Differential Collagen Biosynthesis by Human Neuroblastoma Cell Variants

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ABSTRACT

Two morphologically distinct types of cells have been observed in cultures of human neuroblastoma cells. One, designated N-type, is composed of small neuroblast-like cells; the other, designated S-type, is composed of large, substrate-adherent cells. To obtain additional information on the nature of these two phenotypes, we have investigated the collagen biosynthesis in several clones of human neuroblastoma cells with N-type, S-type, or mixed morphology, using acrylamide gel electrophoresis, ion exchange chromatography, and Northern and Southern blots. A direct correlation between the proportion of cells with S-type morphology and the amount of collagen secreted was observed, with the largest amount of collagen being produced by clones composed exclusively of S-type cells. Type I trimers and type III collagens were the two major collagen biosynthesis in several clones of human neuroblastoma cells.

INTRODUCTION

Human neuroblastoma is a childhood tumor which derives from the neural crest (1). In vivo this tumor commonly expresses diverse morphological and biochemical attributes characteristic of neural crest-derived cells such as neuronal cells, Schwann cells, and melanocytes (2). Recently a similar diversity has been demonstrated in several human neuroblastoma cell lines that can express two morphologically distinct phenotypes (3, 4). One type, designated N (for neuroblast-like), is composed of small round cells with short neuritic processes that grow poorly substrate adherent and express enzyme activities for neurotransmitter synthesis. Cells of the second type, designated S (for substrate adherent), have an epithelial-like morphology, are strongly substrate adhesive, do not show neuritic processes, and have little or no neurotransmitter synthetic activity. Karyotype analysis has indicated a common cellular origin of these two morphological variants (3). Furthermore, interconversion from one type to the other has been demonstrated (3). These two phenotypes also differ in expression of cell surface antigens and cell surface receptors for growth factors (5). The presence of high tyrosinase activity, a melanocyte marker enzyme, in some substrate adherent neuroblastoma cells also suggests that neuroblast cells can undergo differentiation into cells with a melanocyte phenotype (6).

We have recently demonstrated the production of various collagen isotypes including type I, type I trimers, type III, and type IV collagens in several human neuroblastoma cell lines (7). These results suggest that these cells may express biochemically similar properties of glial-like cells. Since a small number of S-type cells were observed in collagen-synthesizing cell lines and since a neurilemmal (Schwann cell) origin for these cells has been suggested, we postulated that S-type morphological variants could be the collagen-synthesizing cells in neuroblastoma.

To explore this possibility several clonal populations composed of N-type, S-type, or both ("mixed") were established and analyzed for collagen biosynthesis. A molecular analysis was also performed using cDNA probes for human interstitial collagens.

MATERIALS AND METHODS

Cell Culture. Cell lines and clones were cultured either in a 1:1 mixture of Eagle's minimum essential medium with nonessential amino acids and Ham's F12 nutrient medium supplemented with 15% heat-inactivated fetal bovine serum (SH-EPI) or in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (SK-N-SH-B, LAN-1, LA1-5s). Media were supplemented with 100 µg of streptomycin per ml and 100 IU of penicillin per ml. Cells were reseeded every 2–3 days. SK-N-SH-B clones were derived from the SK-N-SH cell line (8) at Children's Hospital of Los Angeles by plating SK-N-SH parent cells at 10^6 and 10^5 cells in 100-mm tissue culture dishes and isolating single colonies by trypsinization in a sterile O ring. SH-EPI, a twice cloned subline of SK-N-SH, and LA1-5s, cloned from LAN-1, were isolated by limiting dilution methods as previously described (9). The percentage of S-type or N-type cells in each clone was determined microscopically (3).

Cell Labeling and Collagen Analysis. Cells were plated at 2 × 10^5 to 60-mm plastic dishes (Falcon Plastics, Oxnard, CA) and grown for 7–10 days. Cultures were then labeled with 10 µCi/ml of L-[3,4-3H]proline (50 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, CA) in serum-free medium supplemented with ascorbic acid (10 µg/ml) and β-aminopropionitrile (65 µg/ml). After 20–24 h, the culture medium was harvested, centrifuged at 1,000 × g for 10 min at 4°C, and analyzed for collagen. Quantitative analysis was performed by collagenase treatment according to Diegelman and Peterkofsky (9) and qualitative analysis was performed by SDS polyacrylamide gel electrophoresis and carboxymethylcellulose chromatography as previously described (7).

