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Cell Cycle-dependent Changes in Tissue Transglutaminase mRNA Levels in Bovine Endothelial Cells

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SUMMARY: The pattern of transglutaminase gene expression through the cell cycle was examined by Northern blot analysis using cultured bovine endothelial cells and a cDNA probe. When the cells reached confluency or were arrested in G_0/G_1 phase by nutrition deprivation, transglutaminase mRNA rose to a very high level; S- and M-phase extracts showed high and low levels, respectively. Subcellular localization studies by sucrose gradient centrifugation and immunostaining demonstrated that the majority of transglutaminase is present in cytosols throughout the cycle. The cell cycle-dependent changes in the transglutaminase mRNA levels strongly support the implicated involvement of the enzyme in cell growth, differentiation, and senescence.

Transglutaminase (EC 2.3.2.13) is an enzyme that catalyzes the covalent incorporation of polyamines into proteins and formation of glutamine-lysine cross-links between proteins (1-3). Various forms of transglutaminase with different physiological roles have been demonstrated in a wide variety of tissues and body fluid and classified into two groups: extracellular and intracellular transglutaminases; the latter is called tissue transglutaminase. Although exact physiological functions of tissue transglutaminases, except keratinocyte (4) and hair follicle (5) transglutaminase, are unclear, its ubiquitous distribution suggests that they are involved in certain fundamental cellular processes. Evidence suggesting that transglutaminase activities are associated with cell growth (6-8), differentiation (9,10), and malignant transformation (6,11) is currently being accumulated.

In the present study, using a transglutaminase cDNA previously cloned from bovine cultured vascular endothelial cells (12), we examined changes in transglutaminase mRNA levels during cell cycle and found that cells arrested in a resting phase by nutritional deprivation or contact inhibition possessed dramatically increased levels of the mRNA than actively proliferating counterparts; S phase cells also exhibited increased levels of the transcript.

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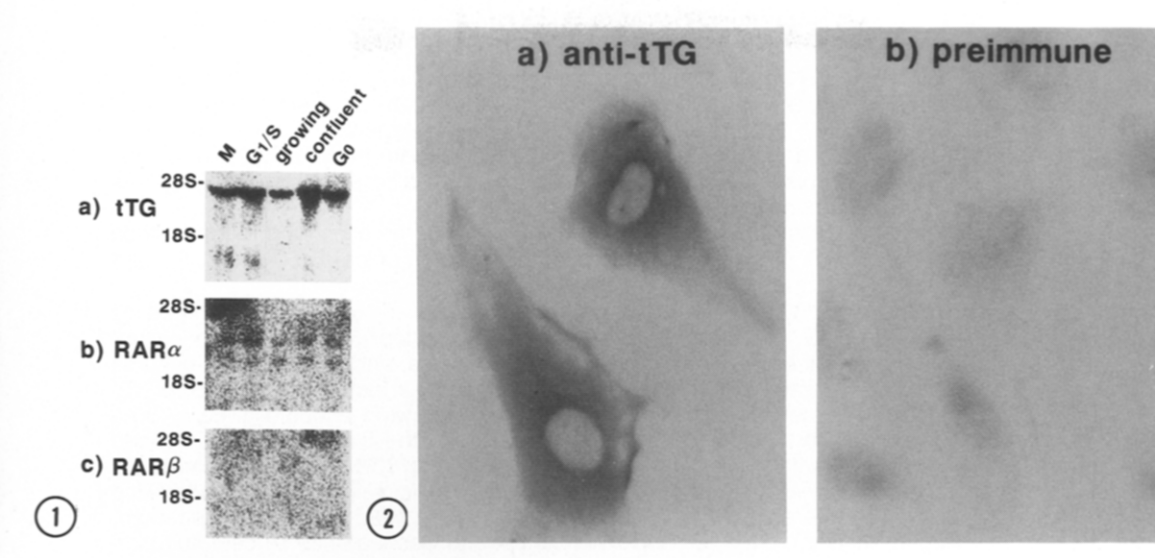

Fig. 1. **Cell cycle-dependent changes in transglutaminase mRNA levels in bovine cultured endothelial cells.** Each lane contains 20 μg of total RNA. Actin mRNA levels also showed significant variation through the cell cycle (with a maximum level at the M phase, not shown); therefore, its levels could not be used to normalize the amounts of RNA applied onto each lane. Northern blot analysis was carried out using a) transglutaminase (tTG), b) retinoic acid receptor (RAR) α, and c) RARβ cDNA probes.

