth regulation. Indeed previous studies have demonstrated that malignant cells have reduced tTG activity as compared to normal cells and that this reduced tTG activity is associated with cell proliferation, tumor promotion and metastatic potential [Birckbichler et al., 1976; Barnes et al., 1985; Knight et al., 1990, 1991]. However, the function and the molecular mechanism determining the cytosolic tTG levels in neoplastic cells are largely unknown [Fesus and Thomazy, 1988].

Various factors have been shown to modulate tTG expression in different cell systems. For example, RA has been shown to induce tTG expression and differentiation in human promyelocytic leukemia HL60 cells [Davies et al., 1985] and in mouse peritoneal macrophages [Moore et al., 1984; Chiocca et al., 1988]. Sodium butyrate induces tTG in human lung fibroblast cells [Birckbichler et al., 1983]. Dimethyl sulfoxide (DMSO) and n-butyric acid increase tTG activity in Friend erythroleukemia cell line GM979 [Hsu and Friedman, 1984]. Enhanced expression of tTG by TGF-β1 is also reported in rabbit tracheal epithelial cells [Jetten et al., 1986] and human epidermal keratinocytes [George et al., 1990]. Taking the analogy of keratinocytes and leukemic cell lines, it is worth investigating the possible involvement of tTG induction and activation in the differentiation and/or growth inhibitory pathways in hepatoma cell lines.

We investigated the regulatory mechanism of tTG levels and its relation to growth regulation in hepatoma cells. Rat hepatoma McA-RH7777 and McA-RH8994 cell lines were used in this study. These two rat hepatoma cell lines have been relatively well characterized [Cook and Chiu, 1986; Cook et al., 1989; Fukuda et al., 1991]. For example, the growth rate of McA-RH8994 cells is much slower than McA-RH7777 cells, with generation times of 40 h and 27 h, respectively [Cook et al., 1989]. DEX increases α-fetoprotein (AFP) production in McA-RH8994 cells, while DEX decreases AFP production in McA-RH7777 cells [Cook and Chiu, 1986; Cook et al., 1989]. Cell growth and morphology are also modulated bidirectionally by dexamethasone (DEX) in these cell lines [Fukuda et al., 1991].

We have established a hybridoma clone that produces a monoclonal antibody against liver tTG and used this to investigate the effects of various growth and differentiation factors on the tTG levels in rat hepatoma McA-RH7777 and McA-RH8994 cell lines. We have demonstrated here that TGF-β1 and RA significantly increased the tTG levels in both cell lines. However, DEX showed differential effects on the tTG levels. Synergistic and antagonistic effects of these factors were also demonstrated both on the tTG levels and growth regulation.

MATERIALS AND METHODS

Cell Cultures and Treatment

Two Morris hepatoma-derived rat hepatoma cell lines [Becker et al., 1976], McA-RH7777 and McA-RH8994, were obtained from the American Type Culture Collection (Rockville, MD) and were maintained at 37°C in an atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 μg/ml of streptomycin and 100 U/ml of penicillin). The hepatoma cells were seeded at $1 \times 10^4/cm^2$ in plastic tissue culture dishes (FALCON 3003, Becton Dickinson Labware, Lincoln Park, NJ) and preincubated for 24 h before the treatment. For experimental treatment, the medium was changed, and the cultures were exposed to various growth and/or differentiation factors at various concentrations. After the treatment for 72 h, adherent and floating cells were counted separately with an electric particle counter (Coulter Counter, Model ZM, Coulter Electronics, Ltd). The percentage of treated cells relative to control cultures was determined from four dishes. Data were analyzed statistically by one group *t*-test. $P < 0.05$, two-tailed, was considered statistically significant.

Reagents that are able to modulate growth rate and/or gene expression were studied for their effects on the tTG induction. These include transforming growth factor-α (TGF-α), TGF-β1, hepatocyte growth factor (HGF), dexamethasone (DEX), dimethyl sulfoxide (DMSO), all-*trans*-retinoic acid (RA), and protein kinase C activator 3-(N-acetylamino)-5-(N-decyl-N-

methylamino)benzyl alcohol (ADMB). Human recombinant TGF-α and TGF-β1 were obtained from King Brewing Co. Ltd. (Kakogawa, Japan). HGF was obtained from Becton Dickinson Labware (Lincoln Park, NJ). DEX, DMSO, RA, were purchased from Sigma Chemical Co. (St. Louis, MO). ADMB was obtained from Funakoshi Co. Ltd. (Tokyo, Japan). Stock solutions of RA and ADMB were made up at a concentration of 20 mM or 10 mg/ml in DMSO, respectively. DEX was dissolved at 10 mM in ethanol. The highest amount of DMSO or ethanol added to the cultures with the compounds was 0.1%, a concentration that did not affect any of the phenotypes tested.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Immunoblot Analysis

