Undifferentiated F9 embryonal carcinoma cells produce a short-chain collagen molecule

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The undifferentiated F9 embryonal carcinoma cells produce a unique collagen that decreases in amount during retinoic acid-induced differentiation of F9 cells into basement-membrane parietal endoderm. A bacterial-collagenase-sensitive protein of approx. 60,000 Da was resolved on polyacrylamide-gel electrophoresis. After pepsin digestion, two pepsin-resistant fragments containing hydroxyproline were demonstrated, suggesting that a portion of the molecule has a stable triple helix. The mRNA from the undifferentiated F9 cells translates a collagen-sensitive protein with a molecular mass consistent with the 60,000 Da collagenous protein produced by undifferentiated F8 cells.

INTRODUCTION

Collagens are a family of proteins traditionally considered as molecules providing mechanical stability to tissue. Recently it has become clear that these molecules play an active role influencing cell behaviour and embryonic morphogenetic events [1].

Most collagens are synthesized at an early stage in development and persist in adult tissue. Type IV collagen first appears in the embryo beneath the primitive endoderm, and is found in larger amounts after differentiation of primitive endoderm to parietal and visceral endoderm. This collagen becomes part of the basement membrane, along with laminin, other glycoproteins and proteoglycans, separating endoderm from underlying mesoderm [2].

Embryonal carcinoma cells are a useful well-characterized system to investigate the events occurring in early development [3]. The undifferentiated F9 cells are similar to embryonal ectoderm. These cells can be induced to differentiate into primitive, parietal or visceral endoderm cells, depending on culture conditions, by the use of retinoic acid and increased concentrations of cyclic AMP [4,5]. Typical markers of the F9 differentiation process are extracellular basement-membrane components, such as type IV collagen [4–6], which seems to be regulated by a pre-translational mechanism [7,8].

The undifferentiated cells appear to be totipotent when inserted into embryos, and can cause tumours when injected into adult animals. The cells are known to express undifferentiation-specific genes such as the cellular homologue of the E1A gene [9] or that for the heat-shock protein HSP 70 [10]. These proteins may be important at very early stages of development or in tumorigenesis. In the present paper we demonstrate that a short collagenous protein is synthesized by undifferentiated F9 cells. This new collagen disappears with differentiation at the same time that type IV collagen appears.

MATERIALS AND METHODS

Cell culture and metabolic labelling

F9 cells were maintained in minimal essential medium containing Earle's salts (Irvine Scientific, Santa Ana, CA, U.S.A.), supplemented with non-essential amino acids, glutamine (2 mm), sodium pyruvate (1 mm), penicillin (50 i.u./ml), streptomycin (50 mm), glucose (4.5 mg/ml) and Nuserum (10 %, v/v) (Collaborative Research, Lexington, MA, U.S.A.). Cells were passaged biweekly with 200 mm-EDTA in phosphate-buffered saline (140 mm-NaCl / 3 mm-KCl / 8 mm-NaH2PO4 / 15 mm-KH2PO4, pH 7.4) and plated at a density of 1000–2000 cells/cm2 on gelatinized flasks coated with bacteriological-grade gelatin [11].

To induce differentiation, F9 cells were treated with retinoic acid (0.5 μM) (Eastman, Rochester, NY, U.S.A.). To potentiate further the differentiation with increased concentrations of cyclic AMP, cholerat toxin (1 μg/ml) (List Biologicals, Campbell, CA, U.S.A.) and 3-isobutyl-1-methylxanthine (2 mm) (Sigma Chemical Co., St. Louis, MO, U.S.A.) were added in accordance with the methods of Strickland et al. [11]. In brief, cells were plated at a density of 2000/cm2, and grown in maintenance medium for 1 day followed by incubation for 1, 2 or 3 days with retinoic acid (0.5 μM) (RA cells) or with retinoic acid in combination with cholerat toxin (1 μg/ml) and 3-isobutyl-1-methylxanthine (2 mm) (RACT cells).

Nearly confluent cells were incubated with 2 μCi of [3H]proline/ml (0.007 nm) (New England Nuclear, Boston, MA, U.S.A.) for 6–8 h in serum-free Dulbecco's minimal essential medium (Irvine Scientific) without glutamine and pyruvate but supplemented with sodium ascorbate (50 μg/ml) and antibiotics. In some experiments cells were also radiolabelled with 2 μCi of [3H]glycine/ml (0.017 mm) (New England Nuclear) in addition to proline to detect the collagen better on polyacrylamide-gel electrophoresis. At the time of har-
vest, the medium was removed, and proteinase inhibitors [10 \(\mu\)M-phenylmethylsulphonyl fluoride (Sigma Chemical Co.), 1 mM-\(p\)-chloromercuribenzoinoate (Sigma Chemical Co.) and 20 mM-EDTA] were added. The cell layers were scraped in phosphate-buffered saline with the same proteinase inhibitors and processed separately.