Fig. 2. **Cytoplasmic localization of transglutaminase demonstrated by immunostaining.** Cultured endothelial cells were stained with an antiserum to transglutaminase (anti-tTG, *left*) and a control serum (preimmune, *right*), and the location of transglutaminase was visualized by the avidin-biotin-peroxidase complex method.

aphidicolin-treated, and TN-16-treated cells and hybridized with the 436-bp *Hin*fI fragment containing a central part of the transglutaminase coding sequence where the active site cysteine resides (12). Aphidicolin, a potent and specific inhibitor of DNA polymerase, was used to arrest the cell cycle in the S phase; TN-16, like colchicine, halts the division of cells thereby causing the random population to accumulate at the M phase. The cDNA probe hybridized to a 3.9-kb mRNA species, whose amount varied according to the cell cycle (Fig. 1a). The highest levels of transglutaminase expression was observed in the resting cells, with high and low levels seen in the G_1/S and M phase, respectively. This result suggests that the transglutaminase message rises sharply during G_1, attains a maximal level at S, and then drops rapidly at the G_2/M boundary. Retinoic acid receptor (RAR) α mRNA levels paralleled with the cycle-dependent changes in the transglutaminase transcripts (Fig. 1b) as expected from the earlier observations that transglutaminase gene expression is under control of retinoic acid (17); RARβ mRNA was not detected (Fig. 1c).

The induction of transglutaminase in the S phase and the presence of nuclear targeting-like sequence (RRWKR---RVK, residues 262-273) in the tissue-type transglutaminases (12) prompted us to examine its nuclear localization. Nuclei were isolated from cultured endothelial cells by density gradient centrifugation and assayed for transglutaminase activity. Contrary to our expectations and the reports from other laboratories (18,19), only very low levels of the activity were detected in the nuclei even in the S phase: total

MATERIALS and METHODS

Materials--Aphidicolin and 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione (TN-16) were obtained from Wako; ^{32}P-labeled nucleotides were from Amersham; a random primer DNA labeling kit was from Takara; nylon membranes were from Stratagene. Eagle's minimum essential medium (MEM) and antibiotics (penicillin-streptomycin) were from Gibco; fetal bovine serum (FBS) was from Mitsubishi Chemical Industries. Retinoic acid receptor (RAR) cDNAs were a gift from Dr. P. Chambon.

Cell Cultures--Endothelial cells of bovine carotid artery were cultured according to the method of Hagiwara *et al.* (13) at 37°C in MEM containing 10% FBS, penicillin (50 units/ml), and streptomycin (50 μg/ml) in a humidified 5% CO_2/95% air atmosphere.

Synchronization of Cultured Cells--Synchrony of the cell cycle was chemically induced as follows: 1) Cells in the G_0/G_1 phase were obtained by serum deprivation from the culture medium, namely bovine endothelial cells were grown to confluence, and the confluent cultures were placed and maintained for 2 days in serum-free medium to arrest the cells in G_0/G_1; 2) synchronization in the M phase was achieved by treating the growing endothelial cells (80% confluent) with 1 μM TN-16 for 8 h; and 3) synchronous cells in the S phase were produced by blocking DNA replication with aphidicolin (1 μg/ml; 20 h) using 80%-confluent cultures.

Preparation of Cells for Northern Analysis--Cells cultured on plastic dishes were washed twice with ice-cold phosphate-buffered saline (PBS), scraped lightly with a rubber policeman, suspended with PBS (1 ml/dish), collected into 15-ml tubes, and washed once by centrifugation at 700 X *g*. The pellets were stored at -80°C in aliquots corresponding to cell numbers from 10 dishes (about 8 X 10^6 cells).

Preparation of ^{32}P-labeled cDNA probes--A probe consisting of the 0.44-kb *Hin*fI restriction fragment of transglutaminase cDNA (12), a 137-bp *Alu*I/*Msc*I restriction fragment of mouse RARα cDNA (14), and a 138-bp *Fok*I/*Taq*I fragment of RARβ cDNA (14) were purified by gel-permeation chromatography and labeled by random priming.