Cultured cells were harvested by centrifugation, the cell pellet was dissolved in SDS-sample buffer (62.5 mM Tris–HCl, pH 6.8, 10 mM EDTA, 5% β-mercaptoethanol, 2% SDS) at concentration of 5×10^6 cells/ml, sonicated for 10 s, and then heated to 100°C for 5 min. Proteins were separated on 9% polyacrylamide slab gels, followed by immunoblot analysis as previously described [Fukuda et al., 1990]. Hybridoma clones that produce monoclonal antibodies (MoAb) against guinea pig liver tTG were prepared by fusion of FO-1 myeloma cells with lymphocytes of inguinal lymph nodes from the mice immunized with purified guinea pig liver tTG which was obtained from Sigma (St. Louis, MO). Immunization and cell fusion was carried out essentially according to the procedure reported by Orlik and Altaner [Orlik and Altaner, 1988]. MoAbs against guinea pig liver tTG were further screened for the cross-reactivity with rat liver tTG. One hybridoma clone, TG100, was shown to produce MoAb that reacts with rat tTG and guinea pig liver tTG with high affinity. Specificity of the MoAb is shown in the results.

Determination of Relative Cytosolic tTG Levels in Culture Cells

The cytosolic tTG level in each treated culture relative to that of control culture was expressed as a fold increase. Serially diluted (1:1) protein samples, starting from 256×10^3 cells/lane, from control and treated cells were subjected to the immunoblot analysis on the same blot. Approximate figures of the fold increase were determined by comparing the intensity of immunoreactive bands of serially diluted protein samples with those of control culture on the same blot. The intensity of the immunoreactive bands were compared by densitometry with a Shimadzu chromatoscanner CS-930 (Shimadzu Co., Kyoto, Japan) in the reflectance mode, and the figures presented were obtained from two separate immunoblots.

RNA Preparation and Northern Blot Analysis

Total cellular RNA was isolated from cell monolayers by the acid guanidinium thiocyanate–phenol–chloroform extraction method as described by Puissant and Houdebine [Puissant and Houdebine, 1990]. Northern blot hybridization was carried out essentially as described by Sambrook et al. [1989]. Briefly, samples of 20 μg each of total RNA were denatured and subjected to electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde. After the electrophoresis, the gel was stained with ethidium bromide and photographed under UV illumination to verify even loading and to reveal the positions of ribosomal RNAs. The RNAs were then transferred to Duralon-UV nylon membranes (Stratagene, La Jolla, CA), fixed by UV irradiation using a Stratalinker UV crosslinker (Stratagene). The membranes were prehybridized at 42°C for 4 h in a solution of 5 × SSC (1 × SSC = 0.15M NaCl/0.015M sodium citrate)/50% (v/v) formamide/0.1% Ficoll/0.1% polyvinyl pyrrolidone/0.1% bovine serum albumin/0.2% SDS/50 mM sodium phosphate, pH 7.0 containing 100 μg/ml of denatured salmon sperm DNA. Hybridization was performed at 42°C for 16 h in an identical solution containing a denatured ^{32}P-labeled DNA probe. After hybridization, the membranes were washed at 55°C in solutions containing 0.1% SDS and decreasing concentrations of salt (2×, 1×, 0.5× SSC). They were then exposed to Fuji RX-film (Fuji Photo Film Co. Ltd. Kanagawa, Japan) with intensifying screens at −80°C. Cloned fragments encoding partial cDNA sequence of mouse tTG (pmTG700) [Chiocca et al., 1988], kindly provided by Dr. Peter J.A. Davies (University of Texas Medical School, Houston), were radiolabeled with ^{32}P using a multiprime DNA labeling system (Amersham, UK) and used for hybridization probes.

RESULTS

Effects of Various Reagents on the Cytosolic tTG Levels in Rat Hepatoma Cells

Several reagents that are able to modulate growth rate and/or gene expression were studied for their effects on the levels of tTG. Some of

the reagents used in this study were shown to be cytotoxic to hepatoma cells when high dosages of the drugs were applied, and the cytotoxic dosage was different depending on the cell lines investigated. For example, cytotoxic effects of RA were significant in 72 h culture at concentrations higher than 50 μM and 10 μM for McA-RH7777 and McA-RH8994 cells, respectively. McA-RH8994 cell line was highly sensitive to the cytocidal effect of TGF-β1: more than 80% of culture cells died within 72 h in the presence of TGF-β1 at concentrations higher than 0.5 ng/ml. Thus, the optimal concentrations of the reagents for each hepatoma cells was determined from preliminary experiments based on their effectiveness on gene expression and cytotoxicity. The rat hepatoma cell lines were cultured in the presence of optimal concentrations of these agents for 72 h and the total cell extracts were analyzed by immunoblotting using the MoAb against liver tTG. Figure 1 shows the representative immunoblots demonstrating the tTG levels in cells under various conditions. TGF-β1 and RA increased the tTG levels in both cell lines (lanes 4 and 6 respectively). DEX showed differential effects on the tTG induction: DEX increased tTG level in McA-RH8994 cells but not in McA-RH7777 cells (lane 7). Other treatments (HGF, TGF-α, DMSO, ADMB, and serum starvation) did not show significant changes in the tTG levels in both cell lines indicating the specificity of the TGF-β1, RA, and DEX effects. Because the basal levels of tTG in both cell lines cultured in 15% FBS-containing medium were maintained at low level, it was difficult to identify suppressive effects. At this time, we only focused on the reagents that significantly increased the level of tTG. By comparing the intensity of immunologically detected tTG protein bands on immunoblot analysis of 1:1 serially diluted samples of experimental and control cultured cells, as shown in Figure 2A, TGF-β1 and RA increased the level of tTG in both cell lines from 8- to 32-fold over that of control culture. Dex increased the tTG protein amount in McA-RH8994 cells approximately 16- to 32-fold at maximum stimulatory concentrations (Fig. 2B). However, Dex alone showed little effect on the tTG level in McA-RH7777 cells at concentrations investigated (Fig. 2B).

