Rapid Publication

Expression of the *ErbA*-β Class of Thyroid Hormone Receptors Is Selectively Lost in Human Colon Carcinoma

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Abstract

Members of the *erbA* gene family are involved both in control of differentiation and in neoplasia. V-*erbA*, a retroviral oncogene, blocks avian erythroid differentiation. V-*erbA*-related transcripts are physiologically expressed in multiple normal tissues. They encode a family of transcriptional regulatory factors, some of which are thyroid hormone receptors. In man, two genes, *erbA*-α and *erbA*-β, are transcriptionally active. We examined expression of *erbA*-related transcripts in normal and neoplastic colon. In normal colon mucosa, as well as in a colon polyp and in a colon polyp cell line, three characteristic *erbA*-related transcripts were consistently found. One transcript of 6 kb was *erbA*-β related. Two transcripts of 2.7 and 5.2 kb were *erbA*-α related. In eight patients' colon carcinomas expression of the 6-kb *erbA*-β transcript was absent or markedly diminished when compared with the same patients' noninvolved mucosa. In contrast, expression of the two *erbA*-α transcripts was the same in both colon carcinoma and noninvolved mucosa. No evidence was found of *erbA*-β gene deletion in any of the tumors lacking *erbA*-β expression. These data suggest that selective loss of normally present *erbA*-β gene expression accompanies malignant transformation of the colonic epithelial cell.

Introduction

Members of the *erbA* gene family are candidates to play an important role in both differentiation and neoplasia. V-*erbA* is an avian retroviral oncogene that blocks erythroid differentiation and accelerates avian erythroleukemias (1). A wide variety of animal tissues express a number of diverse *erbA*-related products, all of which include structural motifs common to known transcription factors (2–13). Some of the *erbA* products have been demonstrated to encode a family of multiple different thyroid hormone receptors (2–13). Thus, *erbA* gene products probably mediate thyroid hormone induction of tissue-specific genes necessary for differentiation of organs such as brain, heart, pituitary and liver (14–17). Finally, *erbA* products may mediate the documented activity of thyroid hormone as a potentiator of carcinogens in some systems (18, 19).

In man two active *erbA*-related loci, *erbA*-α and *erbA*-β, have been defined (2–6). One human *erbA*-β- and two different human *erbA*-α-encoded cDNAs have been cloned and shown to encode different thyroid hormone–binding proteins (2–6). Each of these *erbA* cDNAs hybridizes to multiple different size mRNA transcripts, which are differently expressed in different tissues (2–6). Human *erbA*-β transcripts of 2 and 6 kb, and human *erbA*-α transcripts of 3.2, 2, 2.5, and 5 kb, have all been identified (3, 5–7).

This study was undertaken to determine the pattern of *erbA* products expressed in both normal and neoplastic colon. We found that in normal colon mucosa one *erbA*-β- and two *erbA*-α-related transcripts were expressed. The same transcripts were also expressed in tissue from one patient's colon polyp and in adenomatous epithelium cultured from a second patient's colon polyp. In contrast, in colon carcinoma tissue expression of the *erbA*-β transcript was markedly and specifically diminished. Loss of *erbA*-β expression was not accompanied by *erbA*-β gene deletion. Loss of *erbA*-β expression is a novel marker of malignant transformation in the colon and may play a role in mediating the transformed phenotype of the colon carcinoma cell.

Methods

Cell lines. The VACO 330 cell line was maintained in MEM supplemented with 2% fetal bovine serum and hormones as described (20).

Tissue samples. Colectomy samples were received on ice directly from the operating room. Tissue sections of grossly obvious neoplasm were immediately dissected by the attending pathologist. Adjacent sections were submitted for histologic analyses and microscopic confirmation that the tissue contained predominantly neoplastic cells. Samples of noninvolved mucosa were immediately removed from the resection margin of the surgical specimen by blunt dissection of the mucosa off the muscularis mucosa.

Nucleic acids. Tissue samples were immediately homogenized in guanidine isothiocyanate using an electric tissue grinder (21). RNA was also extracted from the VACO 330 cell line by dissolving the cells in guanidine isothiocyanate (21, 22). Total RNA and DNA were separated by ultracentrifugation of the extract through a cesium cushion (21, 22). Total RNA was resolved by electrophoresis on agarose-formaldehyde gels and judged to be undegraded based on the relative ethidium staining of 28S and 18S subunits (21, 22). Polyadenylated mRNA was selected by chromatography on oligo-dT cellulose (21, 22). DNAs were dissolved in SDS, digested with proteinase K, and extracted with phenol plus chloroform (21, 22).

