Retinol-induced Morphological Changes of Cultured Bovine Endothelial Cells Are Accompanied by a Marked Increase in Transglutaminase*

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Retinol, a morphogen, has been shown to induce morphological changes in vascular endothelial cells, accompanied by an acute and specific accumulation of an 80-kDa protein; purification and characterization of this retinol-induced protein (RIP) have revealed that it is a transglutaminase. Endothelial cells from bovine carotid artery were cultured, treated with retinol, and examined for changes in morphology and protein profiles. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of extracts prepared from retinol-treated cells which had undergone a remarkable change in shape (from a cobblestone-like to a spindle-like shape) indicated that the retinol-induced morphological change is accompanied by a marked increase of an 80-kDa protein. Similar changes were also induced by retinoic acid. The 80-kDa RIP was purified by anion exchange and hydroxyapatite column chromatography. Amino acid sequencing of tryptic fragments of the purified RIP revealed a high degree of homology between the sequence of bovine RIP and that of guinea pig liver transglutaminase, suggesting that RIP is a transglutaminase. This was confirmed by activity measurements; RIP exhibited transglutaminase activity, and an antiserum against RIP immunoprecipitated the activity. These results suggest that transglutaminase plays important roles in the maintenance of morphology and the control of endothelial cell functions.

Retinol (vitamin A) and its derivatives (retinoids) have a wide range of developmental effects; they are essential for normal mammalian morphogenesis, including bone, eye, and epithelial development (1-12). Retinoids are also required for normal growth and differentiation of a variety of cells. Furthermore, they can exert potent effects on the differentiation of certain malignant tumor cells (13-18). The ability of retinoids to alter fundamental processes in cell differentiation and proliferation has attracted widespread attention, and their molecular mechanisms of action are presently the subject of active investigation (19). We have recently demonstrated that retinol can cause a morphological alteration of cultured bovine endothelial cells from a cobblestone-like shape to a spindle-like shape. This change was accompanied by a remarkable accumulation of an 80-kDa protein to a level that is visible on Coomassie-stained SDS-polyacrylamide gels. In addition to the accumulation of tremendous amounts of the 80-kDa protein, retinol stimulated the synthesis and secretion of plasminogen activator and the phosphorylation of certain proteins (20).

In the present study, as a first step in elucidation of the mechanisms of retinol-induced morphological and functional changes in endothelial cells, we isolated the 80-kDa RIP, named RIP, and raised an antiserum against it. Immunochromatographic and biochemical characterization of RIP demonstrated it to be a transglutaminase. Other examples of retinoid induction of transglutaminase have been identified in macrophages (21, 22) and other myeloid cells (23, 24); however, the level of enzyme accumulation in endothelial cells was much higher than in other cells, making the endothelial cell an ideal system for study of the mechanisms of retinoid action.

**Experimental Procedures**

Materials—[3H]Putrescine dihydrochloride (17.9 Ci/mmol) was purchased from Du Pont-New England Nuclear; putrescine, monodansylcadaverine, N,N'-dimethyl casein, all-trans-retinol, retinoic acid, retinyl palmitate, and TPCK-treated trypsin were from Sigma; Ficoll-Conrat (25); affinity-isolated goat anti-rabbit IgG was from Tago; Eagle's minimum essential medium and antibiotics (penicillin/streptomycin solution) were from Gibco; fetal bovine serum was from Mitsubishi Chemical Industries; an anion exchange column (Prepack Mono Q HR 5/5) and protein A Sepharose CL-4B were from Pharmacia LKB Biotechnology Inc. A hydroxyapatite column (Cica-Merck, Hiber RT 100-8 Hydroxyapatite-PM) was from Kanto Chemicals. A reverse-phase HPLC column (Waters μBondapak C18, 3.9 mm × 15 cm) was from Millipore.

