The Distribution of the Deleted in Colon Cancer (DCC) Protein in Human Tissues

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ABSTRACT

A gene called deleted in colon cancer (DCC) has been identified on a region of chromosome 18, which is deleted in 70% of colorectal cancers. The DCC gene encodes a protein belonging to the immunoglobulin superfamily with similarity to the N-CAM transmembrane proteins and is a putative tumor-suppressor gene. Alternative splicing of transcripts of transmembrane proteins, including N-CAM, is known to occur, resulting in different isoforms of the protein. Using five antibodies against the DCC gene product (three monoclonal antibodies raised in our laboratory, one commercially available antibody, and a rabbit polyclonal antibody), we have demonstrated by immunostaining a DCC protein isoform in reticulendothelial cells in human thymus, tonsil, and lymph node. This can be distinguished from another isoform described in normal colonic epithelium, because this latter is not demonstrable with the antibodies we have used. It could not be detected in normal colonic epithelium, polyps or colorectal carcinomas. This restrictive distribution suggests that not all DCC gene products are important in colonic cancer.

INTRODUCTION

Tumors originate from the accumulation of genetic defects causing either activation of genes, promoting growth or survival, or inhibition of genes, repressing growth or inducing cell death. These latter genes are called tumor suppressors, and the proteins they encode are either inactivated or not expressed as a consequence of genetic damage in a variety of tumors (1, 2).

One putative suppressor gene, DCC, has been identified and cloned (3) from a region of the long arm of chromosome 18 that is deleted in 70% of colorectal tumors. This gene encodes for a membrane-bound protein with immunoglobulin and fibronectin domains and a unique cytoplasmic domain. The first two domains are characteristic of an adhesion molecule belonging to the immunoglobulin superfamily, the members of which are expressed mainly on cells of the nervous and immune systems (4). As has been shown for other members of this family of genes, alternative splicing can occur, resulting in the expression of related but structurally different proteins (5, 6). These isoforms can have different biological characteristics, cellular localization, and tissue distributions. Sometimes such differences can be caused by minimal variations in the sequence leading to major changes in glycosylation, which will affect the reactivity of antibodies. Investigation of proteins coded by genes such as DCC, therefore, can be complex.

The DCC gene seems to be no exception. Using one panel of antisera in a Western blot analysis, two forms with Mr of approximately 170,000 and 180,000 have been demonstrated recently (7, 8). Using immunohistochemistry, these antisera stained goblet cells in colon and neurons (7, 8). Reale et al. (9), with a different panel of antibodies (including the antibody 723 used in our study), have demonstrated, in human and rodent cells, proteins ranging in molecular weight from Mr 175,000–210,000. A commercial monoclonal antibody from Oncogene Science has been shown to recognize a Mr 180,000 protein.

Preliminary immunocytochemical studies using rabbit polyclonal antibodies to recombinant DCC protein and parallel in situ hybridization suggested that both the DCC protein and mRNA were present on colonic goblet cells (8). However, no further information was given on the tissue distribution of DCC, nor was its pattern of loss in colonic carcinoma studied in detail. We, therefore, considered that it would be important to extend these early studies by raising and characterizing monoclonal antibodies against the protein(s) coded for by the DCC gene. Our aim was to confirm these previous, more limited studies and to identify whether other normal cells in addition to colonic cells express the DCC protein and to provide supportive evidence for a tumor suppressor function by demonstrating protein loss in colonic and possibly other carcinomas.

The antibodies raised reacted strongly with COS cells transfected with full-length DCC cDNA but did not give the expected expression on other tissues tested. We therefore tried to obtain other DCC antibodies to compare with these results. We were able to look at two other antibodies from two different sources and found that they gave similar results to that of our mouse antibodies.