Synergistic or Additive Effects by TGF-β1, RA, and DEX on the Levels of tTG

In order to characterize further the effects of TGF-β1, RA and DEX, the hepatoma cells were

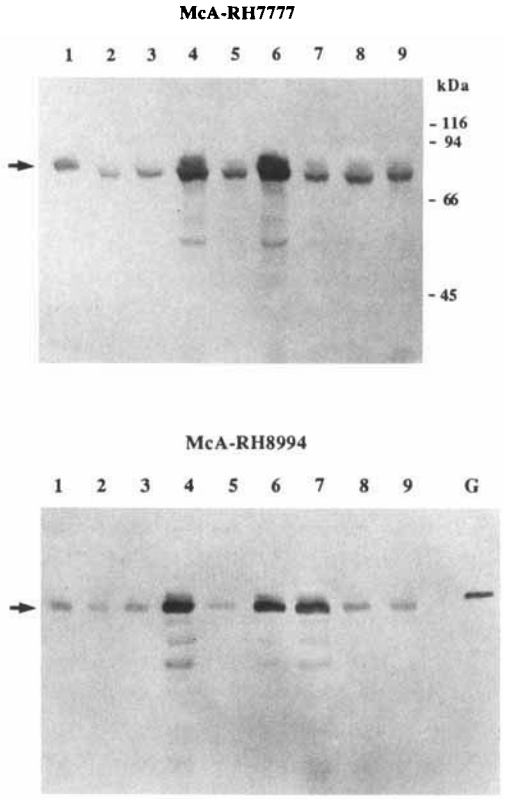

Fig. 1. Representative immunoblots showing the effects of various bioactive reagents on the levels of cytosolic tTG in McA-RH7777 and McA-RH8994 cells. Hepatoma cells were cultured for 72 h in the presence of various growth and differentiation factors as indicated below. Total cell extracts from 2×10^5 cells were applied in each lane and subjected to 9% polyacrylamide slab gel electrophoresis followed by immunoblotting using MoAb TG100 against liver tTG. Specificity of the MoAb TG100 to liver tTG from guinea pig **(lane G)** was also demonstrated. Arrows mark the position of rat 80 kDa tTG bands. **Lanes: 1,** control culture in DMEM with 15% FBS; **2,** serum starved culture; **3,** DMSO (2% v/v); **4,** TGF-β1 (5 ng/ml for McA-RH7777 cells or 0.1 ng/ml for McA-RH8994 cells); **5,** TGF-α (100 ng/ml); **6,** retinoic acid (10 μM for McA-RH7777 cells or 2 μM for McA-RH8994 cells); **7,** dexamethasone (10^{-6} M); **8,** hepatocyte growth factor (10 ng/ml); **9,** protein kinase C activator ADMB (10 μg/ml).

cultured in the presence of either two of these agents and the levels of tTG were analyzed 72 h after the treatment. Representative immunoblots were shown in Figure 3. RA slightly enhanced the TGF-β1-induced increase of tTG level in both cell lines. DEX also enhanced the TGF-β1-induced tTG level in McA-RH8994 cells, while it decreased the TGF-β1-induced tTG induction in McA-RH7777 cells. However, when cells were exposed simultaneously to DEX and RA, a significant enhancement of tTG induction was observed. Using densitometric scanning the intensity of immunoreactivity on the immunoblots, a greater than 50-fold increase of tTG