Cell Culture and Cytosol Preparation—Endothelial cells of carotid artery were obtained from adult bovines and were cultured at 37°C in Eagle's minimum essential medium containing 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). The present study was performed using cells which had been passaged 7-24 times (8-25 generations). Retinol was dissolved in ethanol and added to the culture medium for a final concentration of 10 μM. After 2 days incubation in the presence of retinol, the cells were scraped and washed three times with cold washing buffer (10 mM Hepes, pH 7.5, containing 150 mM NaCl, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride). The washed cells were homogenized in cold homogenization buffer (10 mM Hepes, pH 7.5, containing 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride) with a Teflon homogenizer on ice. The homogenates were centrifuged at 100,000 × g for 1 h at 4°C.

* This work was supported by grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, Chichibu Cement, and the Mombusho International Scientific Research Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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resulting supernatant (cytosol) was stored at −40 °C until use. The cytosol (1.2 ml) contained approximately 2 mg of protein.

Preparation of Antigen by Preparative SDS-PAGE and Immunization—The cytosol was mixed with Laemmli sample buffer containing 1% β-mercaptoethanol, heated to 60 °C for 10 min, and electrophoresed on a 2-mm thick 7.5% polyacrylamide slab gel containing 0.1% agarose. The 80-kDa band of RIPC, located by Coomassie Brilliant Blue staining of the gel, was excised from the stained gel with a razor blade. The gel slice containing RIP (40 μg) was homogenized and emulsified with an equal volume of Freund's complete adjuvant. After 1 week the emulsion was aliquotted and stored at −40 °C.

Brilliant Blue staining of the gel, was excised from the stained gel and the insoluble clot was removed by centrifugation. The resulting serum was aliquotted and stored at −40 °C.

SDS-PAGE and Immunoblot Analysis—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (26), using a 3.5% stacking gel and a 6.0 or 7.5% resolving gel. Gels were fixed with methanol/acetic acid and stained with Coomassie Brilliant Blue. Immunoblotting was used to establish the specificity of the antiserum. After SDS-PAGE of the cytosol of endothelial cells, the polypeptides present in the gels were electrophoretically transferred onto nitrocellulose filters and were saturated with nonfat dry milk in 20 mM Tris, pH 7.5, 137 mM NaCl, and 0.1% Tween 20 (TBS/Tween) for 1 h at room temperature. The filters were then incubated overnight at 4 °C with a 1:2500 dilution of antiserum to RIP in TBS/Tween. After washing, the filters were incubated with rabbit anti-rabbit IgG at a dilution of 1:5000 in TBS/Tween. After washing four times with TBS/Tween, the filters were incubated for 4 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG at a dilution of 1:5000 in TBS/Tween. After a thorough washing, the filters were developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in alkaline phosphatase assay buffer (100 mM Tris, pH 9.5, containing 100 mM NaCl and 5 mM MgCl2).

Dot Blot Analysis—Aliquots (5 μl) of fractions obtained by chromatography were spotted onto nitrocellulose filters and examined for the presence of RIP in a similar manner as above.

Transglutaminase Assay—Transglutaminase activity was measured by two different methods. In the first, activity was determined by fluorometric measurement of the Ca2+-dependent incorporation of monodansylcadaverine into acetylated casein (27). This reaction was carried out at 37 °C in 2 ml of buffer containing 50 mM Hepes, pH 7.5, 5 mM dithiothreitol, 5.5 mM CaCl2, 6.25 μM monodansylcadaverine, 0.2% acetylated casein, and the sample. The fluorescence intensity was measured with a Hitachi fluorescence spectrophotometer model 850, using excitation and emission wavelengths of 350 and 480 nm, respectively. In the second method, transglutaminase activity was assayed as the Ca2+-dependent incorporation of [3H]putrescine into dimethyl casein (28). The reaction mixture consisted of 50 mM Hepes, pH 7.5, 20% (v/v) glycerol, 1 mg/ml N,N′-dimethyl casein, 250 μM putrescine, 1 μCi of [3H]putrescine, 20 mM dithiothreitol, 1 mM calcium chloride, and the sample, in a final volume of 100 μl. The reaction mixtures were incubated for 1 h at 37 °C. Reactions were stopped by addition of 0.9 ml of 50% trichloroacetic acid. The sediments were collected on glass filters and washed three times with 3 ml of 50% trichloroacetic acid. Radioactivity was measured by liquid scintillation counting. Transglutaminase activity was expressed as micromoles of putrescine incorporated into casein 1 h at 37 °C.