MATERIALS AND METHODS

Fresh frozen tissue was obtained via the routine diagnostic histopathology laboratories at the John Radcliffe Hospital. These included lymph node, tonsil, thymus, spleen, liver, kidney, pancreas, brain, testis, ovary, lung, and colon. Twenty-seven colorectal carcinomas were also selected from the tumor tissue bank. Frozen samples were stored at −70°C until use. Cryostat sections were cut at a thickness of 8 μm, and the slides were dried overnight at room temperature and were fixed in acetone for 10 min at room temperature prior to staining or storage at −20°C. Cytosin preparations of the transfected COS cells were made and treated in the same way as the sections. Other fixatives, including 3.7% formalin in PBS and 50:50 acetone methanol, were investigated on colon, thymus, and the transfectant cytosins.

Immunostaining was performed using the DAKO Strept avidin-biotin complex horseradish peroxidase kit (code K377; Dakopatts A/S, Copenhagen, Denmark) or Strept II avidin-biotin complex AP (Dakopatts K391). The peroxidase reaction was developed using dimethylaminobenzene substrate tablets (Sigma Chemical Co.), and the alkaline phosphatase reaction was developed using Naphol-AS-MX-phosphate and fast red (TR-Salt; Sigma) as substrate. The slides were then counterstained with hematoxylin, washed, and mounted in aqueous mountant (Aquamount; BDH, Inc.).

Monoclonal antibodies were raised as previously described (10) against recombinant DCC protein produced by transfecting COS cells with a pIG1 vector containing a part of the DCC gene region coding for the three immunoglobulin domains fused to the Fc domain of human immunoglobulin; the protein being harvested from the supernatant and affinity purified. The fusion protein being harvested from the supernatant and affinity purified. The fusion

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4 The abbreviation used is: DCC, deleted in colon cancer.
**RESULTS**

Characterization of Anti-DCC Monoclonal Antibodies and Immunostaining of Normal and Neoplastic Tissues. We have produced three monoclonal antibodies, DC3/l30, DC3/l59, and DC3/l68. The evidence for the specificity of these antibodies comes from transfection studies. All three antibodies give strong membrane and cytoplasmic staining of COS cells transfected with the full-length DCC cDNA (Fig. 1) similar to that obtained with the commercial antibody AF5. There was no staining of control COS cells or of those transfected with the vector alone or other cDNAs (CD31 and CD68).

NIH3T3 cells transfected with full-length DCC cDNA showed strong cytoplasmic and membrane staining in 20% of the cells with all five antibodies. This result was not affected by the different fixation schedules tested. The monoclonal antibodies produced in our laboratory do not function in Western blot analysis or by immunoprecipitation either from the COS cDNA transfecants or thymus tissue cell extracts.

Staining was performed on a series of normal tissues and colorectal carcinomas using the monoclonal antibodies DC3/130, DC3/159, and DC3/168, and the commercial antibody AF5. No staining was observed on normal colon (15 samples), hyperplastic polyps (10 cases), or invasive colorectal carcinomas (27 cases). Staining obtained was mainly of scattered reticulum cells in lymphoid tissues, including lymph node, spleen, thymus, and tonsil (Fig. 1). The staining of the thymus was of an elongated, cohesive meshwork of reticulum cells in the medulla with no staining in the cortex. In the tonsil, staining was seen only in scattered, single elongated cells with small, eccentric nuclei and abundant cytoplasm. These cells were found in the interfollicular areas, on the margins of the germinal centers, and occasionally in the overlying epithelium. In the red pulp of the spleen, there were numerous positive mononuclear cells. The white pulp appeared negative. Labeling was also seen of mesangial cells in renal glomeruli and scattered endothelial cells in the brain and spleen (Fig. 1).

No staining was seen on any definitive epithelial cells or in axons of the peripheral or central nervous system with any of the antibodies. Different fixation methods had no effect on staining in the colon and thymus.

The rabbit polyclonal antibody 723 was not as extensively studied due to the small sample size available to us. It stained both COS cells and NIH3T3 cells transfected with the full-length DCC cDNA in a manner identical to that shown by the monoclonal antibodies and AF5. On samples of tonsil, kidney, spleen, thymus, brain, spinal cord, and colon, antibody 723 gave results similar to those reported above. In particular, no staining of colon or nervous tissue was seen.