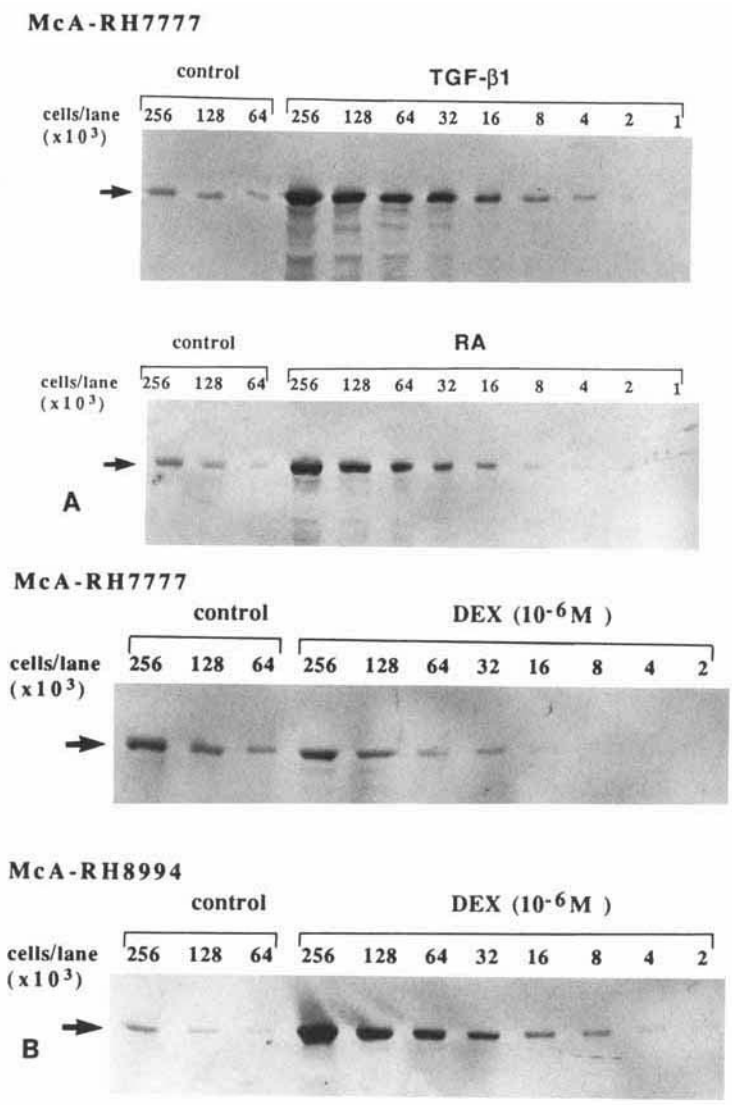

Fig. 2. **A:** Semiquantitati inoreactive bands. The 80 McA-RH7777 cells treated with TGF-β1 or retinoic acid (RA). Protein samples from cells cultured for 72 hr in the absence or presence of TGF-β1 (5 ng/ml) or RA (10 μM) were serially diluted and analyzed by immunoblot using MoAb TG100. Antigen amounts relative to that of control culture were determined kDa tTG band is identified by arrow mark. **B:** Immunoblots demonstrating the relative tTG levels in McA-RH7777 and 8994 cells treated with 10^{-6} M of dexamethasone for 72 h. Serially diluted samples were analyzed as indicated above.

level was observed in the cells that were simultaneously treated with DEX and RA (Fig. 3). The synergistic effect by DEX and RA was further confirmed by analyzing serially diluted samples (Fig. 4) and cells treated with various concentrations of each agent (Fig. 5). As shown in Figure 5, DEX did not increase the tTG level at concentrations between 10^{-8} to 10^{-5} M and did not enhance the TGF-β1-induced tTG induction in McA-RH7777 cells. TGF-β1 and RA showed additive effects on tTG levels, while DEX remarkably enhanced the RA-induced tTG increase by dose-dependent manner, indicating the synergistic effect on the tTG induction. These results are summarized in Table I. Several smaller bands, recognizable by the MoAb on the immunoblots shown in Figures 1–5, represent proteolytic degradation of tTG polypeptides. This notion was proved by the following findings: (1) the appearance of the smaller immunoreactive bands is correlated with the intensity of the 80-kDa tTG bands; (2) the molecular weight of the smaller fragments detected by the MoAb is similar among the immunoblots, indicating limited proteolysis at specific sites of the tTG polypeptides; and (3) the bands cannot be due to nonspecific binding to unrelated proteins, because immunoreactive bands were not observed in the high-molecular-

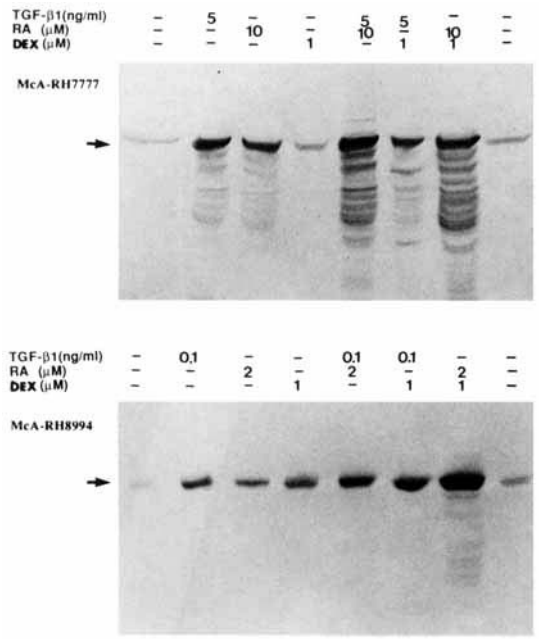

Fig. 3. Synergistic or additive effects by TGF-β1, RA, and DEX on the cytosolic tTG levels in hepatoma cells. McA-RH7777 and McA-RH8994 cells were cultured in the absence or presence of TGF-β1, RA, DEX, or combinations of these reagents at indicated concentrations for 72 h. Total cell extracts from 2×10^5 cells were applied in each lane and analyzed by 9% SDS–PAGE followed by immunoblot analysis using MoAb TG. The 80-kDa tTG band is identified by arrow mark.