Northern analysis. 2 μg of polyadenylated mRNA was resolved by electrophoresis on agarose-formaldehyde gels (22). RNA was transferred to nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, NH) following the manufacturer's instructions. Double-stranded

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cDNA probes were labeled with ^{32}P by the random primer technique (23). Hybridization of probe to filters was at 42°C in 50% formamide, 5× SSPE, 2× Denhardt's, 0.1% SDS, and 0.1 mg/ml salmon sperm DNA. Final washes were in 0.1× SSC and 1% SDS. Blots were exposed on XAR film (Eastman Kodak Co., Rochester, NY) with an intensifying screen.

Southern analyses. 10 μg of genomic DNA was cut with restriction enzymes, resolved by agarose gel electrophoresis, and transferred to nitrocellulose membranes (21, 22). Labeling of probe and hybridization of blots were as described above.

Probes. Northern probes constructed from human *erbA-β* placental clone pheA12 (3) included: probe 1, a near full-length 1.3-kb Eco RI-Bgl II fragment spanning all but the carboxyl terminus of the coding region; probe 2, a 0.3-kb Eco RI-Sst I fragment spanning the NH_2 terminus of the coding region; and probe 3, a 0.15-kb Bgl II-Eco RI fragment spanning the COOH terminus of the coding region. An additional *erbA-β* probe, pBH302, detects several restriction fragment length polymorphisms (RFLPs)[1] at the 5' end of the *erbA-β* gene (24–26). The *erbA-α* probe used was a Bgl I-Hinc II fragment of rat brain clone rbeA12 (8) spanning all but the carboxyl terminus of the coding region. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe used was a Pst I fragment from a chicken muscle cDNA clone (27). Stringencies for washing blots were: *erbA-β* probes, 65°C; *erbA-α* probe, 60°C; and GAPDH probe, 56°C.

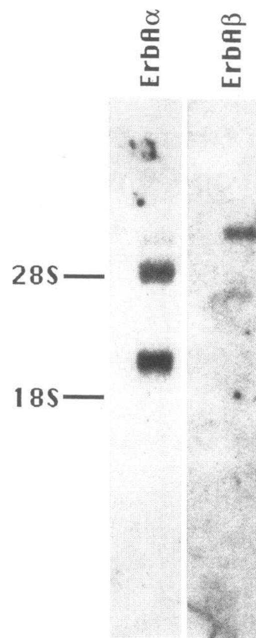

Figure 1. Expression of *erbA*-related transcripts in the VACO 330 human colonic epithelial cell line. Shown is Northern analysis of *erbA*-related messages in 2 μg of polyadenylated RNA from the VACO 330 cell line. Duplicate blots were hybridized with either a human placental *erbA-β* or a rat brain *erbA-α* cDNA probe. The positions of the 28S and 18S ribosomal RNAs are shown.

Results

Three erbA-related transcripts are expressed in cultured colonic adenomatous epithelium. We initially characterized *erbA* transcripts expressed in VACO 330, a cell culture model of the nonmalignant colon epithelial cell. VACO 330, an immortal culture established from a human colonic tubular adenoma, grows as a differentiated epithelial sheet with basal nuclei, apical microvilli, and tight junctions (20). The cell line does not clone in soft agar and is not tumorigenic when injected into the nude mouse (20). VACO 330 thus provides a unique culture model of the nonmalignant human colonic epithelial cell.

Fig. 1 shows the expression of three *erbA*-related transcripts detected by Northern analysis of RNA from VACO 330. Hybridization of 2 μg of VACO 330 polyadenylated mRNA to *erbA-β* probe 1, a 1.3-kb fragment of human placental *erbA-β* cDNA pheA12 (3), detected in VACO 330 a 6-kb transcript. Two additional transcripts of 5.2 and 2.7 kb were identified by hybridization of an identical sample to a rat brain *erbA-α* cDNA (8). The 5.2-kb transcript detected in VACO 330 polyadenylated RNA was barely detectable in VACO 330 total RNA (data not shown). It therefore represented an authentic *erbA-α* transcript and not hybridization to the 28S ribosomal RNA.