Purification of RIP from the Cytosol of Endothelial Cells—The cytosol of endothelial cells was chromatographed on a Mono Q column which had been equilibrated with 20 mM triethanolamine, pH 7.5. The column was developed with a linear gradient from 0.2 to 0.35 M NaCl at a flow rate of 0.8 ml/min, using a Pharmacia FPLC system. The column eluates were monitored at 280 nm. Fractions containing RIP were identified by dot blot analysis using anti-RIP antibody, and were assayed for transglutaminase activity. The fractions containing transglutaminase activity were pooled and applied to a tritiatedfluorescence spectrum of 116- and 97-peak columns, respectively. The bound proteins were eluted with a linear gradient between 10 and 100 mM phosphate buffer, pH 6.8, containing 0.05% NaN3 and 0.05 mM CaCl2. The bound proteins were eluted with a linear gradient between 10 and 100 mM phosphate buffer, pH 6.8, containing 0.05% NaN3 and 0.05 mM CaCl2. The bound proteins were eluted with a linear gradient between 10 and 100 mM phosphate buffer, pH 6.8, containing 0.05% NaN3 and 0.05 mM CaCl2. The bound proteins were eluted with a linear gradient between 10 and 100 mM phosphate buffer, pH 6.8, containing 0.05% NaN3 and 0.05 mM CaCl2.

Tryptic Digestion and Amino Acid Sequencing—Purified RIP was dialyzed against 0.1 M ammonium bicarbonate, lyophilized, and dissolved in 50 μl of 0.1 M ammonium bicarbonate. TPC-researched trypsin was added at a ratio of 1:100 (w/w). After incubation at 37 °C for 12 h, a further identical aliquot of TPCK-treated trypsin was added, and the incubation was continued for an additional 12 h. After removal of the salt by lyophilization, the digestes were dissolved in 100 μl of 0.1% trifluoroacetic acid and fractionated on a C18 reverse-phase HPLC column in a gradient from 0 to 45% CH3CN containing 0.1% trifluoroacetic acid, using a Waters 600 HPLC system. Elution was monitored at 220 nm. Amino acid sequences of the proteolytic peptide fragments isolated by HPLC were determined with an Applied Biosystems protein sequenator, model 470A/120A.

Immunoprecipitation—The cytosol of the retinol-treated endothelial cells was incubated overnight at 4 °C with rabbit preimmune or anti-RIP antibody, purified by affinity chromatography on protein A-Sepharose CL-4B, in a total volume of 275 μl. Immune complexes were precipitated by addition of 80 μl of a 50% suspension of protein A-Sepharose CL-4B. The supernatants were removed after centrifugation, and 200-μl aliquots were assayed for remaining transglutaminase activity.

Protein Assay—Protein concentration was measured as described by Bradford (29), using bovine serum albumin as the standard.

RESULTS

Morphological Change and Accumulation of RIP—Endothelial cells of bovine carotid artery were cultured, treated with 10 μM retinol for 2 days, and examined for morphology. As shown in Fig. 1A, clear morphological alterations in endothelial cells were observed after exposure to retinol. To examine whether these changes were accompanied by any change in protein profiles, we performed SDS-PAGE analyses of extracts prepared from the control and the retinol-treated cells. Fig. 1B shows the result. Specific induction of an 80-kDa protein is evident; the levels of the other major proteins that could be seen on the gel were not significantly affected.