Analytical methods

Radiolabelled proteins synthesized by F9 cells were precipitated by ethanol in a 4:1 (v/v) ratio of ethanol to sample. In some experiments the precipitated proteins were resuspended in electrophoresis buffer and separated on 7.5\%-polyacrylamide-gel or on 5–15\%-gradient-polyacrylamide-gel electrophoresis [12]. Samples were reduced with 2 mM-dithiothreitol before electrophoresis except where indicated. Gels were impregnated with Autofluor (National Diagnostics, Somerville, NJ, U.S.A.), dried and exposed to Kodak X-omat X-ray film at \(-70^\circ\)C for various times. Quantitative determination of protein was achieved by absorbance scanning of autoradiograms with an LKB laser scanner. Several exposures were scanned to ensure linear conditions.

Collagenase digestions were performed by incubating resuspended proteins with purified bacterial collagenase (50–60 units) (form III; Advance Biofactures, Lynbrook, NY, U.S.A.) for 4–16 h at 37 \(^\circ\)C in 50 mM-Hepes buffer, pH 7.4, containing 0.1 mM-Ca\(\text{Cl}_2\) and 0.5 mM-N-ethylmaleimide [13]. Concentrated electrophoresis sample buffer was added, and the proteins were loaded directly on polyacrylamide gels as described above. For quantitative determination of collagen production, the proteins were re-precipitated after digestion. The amount of radioactivity remaining in the supernatant following re-precipitation was determined and expressed as a percentage of the total incorporated radioactivity.

Quantitative determination of collagen was also performed by determining the hydroxyproline content of precipitated proteins. Samples were freeze-dried, then hydrolysed in 6 M-HCl for 24 h at 100 \(^\circ\)C. Proline and hydroxyproline were separated on Ultrasphere ODS h.p.l.c. columns (15 cm x 4.6 mm) (Beckman) by the method of Dydek & Kehrer [14]. Determination of hydroxyproline/proline ratios in proteins extracted from gel bands was performed essentially as described previously [15].

Data were normalized for DNA content as determined by the diphenylamine colorimetric method.

RNA extractions and cell-free translation

Total cellular RNA was extracted by the guanidinium chloride method as previously described [16]. Equal amounts (2–10 \(\mu\)g) of RNA were added to a rabbit reticulocyte lysate (Bethesda Research Laboratories, Bethesda, MD, U.S.A.) in the presence of 2,3,4,5,6\(^{3}H\)-proline (100 mCi/mmol; 100 \(\mu\)Ci/reaction) or \(^{35}S\)-methionine (10 \(\mu\)Ci/reaction). The lysate was adjusted to 100 mM-potassium acetate in order to increase translation of high-molecular-mass proteins. The samples were incubated with and without collagenase and subjected to polyacrylamide-gel electrophoresis as described above.

RESULTS

The first experiment examined the time course for the appearance of type IV collagen with the use of retinoic acid induction compared with retinoic acid and cholera toxin. F9 cells were incubated with retinoic acid (RA cells) or retinoic acid plus cholera toxin (RACT cells) for 0, 1, 2 or 3 days before the addition of \([^{3}C]\)proline. Samples were subjected to polyacrylamide-gel electrophoresis with and without collagenase treatment. Many of the cell-layer samples from cells treated with retinoic acid for 0–2 days contained a short-chain collagenase-sensitive protein (as shown in Fig. 1 for the 2-day RA-cell layer) that was approx. 60 000 Da compared with collagen standards prepared from CNBr-digested type I collagen.

The time course shown in Fig. 2 indicated that the short-chain collagenase-sensitive protein decreased in the cell layer with differentiation. The production of type IV collagen was quantified by densitometric scanning of autoradiograms similar to Figs. 1 and 2. The collagenase-treated samples were used to correct for background caused by bands co-migrating in the position of the collagen bands. Absorbance values from cell layer and media were totaled and graphed in Fig. 3. The short-chain collagen decreased after treatment with retinoic acid for 1 day, with a further decrease to almost undetectable amounts at 3 days. The rate of decrease was similar for both the RA and RACT cells, indicating that cyclic AMP does not influence the F9 cell’s production of this form of collagen. Type IV collagen production increased steadily for 3 days with retinoic acid treatment. Production of type IV collagen was always 2–4-fold greater by the RACT cells compared with the RA cells,
suggesting an alteration in type IV collagen in the presence of increased concentrations of cyclic AMP. The media in these experiments contained no indication of a secreted short-chain collagen, whereas type IV collagen was demonstrated in the media from RA or RACT cells.