Human placental *erbA-β* cDNA pheA12, a 1.7-kb cDNA, was previously shown in HeLa and MCF7 cell lines to hybridize to a 2-kb transcript, and, in human placental RNA, to hybridize to multiple transcripts of 5, 3, 2.5, and 2 kb (3). A 6-kb transcript was detected in VACO 330 using as a hybridization probe either *erbA-β* probe 1, which spans nearly the complete coding region from placental cDNA pheA12 (Fig. 1), or using *erbA-β* probes 2 and 3, derived from the amino and carboxyl terminus of the placental *erbA-β* coding region (data not shown). Thus the colonic 6-kb transcript shares homology with the placental *erbA-β* cDNA throughout the coding region, but clearly must contain additional sequence information.

Two erbA-α transcripts and one erbA-β transcript are expressed in normal colonic mucosa. Expression of the *erbA-β*-related transcript is specifically lost in colon carcinoma. To determine if *erbA* transcripts expressed in VACO 330 are representative of *erbA* transcripts expressed in intact human colon mucosa, we next characterized *erbA* expression in normal and neoplastic colon tissue. Matched samples of neoplastic and noninvolved mucosa were obtained from colectomy specimens from nine patients. As shown in Table I, eight patients had colon cancer, which in seven cases had spread to regional lymph nodes or distant sites at the time of initial surgery. Fig. 2 shows expression of *erbA-α*- and *erbA-β*-related transcripts in these samples examined by Northern analysis on duplicate filters. Fig. 2 shows that the 6-kb *erbA-β* transcript expressed by VACO 330 was also expressed in noninvolved colon mucosa from each of eight patients; interestingly, however, this transcript was absent or markedly diminished in each of these patients' colon cancers (patients 2–9). In con-

Table I. Patient Characteristics

Patient No.	Age	Sex	Neoplasm	Site	Stage
1	45	F	P	R	V
2	81	M	T	R	B2
3	74	F	T	R	C2
4	31	F	T	R	C2
5	77	M	T	R	C2
6	68	F	T	L	C2
7	83	M	T	L	C2
8	74	F	T	L	D
9	42	F	T	L	D

M, male; *F*, female; *P*, polyp; *T*, cancer; *R*, right colon; *L*, left colon; *V*, villous adenoma; tumor stages A–D as defined by Astler and Coller (38).

1. *Abbreviations used in this paper:* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RFLP, restriction fragment length polymorphism.