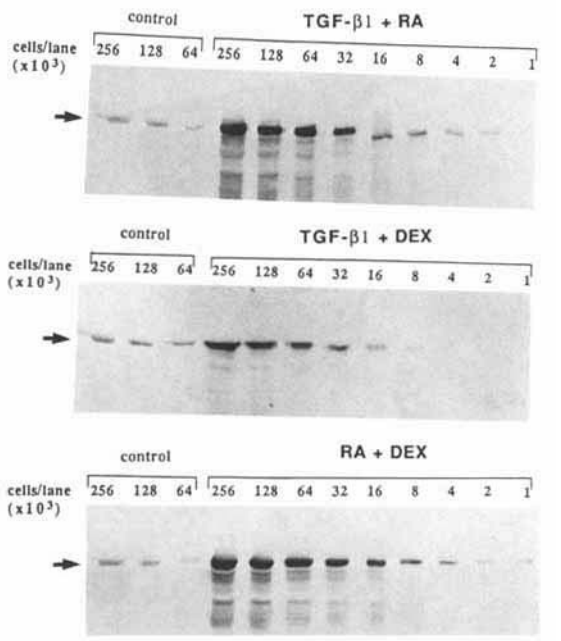

Fig. 4. Semiquantitative analysis of the synergistic or additive effects of TGF-β1, RA, and DEX on the cytosolic tTG induction in McA-RH7777 cells. Protein samples from cells cultured for 72 h in the presence of two of three reagents were serially diluted and analyzed by immunoblot using MoAb TG100. The dosages of each reagents are same as Figure 3.

weight region larger than 80 kDa. The existence of these proteolytic fragments, in spite of rapid disruption of cells in SDS-sample buffer, indicates that tTG polypeptides are constantly degraded in the cells probably due to rapid turnover of the enzyme.

Northern Blot Analysis

Expression levels of tTG mRNA were analyzed by Northern blot hybridization using ^{32}P-labeled mouse tTG cDNA (pmTG700) as a probe. Figure 6 shows the levels of tTG transcript in culture cells which were incubated for 48 h in the absence or presence of TGF-β1, RA, or DEX. Transcript levels of tTG were maintained at low level in both cell lines when cultured in 15% FBS-containing control medium. Treatment with TGF-β1 or RA resulted in the marked increase of tTG transcript in both cell lines. DEX slightly increased the transcript level in McA-RH8994 cells but not in McA-RH7777 cells.

Effects of TGF-β1, RA, and Dex on the Growth Rate and Induction of Cell Death

To investigate whether tTG induction is closely associated with growth rate, we exam-

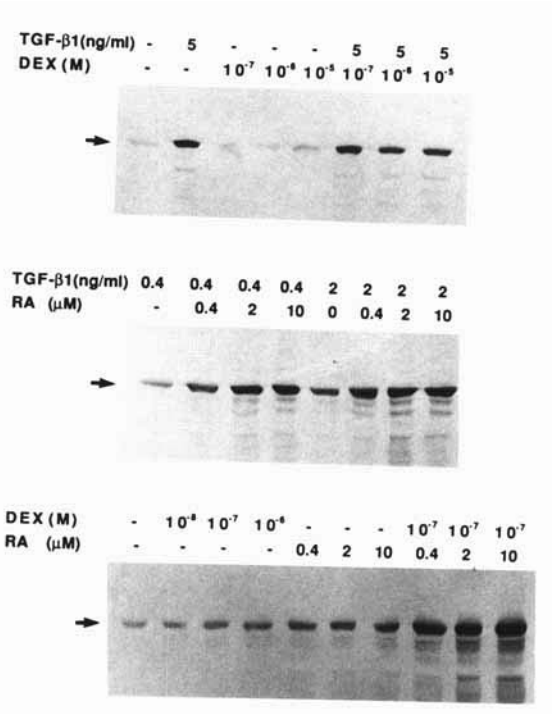

Fig. 5. Synergistic or additive effects of TGF-β1, RA, or DEX at various concentrations on the cytosolic tTG levels in McA-RH7777 cells. The 80-kDa tTG band is identified by arrow mark.