Preparation of 32P-Labeled Probes. Human collagen cDNA probes were provided by Dr. F. Ramirez (Rutgers Medical School, Piscataway, NJ). Hfi1131 clone contains a 1.9-kilobase cDNA fragment encoding for the 3' end of the carboxyterminal portion of the pro-α2 chain of type I collagen (10). Hf577 clone contains a 1.8-kilobase cDNA fragment extending from nucleotides coding for amino acid 787 to 270 nucleotides into the 3' untranslated region of the DNA for the pro-α1 chain of human type I collagen (10). Hf577 clone contains a 1.8-kilobase cDNA fragment encoding the carboxyterminal portion of the pro-α1 chain of human type I collagen (11). Hf934 clone contains a 1.3-kilobase fragment encoding the carboxyterminal portion of the pro-α1 chain of type III collagen (12). Inserts from the recombinant DNA clones were excised from the plasmid using restriction endonucleases EcoRI and recovered by electrophoresis through a 1% agarose gel (13). Probes were radiolabeled with [α-32P]dCTP (3000 Ci/ mmol; Amersham, Arlington Heights, IL) to specific activities of >10^6 cpm/µg of DNA according to the procedure of Feinberg and Vogelstein using primer extension with the Klenow fragment (13).

RNA Isolation and Northern Blot. Total cellular RNAs and DNA were isolated from the same cell culture population by the method of Lee et al. (14). RNA samples (20 µg) were electrophoresed in 1.2% agarose (15).
and transferred to Biodyne membrane (ICN) in 20 × SSC (1 × SSC = 0.15 M NaCl plus 0.015 M sodium citrate). Blots were baked for 2 h at 80°C under vacuum and hybridized with the appropriate 32P-labeled collagen probe at 42°C in 5 × SSC. After hybridization for 48 h, blots were washed twice at room temperature in 3 × SSC, 0.2% SDS for 30 min, twice at 60°C in 1 × SSC for 30 min with 3 buffer changes, and finally in 0.3 × SSC at 22°C for 20 min with 3 buffer changes. Filters were then air dried and exposed to Kodak XAR-5 films with intensifying screen for 1–3 days at ~80°C.

Isolation of DNA and Southern Blot. High molecular weight DNA was extracted from cell nuclei (14). Aliquots of DNA (10 µg) were then digested overnight at 37°C with restriction endonucleases under conditions recommended by the supplier (New England Biolabs, Beverly, MA). Samples were fractionated by electrophoresis through 1% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8). Molecular standards (Boehringer Mannheim, Indianapolis, IN) were run in parallel wells. The DNA fragments were transferred to Biodyne membrane according to the procedure of Southern (16).

RESULTS

Cell Culture

Eight cell populations, six clones derived from two cell lines (SK-N-SH and LAN-1) and the parental lines, were selected for analysis (Table 1). Some cultures (SK-N-SH-B5 and LAN-1) were composed of a majority of neuroblast-like cells, whereas others (SK-N-SH-B10, SH-EP1, and LAI-5s) were composed exclusively of epithelial-like cells. The remaining three were composed of a mixture of both morphological variants (SK-N-SH-B uncloned, SK-N-SH-B6, and SK-N-SH-B9). N and S cell clones also differed markedly in their growth kinetics. For example, whereas one N-type clone (SK-N-SH-B5) grew with a doubling time of approximately 65 h, an S-type clone (SK-N-SH-B10) had a much slower growth rate with a doubling time of more than 200 h and after several months ceased to proliferate (loss of immortalization).

Collagen Biosynthesis

Quantitative Analysis. The amount of collagen secreted by these different cell lines clearly correlated with their morphological phenotype (Table 1). Large amounts of collagen (between 7.7 and 16% of the total protein synthesis) were secreted by clones composed entirely of substrate-adherent cells; by contrast, the amount of collagen secreted by two N-type clones was insignificant. Clones composed of a mixture of S-type and N-type variants secreted collagen in amounts ranging from 2.40–4.04% of the total protein synthesis. In these three cultures, higher levels of collagen biosynthesis mirrored higher proportions of S-type cells (Table 1). The presence of the large, substrate-adherent cells in neuroblastoma cultures was therefore clearly associated with a marked increase in collagen biosynthesis, suggesting that these cells are responsible for the production of collagen by neuroblastoma cells in vitro.

Qualitative Analysis. SK-N-SH-B10, SH-EP1, and LAI-5s clones exclusively composed of S-type cells were selected for further analysis of the types of collagen synthesized.

Radiolabeled collagens extracted from the culture medium were analyzed by SDS-polyacrylamide gel electrophoresis in the absence or presence of dithiothreitol (Fig. 1). Pepsin treatment of the samples was used to separate procollagen (pepsin-sensitive) from collagen (pepsin-resistant) chains and collagenase treatment was used to confirm the identity of the radiolabeled proteins as collagen.

In the absence of reducing conditions (Lanes 1–6), a precursor molecule which after treatment with pepsin comigrated with a1(I) was identified in culture medium from LAI-5s and SH-EP1 cell lines. Under these conditions large molecular weight proteins did not penetrate into the gel. After reduction and treatment with pepsin (lanes 7–15), these radiolabeled proteins comigrated with the a1 chain of type III collagen (Lanes 8, 11, and 14).