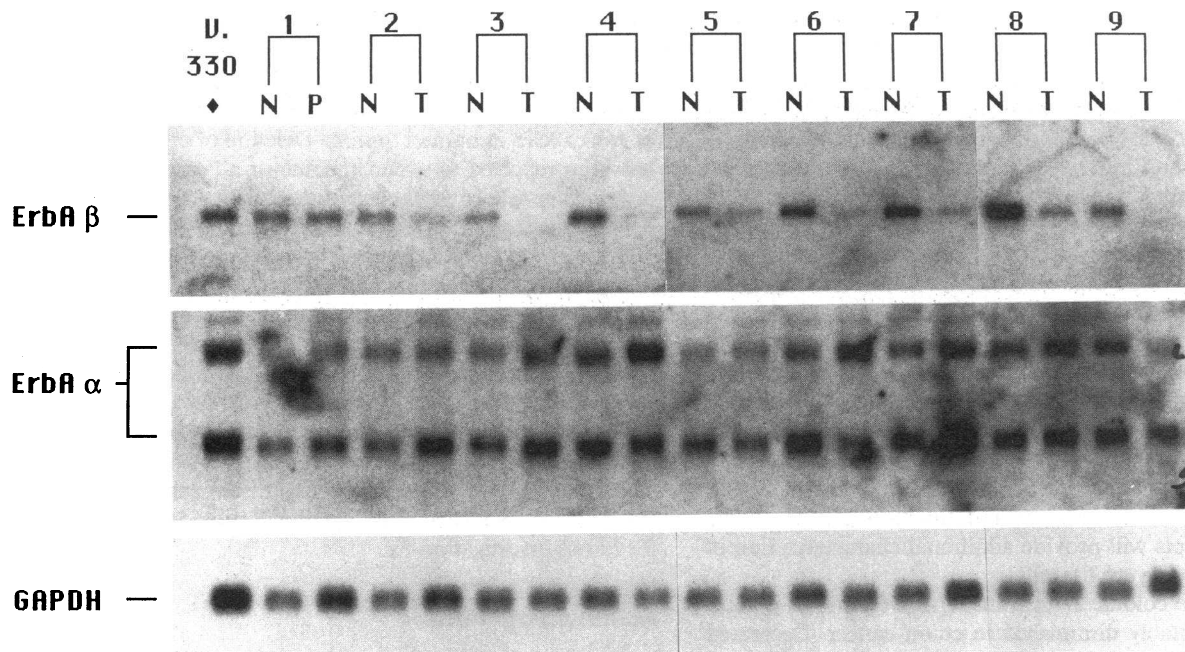

Figure 2. Expression of *erbA*-related transcripts in human colon tissue. Tissue samples were obtained from colectomy specimens from nine patients (*1–9*). Shown is Northern analysis of *erbA*-related messages in 2 μg of polyadenylated RNA extracted from either polyp (*P*) or cancer (*T*), and from accompanying noninvolved mucosa (*N*). 2 μg RNA from the VACO 330 polyp cell line is included for comparison. Duplicate filters were hybridized to a human placental *erbA-β* cDNA or to a rat brain *erbA-α* cDNA. The filters were then rehybridized to a cDNA probe for chicken GAPDH. The pattern of GAPDH hybridization to the *erbA-β* filter is shown.

trast, Fig. 2 shows that the 2.7- and 5.2-kb *erbA-α*-related transcripts expressed by VACO 330 were expressed in all patients equally in carcinoma and noninvolved mucosa.

Also in contrast to the loss of *erbA-β* expression in colon carcinoma, Fig. 2 demonstrates maintenance of *erbA-β* expression in a villous polyp. Expression of *erbA*-related alleles in the polyp tissue from patient 1 is similar to expression of *erbA* alleles in VACO 330, a pure epithelial culture previously established from a colonic tubular adenoma. These data suggest that the loss of *erbA-β* expression in colon carcinoma occurs within the malignant epithelial cell.

To insure that comparable amounts of mRNA from both tumor and noninvolved mucosa were applied, filters were stripped and rehybridized to a cDNA probe for GAPDH (Fig. 2). GAPDH, a "housekeeping enzyme," which is equally expressed in both proliferating and nonproliferating cells (28), was comparably expressed in all pairs of tumor and noninvolved mucosa. Hybridization of the *erbA-α* filter to GAPDH shows it to be loaded identically to the *erbA-β* filter (data not shown). Rehybridization of the filters with a human β-actin probe also demonstrates comparable expression of β-actin message in RNA samples from both carcinoma and noninvolved mucosa, but suggests that in VACO 330 β-actin message is relatively underexpressed compared with GAPDH (data not shown).

ErbA-β gene deletion does not accompany loss of erbA-β gene expression. Chromosomal losses have been demonstrated to occur commonly in colon carcinomas (29). To address whether loss of *erbA-β* expression in colon carcinoma is due to loss or rearrangement of the *erbA-β* genomic locus, we analyzed by Southern blots the integrity of this locus in normal and malignant colon tissue. *ErbA-β* genomic clone pBH302, a fragment from the 5′ portion of the *erbA-β* gene, detected a family of RFLPs produced by digestion with Hind III, Msp I, or Dra I, respectively (24–26). Genomic DNA from colon carcinomas and noninvolved colon mucosa were therefore digested separately with Hind III, Msp I, and Dra I, and compared after Southern hybridization to probe pBH302. Of the eight patients, five were heterozygous for at least one RFLP. Heterozygosity was retained in the tumor tissue in each of the informative patients (data not shown). Potentially, DNA extracted from nonneoplastic cells in the tumor samples might partially mask allelic loss in the neoplastic cells. However, there was no suggestion in any of the heterozygotes of any decrease in relative intensity of an allele in the tumor sample compared with its intensity in the normal control. Additionally, DNA from colon carcinoma and noninvolved mucosa was also digested with Eco RI and examined by Southern hybridization to *erbA-β* cDNA probe 1. The expected four genomic bands (30) were detected and there was no evidence of *erbA-β* gene rearrangement in any of the tumor samples (data not shown).

Discussion

These data identify three *erbA* transcripts that are normally expressed in human colon mucosa, and demonstrate that loss of expression of a 6-kb *erbA-β* transcript is a novel marker for colon carcinoma. These data also provide a structural basis for thyroid-mediated responses in human colon. As three different *erbA* transcripts are detected, human colon mucosa may contain as many as three distinct thyroid hormone receptors.