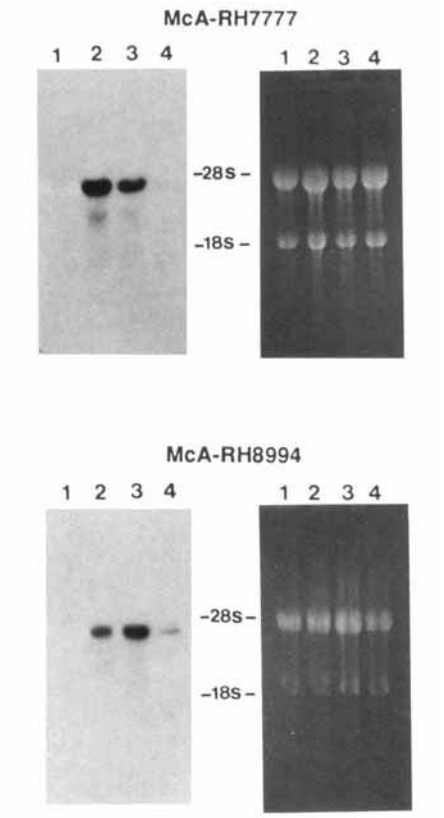

Fig. 6. Northern blot analysis showing the effect of TGF-β1, RA, and DEX on the mRNA levels of tTG. Twenty μg of total RNAs isolated from monolayer cells cultured for 48 h in control medium containing 15% FBS **(lane 1)**, or in the presence of TGF-β1 **(lane 2)**, RA **(lane 3)**, or DEX **(lane 4)** were separated by 1.2% agarose–formaldehyde gel electrophoresis followed by Northern blot hybridization using ^{32}P-labeled mouse tTG cDNA (pm TG700) as a probe. The dosages of each reagents are same as Figure 1. Autoradiographs of Northern blot analysis showing the expression levels of cytoplasmic tTG mRNA in McA-RH7777 and McA-RH8994 cells are demonstrated on the left. Photographs of ethidium bromide-stained gel prior to transfer of RNAs to the nylon membrane are demonstrated on the right to show even loading and the positions of ribosomal RNAs.

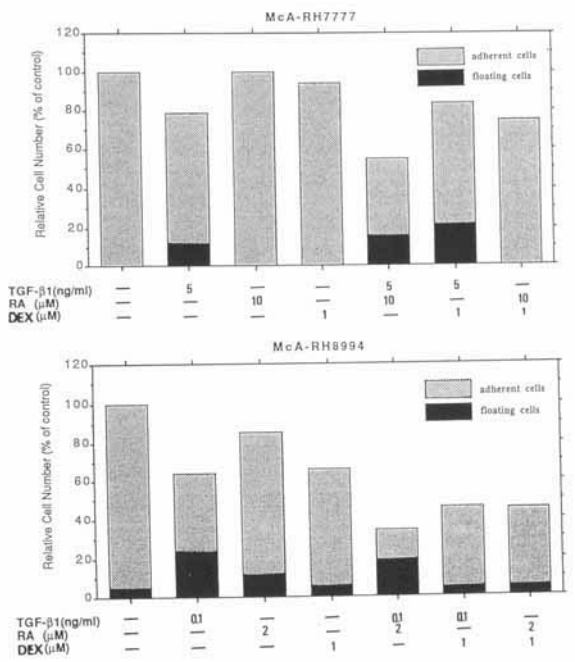

Fig. 7. Effects of TGF-β1, RA, and DEX on the growth rate and induction of cell death. Hepatoma cells were cultured in plastic culture dishes and treated with TGF-β1, RA, DEX or combinations of either two of these reagents at indicated concentrations for 72 h. Floating and adherent cells were separately counted. Total cell number of control culture was set at 100%. Adherent and floating cells are indicated by stippled and black shading columns, respectively. Values in this figure represent the average of 4 culture dishes (bars; ±SD). Asterisks indicate statistically significant ($P < 0.05$) decrease of growth rate compared to the control.

ined the effects of these reagents on cell proliferation. Adherent and floating cells were separately counted after the 72-h treatment and summarized in Figure 7. Floating cells were shown to be dying or dead cells by analyzing the morphology and the existence of extensive DNA cleavage. When McA-RH8994 cells were exposed to TGF-β1 or DEX, growth rate of the cells were suppressed; growth rate was reduced to 60–70% of the control culture at concentrations indicated in Figure 7. TGF-β1 showed a cytocidal effect on these hepatoma cells. When the McA-RH8994 cells were cultured for 72 h in the presence of 0.1 ng/ml of TGF-β1, around 20% of the culture cells detached from the substrate. McA-RH7777 cells appear to be rather resistant to growth regulation of these reagents when compared to McA-RH8994 cells. Although TGF-β1 suppressed the proliferation and induced cell death, the effective dosage in McA-RH7777 cells was much higher than that of McA-RH8994 cells: the growth rate was reduced to around 80% of the control culture and 10% of the total cell number appeared as floating cells (indicated by black shading column in Fig. 7). RA did not show statistically significant growth suppressive effects on both cell lines at concentrations indicated in Figure 7. RA enhanced the proliferative arrest effect of TGF-β1 in both cell lines. On the contrary, DEX decreased the cytocidal effect of TGF-β1 in McA-RH8994 cells. Floating cells decreased to less than 5% of the total cells in the culture at 72 h after the treatment with 0.1 ng/ml of TGF-β1 in the presence of 10^{-6} M DEX. However, DEX did not suppress but rather enhance the cytocidal effect of TGF-β1 in McA-RH7777 cells. Results described above were also summarized in Table I.