An additional pepsin-resistant and collagenase-sensitive radiolabeled protein with a molecular weight of approximately 120,000 was also identified in the culture medium of LAI-5s cells (Lane 11). The exact nature of this collagenous protein is presently unknown but it has been previously observed in other neuroblastoma cell lines (7). The a2(I) chain normally associated with type I collagen was not identified in any of the specimens analyzed.

The delayed reduction method of Sykes et al. (17) was further used to separate a1(I) from a1(III) collagen chains (Fig. 2). Under these conditions, the a1(I) chain secreted by SH-EP1 and LAI-5s cell lines was clearly separated from the a1(III) chain. The absence of a2(I) chain in SH-EP1 and LAI-5s cells was again noted.

We also used carboxymethyl-cellulose chromatography to confirm these observations. Analysis of radiolabeled collagen extracted from the culture medium of SK-N-SH-B10 cells revealed the presence of a single major peak of radioactivity which eluted between the position of a1(1) and a2(I) collagen chains during the NaCl gradient (data not shown).

These data therefore indicate the biosynthesis of two major collagen isotypes in three S-type variant clones from two human neuroblastoma cell lines, a homotrimeric form of type I collagen (a1(I) trimer) and type III collagen. No production of type IV collagen was detected in these cells.

RNA Analysis

Correlation between the production of collagens in these three clones and the presence of specific RNA for these proteins was determined by Northern blot analysis of total cellular RNA using human cDNA probes for a1(I), a2(I), and a1(III) procollagen chains (Figs. 3 and 4). RNA coding for the a1(I) procollagen chain (probe Hf677) was clearly detected in these cell lines as well as in a human fibroblast cell line (T1) which produces a heterotrimer molecule (2 a1 chains and 1 a2 chain). By contrast, only this latter cell line contained a significant amount of RNA corresponding to the a2(I) chain (probe Hf1131). All three neuroblastoma cell lines and the human fibroblast cell line contained RNA for the a1(III) procollagen chain (probe Hf934).

Therefore in the three neuroblastoma cell lines studied, the
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Fig. 1. Autoradiography of SDS-polyacrylamide gel electrophoresis of [3H]proline-labeled collagen secreted by S-type neuroblastoma clones. Labeled collagens secreted into the culture medium were extracted in 0.5 M acetic acid as described in “Materials and Methods.” Samples containing approximately 100,000 cpm were loaded. Electrophoresis was performed at 15 mA/plate. Autoradiography was done after fluorography with Enhance. Lanes 1, 2, 7, 8, and 9, SK-N-SH-B10. Lanes 3, 4, 10, 11, and 12, LAI-5s. Lanes 5, 6, 13, 14, and 15, SH-EP1. Lanes 16 and 17, HT1080 (type IV collagen). Lane 18, human fibroblasts T1 (types I and III collagens). When indicated, samples were incubated prior to electrophoresis with pepsin (1 μg) in 0.5 M acetic acid for 16 h at 4°C or with purified bacterial collagenase (coll) at 37°C for 5 h in 0.05 M Tris HCl, pH 7.5, containing 10 mM CaCl2. For gels run under reducing conditions, dithiothreitol (50 mM) was added to the denaturation buffer prior to electrophoresis. Arrows indicate the migration position of various collagen precursor molecules. PRE, precursor with both N and C terminal propeptides. PC, precursor with C terminal propeptide. PN, precursor with N terminal propeptide.

DNA Analysis

To investigate whether the absence of synthesis of the α2(I) collagen chain in neuroblastoma cells was associated with alteration of the structural gene and, in particular, associated with changes in the degree of methylation of this gene, DNA from LAI-5s and SH-EP1 was analyzed by RFLP using the α2(I) procollagen cDNA probe (Hf1131). Mspl and HpaII restriction endonucleases were selected to investigate the degree of methylation of the internal cytosine residue in the 5′-CCGG-3′ sequences. Whereas both enzymes cleave the 5′-CCGG-3′ sequence if the internal cytosine is methylated (5′-CCMeGG-3′), only HpaII can cleave the sequence if the internal residue is unmethylated (5′-CCGG-3′). DNA from HT1080 human fibrosarcoma cells, which do not produce type I collagen (18), and from human fibroblasts (T1), which produce both α1 and α2 chains, were analyzed simultaneously for comparison (Fig. 5).