Quantitative determination of the amount of collagen produced by undifferentiated F9 cells that produced no visible type IV collagen on gels indicated that only 1.1–1.9% of the total protein was collagenous as judged by either collagenase or hydroxyproline assays. The undifferentiated cells have more collagen in the cell layer. Usually the collagenase-sensitive radioactive material or the hydroxyproline represented only the short-chain collagen, although occasionally these cells will spontaneously differentiate, producing small amounts of type IV collagen, as judged by gel electrophoresis. The RA and RACT cells contained more hydroxyproline owing to the increased amounts of type IV collagen, mostly secreted into the media, just as reported by others [6].

Native collagens in their triple-helical conformation are insensitive to pepsin degradation. When the cell layers and medium fractions from untreated F9 cells were treated with pepsin, only the cell layer contained small amounts of two pepsin-resistant fragments approx. 40000 Da in size. Most (95%) of the proteins were degraded at the bottom of the gel. The pepsin-resistant fragments were collagenase-sensitive. The relative content of hydroxyproline in the pepsin-resistant fragments was determined from excised gel bands. The pepsin-resistant fragments had hydroxyproline/proline ratios similar to those of α1(1) collagen undergoing the same analysis. The molecular masses of these resistant frag-

DISCUSSION

A small amount of a short-chain collagen (approx. 60000 Da) is produced by F9 embryonal carcinoma cells.

**Fig. 2. Alterations in cell-layer proteins during differentiation of F9 cells**

Cells were differentiated with retinoic acid (a), a combination of retinoic acid and cholera toxin (b) or no treatment (c) as described in the Materials and methods section. The cells were radiolabelled for 6 h with [14C]-proline and -glycine at 0, 1, 2 or 3 days after treatment. Cell-layer proteins were resolved by polyacrylamide-gel electrophoresis as described for Fig. 1. Short-chain collagen (SCC) and type IV collagen are identified with arrows on the right, and collagen molecular-mass standards are identified on the left. Each sample represents proteins per 12 μg of DNA.

**Fig. 3. Graph of changes in collagen production during differentiation of F9 cells**

Fluorescence autoradiograms of proteins from both cell-layer and media fractions with and without bacterial-collagenase digestion were scanned with an LKB laser densitometer. The absorbance (A units × mm) of each collagen protein band was calculated by subtracting the background present on the collagenase-treated autoradiograms from the untreated samples. Media and cell-layer values were added together to plot total short-chain collagen (a) and type IV collagen (b) at each time point (0, 1, 2 or 3 days). ○, RACT cells were treated with both retinoic acid and cholera toxin. □, RA cells were treated with retinoic acid.
During differentiation, production of this collagen decreases. Several genes and proteins have been identified as characteristic of the undifferentiated state of F9 cells. These include the cellular homologue of the E1A gene [9], which may be responsible for maintaining cells in the undifferentiated state [17], the gene for the heat-shock protein HSP 70 [10], several unidentified genes [10] and SSEA-1, a cell-surface embryonic antigen [5]. The present paper is the first report of a collagenous molecule produced by the undifferentiated F9 cells.

The F9-cell short-chain collagen may be a collagenous form that is produced before type IV collagen production during deposition of basement membrane. In this case there would be small amounts of this collagen in adult basement membranes. A collagen molecule has been isolated from bovine anterior lens capsule, a tissue enriched in basement membrane [18]. This form of collagen is approx. 55000 Da as judged by gel electrophoresis. Little is known about its distribution or synthesis. Other investigators have isolated a cDNA clone from a basement-membrane-producing cell line HT1080 that represents a collagenous protein of 62000–67000 Da [19]. Since F9 cells produce type IV collagen and other basement-membrane components after differentiation, the undifferentiated F9-cell low-molecular-mass collagen could be identical with these previously reported collagens.

The short-chain F9-cell collagen could also be similar to or identical with other previously described short-chain collagens. Type X collagen, the most characterized of this series of collagens, is similar to the F9-cell collagen in molecular size and pepsin-sensitivity [20]. Since F9 cells are embryonal ectoderm cells before differentiation into endoderm, it might be expected that the F9-cell collagen is first produced at a very early stage of development. Type X collagen, on the other hand, is first produced by hypertrophic chondrocytes [21] in presumptive mineralization zones of hyaline cartilage during bone development [22], a much later stage of development than endoderm differentiation. This implies that the undifferentiated F9-cell collagen and type X collagen are different forms of collagen.

Another, less-characterized, 60000 Da collagen is produced by smooth-muscle cells treated with heparin [23]. This molecule has no interchain disulphide bonds and accumulates primarily in the cell layer. In an attempt to stimulate production of F9-cell short-chain collagen, the undifferentiated F9 cells were treated with heparin. F9 cells do not respond to heparin by producing more collagen (results not shown).

A smaller molecular form of collagen is produced by fetal-calf ligament fibroblasts [24]. It is striking that this collagen protein is also induced by extracellular matrix, and its synthesis appears transiently during development [25]. It is reasonable to assume that collagens with unusual structures increasing transiently during development may play critical roles in cellular recognition and association.

REFERENCES

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