TABLE I. Summary of Semiquantitative Analysis of the Relative Levels of tTG, the Percentages of Growth Inhibition, and Floating Cells After Treatment of Hepatoma Cells With TGF-β1, Retinoic Acid (RA), Dexamethasone (DEX), or Either Reagent[a]

	McA-RH7777			McA-RH8994		
Reagents	Max. tTG induction	Growth inhibition	% Floating cells	Max. tTG induction	Growth inhibition	% Floating cells
None	1	0	0	1	0	5
TGF-β1	24	20	10	24	35	20
RA	12	0	0	12	15	10
DEX	1	10	0	18	33	6
TGF-β1 + RA	40	45	18	36	65	18
TGF-β1 + DEX	12	20	20	24	32	5
RA + DEX	64	27	0	96	35	5

[a]For McA-RH7777 cells, 5 ng/ml of TGF-β1, 10 μM of RA or 10^{-6} M of DEX were used as stimulating dosages. For McA-RH8994 cells, 0.1 ng/ml of TGF-β1, 2 μM of RA or 10^{-6} M of DEX were used. Culture cells were treated for 72 h, and total cell extracts were prepared directly dissolving the cells in SDS sample buffer at concentrations of 5×10^6/ml. The amounts of tTG relative to the control culture were determined by comparing the intensity of immunologically detected bands on immunoblot analysis of 1:1 serially diluted samples as shown in Fig. 2 and 4. The relative amount of tTG in cells grown in control medium were set at 1. The data shown were determined from two separate immunoblots. The details of the experiments on the effects of TGF-β1, RA and DEX on the growth rate and induction of cell death were described in Figure 7. The percentages presented in this table represent the average of 4 culture dishes.

DISCUSSION

While evidence is accumulating that there is a close correlation between tTG activity and cell proliferation, differentiation or cell death, the physiological function of this enzyme remains unclear [Fesus and Thomazy, 1988]. According to the appealing theory of Birckbichler et al. [1981], tTG-mediated isopeptide crosslinks in proteins are maintained at low levels under proliferating conditions and tTG concentration and activity are increased as cells cease to proliferate and stabilize as the result of increased crosslinkage. Recent studies indeed have shown that a reduced tTG activity is associated with cell proliferation and tumor promotion. An inverse relationship existing between the enzyme expression and the metastatic potential of various cell lines was also demonstrated [Birckbichler et al., 1976; Barnes et al., 1985; Knight et al., 1990, 1991]. It seems quite likely that cross-linking of cellular proteins and/or post-translational modification via acyl-transfer reaction by tTG may change the functional states of proteins [Fesus et al., 1985; Zatloukal et al., 1989; Hand et al., 1990; Cordella-Miele et al., 1990; Ando et al., 1991; Aeschlimann and Paulsson, 1991; Borth et al., 1991; Barsigian et al., 1991] and therefore influence cellular behavior such as growth, differentiation, and cell death [Birckbichler et al., 1981; Kannagi et al., 1982; Hsu and Friedman, 1983; Maccioni and Seeds, 1986; Davies et al., 1988; Suedhoff et al., 1990]. However, accumulated data do not entirely support this theory. Although the induction of tTG parallel the initiation of cell differentiation in a number of systems [Hsu and Friedman, 1983; Davies et al., 1988; Suedhoff et al., 1990; Davies et al., 1985; Jetten et al., 1986], there is no evidence of the direct and essential involvement of tTG action in this process. In addition, there are compounds that are able to induce the differentiation processes but do not induce tTG expression [Davies et al., 1985; Goldman, 1985]. This indicates the uncoupling of tTG induction and differentiation processes. Furthermore, increased TG activity observed in lymphocytes after treating cells with mitogens in vitro [Novogrodsky et al., 1978] suggests that the enzyme may have a function during the early phase of growth stimulation.

The levels of tTG appear to be closely correlated to growth regulation induced by either TGF-β1 or DEX in the rat hepatoma McA-RH7777 cells used in our studies. While RA treatment increased tTG level it did not suppress the growth in these hepatoma cells. Besides the above treatment, the reagents that stimulate the growth of hepatoma cells such as TGF-α and HGF appear to have no effect or rather show suppressive effects on the tTG induction (Fig. 1). Serum starved condition suppressed the proliferation of both hepatoma cell lines. When the cells were cultured for 72 h in serum-starved condition, relative cell number to the control culture in 15% FBS-containing me-

dium were about 75% and 35% in McA-RH7777 and McA-RH8994 cells, respectively. Serum deprivation, however, did not increase but rather suppressed the cytosolic tTG level (Fig. 1). Cellular growth is regulated or modulated by multiple pathways and tTG induction may be linked to complicated pathways in the growth regulation. Therefore, elucidation of regulatory mechanisms of tTG induction and its relation to various cellular phenomena should provide new clues for the understanding of some aspects of differentiation processes and growth regulation in hepatoma cells.