Because the sensitivity of SDS-PAGE analysis is low even if combined with silver staining, we decided to raise an antiserum and, by using it, to determine the time course of the appearance of 80-kDa RIP and the dose-response curve. The antiserum was produced by immunizing a rabbit with the Coomassie-stained 80-kDa band after emulsification with Freund's complete adjuvant. The specificity of the antiserum was established by immunoblot analysis (Fig. 2). Fig. 3 shows the time course of the retinol-induced accumulation of RIP monitored by dot blot analysis. A significant induction occurred at 6 h and there was a progressive increase in the level of RIP, which reached a maximum after 20–30 h. Although it is difficult to quantify the morphological alterations in the endothelial cells, microscopic observations, combined with the above dot blot analysis, of the cells exposed to retinol for different lengths of time indicated that the retinol-induced...
either silver-stained transfer to a nitrocellulose filter were electrophoresed on SDS-polyacrylamide gels. The gels were specifically with RIP among many other proteins in the cell extract.

FIG. 2. Immunoblot analysis demonstrating the specificity of anti-RIP. Cytosols from retinol-treated vascular endothelial cells were electrophoresed on SDS-polyacrylamide gels. The gels were either silver-stained (lane 1) or immunostained with anti-RIP after transfer to a nitrocellulose filter (lane 2). The antisera reacted specifically with RIP among many other proteins in the cell extract.

FIG. 3. Time course of retinol-induced accumulation of 80-kDa protein (RIP) in endothelial cells. Cultured bovine carotid artery endothelial cells were treated with retinol for the indicated times and the amount of RIP accumulated was determined by dot blot analysis using anti-RIP, as described under "Experimental Procedures."

accumulation of RIP closely paralleled the morphological changes.

Fig. 4 represents a rough estimate of the dose-response relationships between the amounts of retinoids added and the amount of RIP accumulated. Of the three retinoids tested, retinol was the most potent (Fig. 4A). The threshold concentration of retinol for stimulation of RIP production was about 0.1 μM; a dose range of 2–5 μM caused a maximal response. Like retinol, retinoic acid also elicited morphological alterations in endothelial cells in parallel with the accumulation of RIP. Retinyl palmitate was ineffective in inducing either RIP (Fig. 4C) or morphological changes.

Purification of RIP—On the basis of the above observation, we considered RIP to be a key substance for understanding the actions of retinoid on endothelial cells, and we therefore decided to purify and characterize it. RIP was purified from cytosols prepared from retinol-treated bovine endothelial cells by a two-step procedure, using Mono Q (Fig. 5A) and hydroxyapatite (Fig. 5B) columns. The elution positions of RIP were identified by dot blot analysis. Starting with 5.3 ml of cell extracts containing 12.7 mg of protein, about 200 μg of a homogeneous preparation of RIP (Fig. 5C) were obtained.

Identification of RIP as a Transglutaminase—We first attempted to determine the partial amino acid sequence of RIP by sequencing its peptide fragments obtained by reverse-phase HPLC of tryptic digests of purified RIP. Three well-separated peaks, numbered 1 through 3, were sequenced (Fig. 6). A computer-assisted homology search revealed a high degree of homology between the sequence derived from the bovine RIP fragments and that of guinea pig liver transglutaminase (30). Furthermore, both proteins have a similar Mr of about 80,000. These facts appeared to indicate that RIP is a transglutaminase.

To examine this possibility further, we next measured the transglutaminase activity of RIP. It did exhibit such activity, as shown in Fig. 5A; its specific activity was calculated to be 30.8 pmol of [3H]putrescine/h/mg of protein from curve b. This value is comparable to that of guinea pig transglutaminase commercially available from Sigma. The enzyme requires Ca2+ for its activity; therefore, the eluate from the hydroxyapatite column (Fig. 5B) was not suitable for the enzyme assay, as it contained phosphate which removes Ca2+ from the assay system as insoluble calcium phosphate.

In addition, cultured endothelial cells exposed to 1 μM retinoic acid or 10 μM retinol for 40 h exhibited more than 40-fold higher levels of transglutaminase activity (308 nmol of [3H]putrescine/h/mg of protein) compared to untreated control cells (7.7 nmol of [3H]putrescine/h/mg of protein) (also see Fig. 7, curves a and b); this elevated transglutaminase activity could be completely immunoprecipitated with the anti-RIP antiserum (Fig. 7, curve c). These results firmly establish the identity of RIP.