This study provides the first characterization of the tissue-specific expression of *erbA*-related transcripts in human colon. The 6-kb *erbA-β* transcript detected in VACO 330 and colon mucosa is different in size from transcripts previously detected

in human placenta and HeLa and MCF7 cell lines, but is similar to the previously reported 6-kb *erbA-β* transcript identified in human liver (3, 7). In the rat, alternate splicing of the primary *erbA-β* transcript gives rise to distinct *erbA-β*1 and *erbA-β*2 transcripts (9). Alternate splicing may similarly account for the multiple-sized human *erbA-β* transcripts detected by different laboratories. The relation of the colonic 2.7- and 5.2-kb transcripts to previously identified *erbA-α* transcripts remains to be clarified. To date, two human *erbA-α* class cDNAs, *erbA-α*1 and *erbA-α*2, have been cloned, shown to arise from alternate RNA splicing, and demonstrated to encode thyroid hormone binding proteins (5, 6). In rat, additional *erbA-α*-related cDNAs have been cloned and shown to encode not thyroid receptors but thyroid receptor antagonists (10–12). In man, a 3.2-kb *erbA-α*1 transcript is present in kidney, placenta, tonsil, and spleen (5). *ErbA-α*2 transcripts of 2, 2.5, and 5 kb are expressed in multiple human tissues (6). Further study of the structure and function of the colonic *erbA-α* transcripts will provide additional characterization of the rapidly growing *erbA* family.

We find one colonic *erbA* transcript, a 6-kb *erbA-β* transcript, to be notably diminished in colon cancer. Decreased expression of this transcript is found in colon cancers from each of the eight patients examined in this study. However, seven of the eight primary colon carcinomas examined in this study were already metastatic to regional lymph nodes or distant sites at the time of surgery. Thus, further investigation is required to determine if loss of *erbA-β* expression in colon carcinoma correlates best with stages of local tumor invasion or with tumor metastases.

Decreased *erbA-β* expression in colon cancer probably involves specific regulatory events, as expression of *erbA-α* is the same in cancer and noninvolved mucosa. It is technically possible that in normal mucosa *erbA-β* is mainly expressed in a nonepithelial cell type that is absent in colon tumors. We do not believe this explanation to be likely. We have demonstrated that *erbA-β* is expressed in VACO 330, a cell line model of nontransformed colonic epithelium. Further, expression of *erbA-β* in VACO 330 is similar in magnitude to expression of *erbA-β* in intact polyp tissue or intact normal mucosa. Thus, we believe it is most likely that *erbA-β* expression in these colonic tissues reflects expression in the normal colonic epithelial cell, and that this expression is therefore lost in the malignant epithelial cell.

The mechanism of loss of *erbA-β* expression in colon cancer remains to be defined. We find no evidence for either *erbA-β* gene deletion or rearrangement, consistent with previous reports that losses on chromosome 3p are uncommon in colon cancer (29). Changes in transcription of the *erbA-β* gene or in stability of the *erbA-β* transcript probably account for the diminished *erbA-β* expression in colon carcinoma.

Loss of *erbA-β* expression is a novel marker for colon carcinoma. Previously described markers of colon carcinoma include altered expression of blood group antigens, changes in lectin binding, increased carcinoembryonic antigen expression, increased c-*myc* expression, increased tyrosine kinase activity of the c-*src* protooncogene product, and mutational activation of the k-*ras* oncogene (31–35). The present result contrasts in demonstrating an oncogene-related product whose expression is lost during colon carcinogenesis. Differential probing of a colonic cDNA library recently detected seven unidentified clones whose expression diminished between normal and colon carcinoma tissue (36). *ErbA-β* is, however, the first identified gene whose expression diminishes during colon carcinogenesis.

These observations are the first report of altered expression of *erbA* alleles in human tumors. Deletion of one *erbA-β* allele has been reported to occur in some or all cases of small cell lung cancer as a consequence of the deletion on chromosome 3p which is associated with this disease (30, 37). However, it is unknown if in small cell lung cancer this genomic loss results in altered expression of *erbA-β* in comparison with the normal bronchial neuroendocrine cell. Thus, at present, loss of *erbA-β* expression has been demonstrated in colon cancer only. As the *erbA* family encodes products that regulate gene transcription, it is tempting to speculate that loss of *erbA-β* expression might directly influence important aspects of tumor phenotype such as differentiation, invasiveness, metastasis, or drug sensitivity. The question of whether *erbA-β* expression suppresses any of these tumor-associated phenotypes will provide fertile ground for future investigation.

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