The levels of tTG in the McA-RH8994 cell line correlates with the growth suppression induced by TGF-β1 and DEX. However, McA-RH7777 cells appear to be altered in the DEX-mediated processes regarding tTG induction, growth regulation, and the preventive effects to TGF-β1-induced cell death processes when compared to the responses in McA-8994 cells. RA enhanced tTG expression without inducing growth suppression in both cell lines. This suggests that the induction processes of tTG by RA may not be linked to the growth inhibitory pathways in these cell lines.

Synergistic effect of DEX and RA on the tTG induction is also reported in transformed mouse epidermal cell line PAM212, in which 1α-25-dihydroxyvitamine D_3 and RA caused remarkable synergistic effect [Lee et al., 1989]. RA and DEX also exhibited synergistic effects on the induction of tTG in hepatoma McA-RH7777 and McA-RH8994 cell lines, and both reagents appeared to affect the TGF-β1-mediated tTG induction in an additive manner. However, the former enhanced the cell death process induced by TGF-β1 in both cell lines and the latter suppressed the process in McA-RH8994 cells but not in McA-RH7777 cells. This indicates that RA and DEX have different effects on the process of cell death and that the cell death mechanism induced by TGF-β1 appears to be independent of the tTG induction process. Enhanced TG activity by DEX has been reported in the various myeloid leukemic cell lines of murine and human origin [Goldman, 1985, 1987]. However, DEX was shown to inhibit the DMSO-mediated tTG induction in Friend erythroleukemia cell line GM979 [Hsu and Friedman, 1984]. Stimulation of tTG expression by RA has been well documented in mouse macrophages [Moore et al., 1984; Chiocca et al., 1988] and human promyelocytic leukemia HL60 cells [Davies et al., 1985]. However, Goldman [1987] reported that RA suppressed TG activity in some murine leukemia cell lines, suggesting that the effect of RA on tTG expression is different depending on the cell lines and species specificity. The suppressive effect of RA on tTG expression was also reported in human epidermal carcinoma cells [Thacher et al., 1985]. These data indicate that modulation of TG activity by these agents occur via disparate mechanisms. Alternatively, these conflicting results may reflect the diverging alterations in the regulatory mechanisms and/or signal transduction processes in neoplastic cells, depending on the cell line [Rubin and Rice, 1986].

TGF-β1 is a potent inhibitor of hepatocyte proliferation in vivo and in vitro [Nakamura et al., 1985; Carr et al., 1986; Fausto et al., 1991]. TGF-β1 has recently been shown to induce cell death in several cell types including liver and hepatoma cells [Rotello et al., 1991; Oberhammer et al., 1991; Lin and Chou, 1992]. Although there are several reports indicating that malignant liver cells are resistant to growth inhibition by TGF-β1 [Houck et al., 1989; Huggett et al., 1991], we have demonstrated in the present studies that TGF-β1 is able to induce growth suppression and cell death in McA-RH7777 and McA-RH8994 cells. The observation that DEX can suppress the cytocidal effect of TGF-β1 in McA-RH8994 cells is especially intriguing, since DEX itself can induce apoptosis in some cell types such as thymocytes [Wyllie, 1980; Cohen and Duke, 1984]. We propose that DEX-induced phenotypes may be responsible for the resistance to cytolysis induced by TGF-β1 in McA-RH8994 cells and that this process is altered in McA-RH7777 cells. This suggests that cytocidal effects of TGF-β1 may involve pathways which are counter regulated by DEX in rat hepatoma cells.

McA-RH8994 cells were shown to be highly sensitive to the cytocidal effects of TGF-β1. We have demonstrated that the levels of tTG induced by simultaneous addition of RA and DEX was greater than TGF-β1-treatment alone. However, this treatment did not induce cell death, suggesting that tTG induction is not an invariable prerequisite of cell death. Possible participation of tTG induction during programmed cell death has been suggested in liver cells and several cancer cell lines [Fesus et al., 1987; Piacentini et al., 1991]; however, Suedhoff et al. [1990] concluded that tTG induction in human erythroleukemia cells by RA was not related to cell

apoptosis. The mechanism by which DEX inhibits the TGF-β1-induced cell death is intriguing. This relationship is also important for the evaluation of TGF-β1-effects, since various concentrations of DEX are frequently used as a supplement of culture media for liver cells to maintain the differentiated states.

Our observations indicate that tTG induction is not the direct cause of the growth inhibition and cell death in hepatoma cells although the biochemical pathways involved in the induction of tTG appear to be closely coupled to some aspects of the growth inhibitory processes, and a contributory role of this enzyme to the processes of growth suppression and cell death in certain cell lines may exist. Tissue TG induction appears to be readily modulated via different signals. Although the increase of tTG levels was shown to be, at least in part, due to an increase of the transcript, the detailed molecular mechanisms regulating the tTG expression remains to be clarified. Elucidation of the functions of tTG and the mechanisms determining the levels of tTG may provide clues for the elucidation of regulatory mechanisms of growth and differentiation in hepatoma cells.

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