Isolation and Northern Blot Analysis of RNA--Total RNA was isolated from cultured bovine endothelial cells by the acid guanidinium thiocyanate-phenol-chloroform method (15). For Northern analysis, the RNA was size-fractionated by electrophoresis on a 1% agarose/2.2 M formaldehyde gel, transferred to a nylon membrane, and cross-linked to the membrane by UV irradiation. The blots were hybridized with a random primer-labeled transglutaminase cDNA probe (12) by the standard procedure. Briefly, the membrane was prehybridized for 2 h in 50% formamide/5 X SSPE/2 X Denhardt's/1% SDS containing 100 μg/ml sonicated herring sperm DNA. 5 X SSPE was 0.9 M NaCl, 50 mM sodium phosphate (pH 7.7), 5 mM EDTA. Hybridization was carried out for 20 h at 42°C in the same buffer containing labeled probe. The filters were washed in 2 X SSC/0.1% SDS for 5 min at room temperature, then twice in 1 X SSC/0.1% SDS for 1 h at 55°C. 2 X SSC was 0.3 M NaCl, 0.03 M sodium citrate (pH 7.0). For reprobing with mouse RAR cDNAs, the filters were stripped by washing with 0.2 N NaOH for 1 h at room temperature.

Isolation of Nuclei--Nuclei were isolated according to the method of Blobel and Potter (16). Cultured endothelial cells (25 dishes) were washed with 10 mM Hepes-buffered saline, suspended in 1 ml of 0.25 M sucrose containing 50 mM Tris-HCl (pH 7.5), 25 mM KCl, and 5 mM $MgCl_2$, and homogenized with a teflon homogenizer. Sucrose concentration was adjusted to 1.6 M by adding 2 volumes of 2.3 M sucrose containing 50 mM Tris-HCl, 25 mM KCl, and 5 mM $MgCl_2$ and the sample (3 ml) was layered onto the 2.3 M sucrose solution (6 ml) and centrifuged at 30,000 rpm and 4°C for 2 h in a Beckman SW41Ti rotor. Nuclei were recovered as precipitates, treated with 0.2% Triton X-100 in the 0.25 M sucrose solution, and assayed for transglutaminase activity as described previously (17).

Immunostaining--Endothelial cells were grown on a slide glass, fixed with 2.5% glutaraldehyde in PBS, treated with 1.5% H_2O_2 in methanol, and stained with anti-transglutaminase antiserum (17) at a 1:1000 dilution using a Vectastain *elite* ABC kit.

RESULTS and DISCUSSION

The pattern of transglutaminase gene expression during cell cycle was studied by Northern blot analysis using bovine cultured endothelial cells. To determine the relative amount of transglutaminase mRNA in cells in different stages of cell cycle, total RNA was prepared from proliferating (50% confluent), confluent, nutrition-deprived quiescent,

nuclear activity (0.35 nmol of putrescine incorporated/h) *versus* total cytoplasmic activity (43.9 nmol/h); on the protein basis, 2.0 nmol/h/mg of nuclear protein *versus* 32.0 nmol/h/mg of cytoplasmic protein. This low level was not due to the possible loss of the enzyme from the nuclei during homogenization and centrifugation since immunostaining of the cultured endothelial cells with an anti-transglutaminase antiserum clearly demonstrated that the majority of the enzymes were present in the cytosol (Fig. 2). Fig. 2 was obtained using S phase cells; cells in other phases of the cell cycle also produced similar staining patterns, indicating that the majority of the tissue-type transglutaminase remains in the cytosol throughout the cell cycle.

In the present study, we demonstrated that the levels of tissue transglutaminase mRNA in cultured bovine endothelial cells vary according to the cell cycle. The high levels observed in G_0/G_1 phase are consistent with the earlier report by Korner *et al.* (20) that transglutaminase activities increase in a resting stage. The surge at S phase is an interesting new finding. The observed cell cycle-dependent changes may prove to be a useful clue in continued efforts to unravel the exact physiological role of tissue transglutaminase.

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