DISCUSSION

In the present study, we investigated retinoid-induced morphological alterations in cultured bovine endothelial cells and accumulation of an 80-kDa protein which was termed RIP. Partial sequencing and immunochromatographic characterization revealed that RIP is a transglutaminase. This identification may give a fascinating clue to the morphogenic action of retinoids.

Transglutaminases are a group of enzymes that catalyze covalent cross-linking of protein (31, 32); for example, the
Plasma transglutaminase known as blood coagulation factor XIII catalyzes the cross-linking of fibrin molecules (33, 34); tissue transglutaminases are also known, and have been intensively studied with regard to the cross-linking of membrane proteins, using keratinocytes (35) and erythrocytes (36). Concerning morphogenesis, there is a report that retinoic acid, whether the induced transglutaminase is directly involved in the differentiation process is presently unknown. This question, and the identification of the substrates for endothelial transglutaminase, remain subjects for future study.

Recently, Chiocca et al. (19) have elucidated the mechanism of retinoic acid-induced accumulation of transglutaminase in mouse resident peritoneal macrophages, and have demonstrated that retinoic acid regulates the expression of the transglutaminase gene at the transcriptional level. Consistent with this mode of action, Giguere et al. (37) have identified a specific retinoic acid receptor which bears striking similarity to the steroid and thyroid hormone receptors. A similar mechanism may also apply to the action of retinoids on endothelial cells as reported here.

**FIG. 5. Purification of RIP by HPLC.** A, ion exchange chromatography on Mono Q. Endothelial cell extracts were applied on a Mono Q column and the absorbed proteins were eluted with an NaCl gradient. RIP-positive fractions, identified by dot blot assay, were pooled for further purification. Later, the fractions were also assayed for transglutaminase activity (curve b) since sequence analysis (Fig. 6) suggested that RIP is a transglutaminase. B, hydroxyapatite column chromatography. The pooled fractions from the Mono Q step were loaded on a hydroxyapatite column. After washing, the column was developed with a linear gradient of phosphate buffer. A protein peak corresponding to RIP was identified by dot blot analysis (inset). C, SDS-PAGE profile of purified RIP.

**FIG. 6. Amino acid sequence of tryptic fragments of RIP.** Purified RIP was digested with TPCK-trypsin and the resulting peptide fragments were separated by reverse-phase HPLC on a Waters μBondasphere CL column. Three well-separated peaks were sequenced, and their amino acid sequences are presented as fragments 1, 2, and 3, together with the homologous sequences found by a computer-aided homology search to that of guinea pig liver transglutaminase (gpTG).

| Fragment 1 | Fragment 2 | Fragment 3 |
|------------|------------|------------|
| LSDATEEGAWAándAQ | DNNYADGISP | QYEVTQGQG |
| gpTG(78-94) | gpTG(241-250) | gpTG(156-165) |

**FIG. 7. Retinol-treated cells showed a marked increase in transglutaminase activity which could be neutralized by anti-RIP antiserum (Ab).** Extracts were made from control (c) and retinol-treated endothelial cells (b) and assayed for transglutaminase activity. The enzyme reaction was continuously monitored by measuring the increase in fluorescence intensity that reflects the enzyme-catalyzed incorporation of monodansylcadaverine into acetylated casein. Curve c, inhibition of transglutaminase activity by anti-RIP. The extract (20 μg of protein) used for curve b experiments was preincubated with anti-RIP (20 μg) and subjected to fluorometric assay for transglutaminase. Preimmune serum had no effect. The fluorescence intensity of 1 μM quinine sulfate in 0.5 N H₂SO₄ measured using the same excitation and emission wavelengths, corresponded to 1000 units according to the arbitrary scale used in the figure.

**Acknowledgments**—We thank Yasuko Nagata and Setsuko Satoh for their secretarial assistance. This manuscript was prepared for submission by Boldface Editors, Inc.

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