Digestion of cell DNAs with EcoRI generated three fragments: 4.1, 3.1, and 1.6 kilobases. The Mspl banding patterns of all but the HT1080 cell line were identical, with four major fragments of 6.4, 4.4, 4.1, and 1.5 kilobases. In the case of HT1080, an additional RFLP of 1.9 kilobase was observed. These fragments were not seen with the HpaII restriction endonuclease, indicating the presence of methylcytosine in these 5′-CCGG-3′ sequences. Furthermore, the HpaII pattern indicated a significant degree of heterogeneity among the specimens analyzed. The pro-α2(I) gene was found to be highly methylated in the two S-type neuroblastoma cell clones which did not produce the α2(I) chain and less methylated in the human fibroblasts which synthesize this collagen chain (as indicated by a 5.5-kilobase fragment). However, the gene was even less methylated in HT1080 cells which do not synthesize type I collagen. These data therefore suggest that, in the case of the two neuroblastoma cell lines studied, the absence of synthesis of the α2(I) procollagen chain is associated with a
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Fig. 3. Northern blot analysis of human neuroblastoma cell lines. Total cellular RNA was electrophoresed in 1.2% agarose, transferred to Biodyne membranes, and hybridized with cDNA probes Hf677 [pro-α1(I) collagen] and Hf1131 [pro-α2(I) collagen] as described in “Materials and Methods.” Blots were exposed to Kodak XAR-5 films for 24 h at −80°C.

Discussion

We have been able to demonstrate the production of large amounts of interstitial collagens by cell clones composed solely of S-type variants and to show a direct correlation between the amount of collagen secreted in mixed-type cultures and the relative proportion of large, substrate-adherent cells. These data support the hypothesis that the presence of large, S-type cells in neuroblastoma cultures reflects an ability of neuroblastic cells to transdifferentiate into glial-like cells. Moreover, our results are in agreement with recent observations of Rettig et al. who showed that S-type variants express cell surface antigens common to ectomesenchymal cells such as meningeal cells or fetal and adult fibroblasts (5). Tyrosinase activity has been demonstrated in the SH-EP1 line, suggesting differentiation into a melanocyte-type cell (6). This observation may not be in conflict with our data, however, since melanogenesis has been the biosynthesis of a homo-trimeric form of type I collagen in S-type cells (type I trimer) is an intriguing observation. The production of this collagen by several tumor cells such as gastric carcinoma (23) or Wilms’ tumor (24) has been previously reported. This unusual form of type I collagen is present in other tissues such as virus-transformed tumors (25), embryonic chick tendons and calvaria (26), or inflamed gingiva (27). The biochemical mechanisms involved in the production of this unusual collagen type have remained unclear. In the present study two possibilities were explored: (a) posttranslational modification of the α2 procollagen chain, resulting in lack of incorporation in the triple helix, and (b) absence of transcription due to an increased level of methylation of the structural gene. By demonstrating the absence of α2 procollagen RNA by Northern blot analysis, we have clearly shown that the absence of the α2 chain in the homo-trimeric molecule is not due to posttranslational modification of the protein but rather to absence of transcription. This observation is consistent with a similar observation in chemically transformed rat liver cells (28). Results of RFLP analysis of DNA from two S-type clones producing α1(I) trimer were compared with those from a similar analysis of DNA obtained from cells producing both collagen chains (human fibroblasts) or producing neither of the two chains (HT1080 fibrosarcoma). In both neuroblastoma cultures studied, the α2(I) procollagen gene was found to be highly methylated; this observation is consistent with the concept that hypermethylation locks genes in an “off” position (29). Results obtained with human fibroblasts failed to confirm that hypomethylation of the pro-α2(I) collagen is required for its expression since the MspI digest sites (CC-GG sequences) were shown to remain methylated in these cells. Our data therefore are more consistent with previous observations showing that expression of the pro-α2(I) collagen gene is independent of methylation (30–31).

Finally, our studies provide some additional information on the role of collagen biosynthesis in malignancy. Increased collagen degradation rather than increased collagen biosynthesis

Fig. 4. Northern blot analysis of human neuroblastoma cell lines hybridized with the Hf934 cDNA probe (type III procollagen).
is generally observed during malignant transformation (32) and transformed cells generally synthesize less collagen than their normal counterparts (33-35). In our study, we observed an increased collagen biosynthesis in clones composed of S-type cells, characterized by a slower growth rate associated with a diminished growth potential (immortalization). A similar loss of immortalization associated with a loss of expression of MC25 surface antigen, a putative stem cell marker, was observed with other S-type clones (5). Moreover, preliminary observations suggest that S-type variants have a reduced ability to form colonies in soft agar and tumors in nude mice (36). Increased collagen biosynthesis in S-type cells may therefore reflect a loss of malignant potential as well as be responsible for the well-known substrate adherence of these cells. In vivo, this type of cell is most likely responsible for the large amount of connective tissue that separates and encloses neuronal cells in neuroblastoma specimens showing evidence of maturation into ganglionneuromas. Collagen biosynthesis in neuroblastoma may therefore be an extremely helpful marker for studying specific patterns of transdifferentiation associated with loss of malignant potential.

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