**DISCUSSION**

Immunostaining results obtained with our antibodies, the commercial AF5 monoclonal antibody, and the rabbit antisera 723 are very similar. They demonstrate the presence of the DCC gene product in cells of the reticuloendothelial system in lymphoid tissues, mesangial cells in the kidney, and a subpopulation of endothelial cells. No staining has been seen with any of the antibodies on either normal colonic mucosa or colorectal tumors. This failure to detect DCC in the colon could be accounted for by epitopes being produced by COS cells, which are not produced endogenously in the colon. This could occur because the protein has not been modified appropriately in COS cells by posttranslational changes such as glycosylation. We believe this is, in fact, unlikely, because the two other independently produced antisera also stained the COS and NIH3T3 transfectants in the same way. This interpretation is strengthened by their similar tissue distribution.

It is not immediately obvious to us why only 20% of the NIH3T3 permanently transfected cells were stained, because one would assume that all of the cells would be identical. However, the fact that all of the antibodies gave the same reaction and that the positively stained transfectants were so strongly labeled argues that the results are real and not a result of insensitive antibodies or techniques. This suggests that only 20% of the transfectants are, in fact, expressing the protein at any time.

**A New Family of Reticuloendothelial Cells.** A previous study of DCC protein distribution (8), using a rabbit polyclonal antibody raised to a bacterial fusion protein, reported similarly that it was difficult to detect in cell lines and tissues. However, in normal colon, specific staining of mucus-producing goblet cells was noted. Because these cells are well known to be lost in colonic carcinogenesis, it was thought that this was consistent with DCC loss in this type of tumor.

We have been unable to reproduce this finding with four different monoclonal antibodies and one polyclonal antisera raised against DCC. This difference in staining is intriguing. A later study by Reale et al. (9) uses the same antibody as the previous study but expands this using in situ hybridization to show that the goblet cells and Purkinje cells in brain have message for the DCC protein. It is interesting to note that the antibodies used are against the cytoplasmic domains, as are the probes used for the in situ work, whereas the monoclonal antibodies we have used are against the first three immunoglobulin domains, i.e., extracellular. The polyclonal antibody 723 is against an intracytoplasmic epitope, therefore, this in itself does not explain the different staining we have obtained. Chuong et al. (11) have raised an antibody against a peptide located in the third fibronectin type III domain of the chicken homologue to DCC. They report that DCC is present in all epithelial cells during development but only in proliferating zones in adult epithelia. The antibody also stains some neural tissues, including spinal cord and dorsal root ganglia. This pattern is consistent with the studies by Hedrick et al. (7, 8).

However, Reale et al. (9) have demonstrated recently that alternative splicing occurs in this gene, resulting in different protein products. These data may explain the limited differences in immunostaining we have observed with the panel of antibodies we have used. More importantly, the positive staining reported in colonic goblet cells could be due to the occurrence of splicing, leading to the synthesis of a distinct form of DCC protein in which the epitopes recognized by the present panel have been altered or are absent, making them unavailable for binding of antibodies. This hypothesis is also supported by the Western blot data; goblet cell staining has been demonstrated with the polyclonal antibody, demonstrating a M, 170,000 band, but not with the wider panel of antibodies, recognizing bands in a M 175,000–210,000 range. Unfortunately, we did not have any...
samples of these particular polyclonal antisera to compare the staining results with our own.

Further studies will be necessary to clarify the existence and nature of the splice variant protein products of this gene and their role in colon cancer. Fearon et al. (3) suggest that this gene could be a tumor suppressor gene, because the mRNA and the protein product can be detected in normal colonic mucosa but not in tumors with chromosome 18 deletions (7). A study showing no correlation between the gap junctional communication capacity (which is regulated by adhesion molecules of the N-CAM family) of human colon cancer cell lines with the expression of DCC mRNA using the reverse transcriptase-PCR technique (12) has raised some doubt recently about the antioncogenic role played in colon cancer by this protein.

A better characterization of all the isoforms encoded for by the gene and the demonstration that the expression of at least one isoform can revert or reduce the neoplastic behavior of cell lines will be necessary to demonstrate the role of DCC in colon cancer. This could be accomplished partly by using different deletions within the DCC gene and epitope mapping with relevant antibodies. It also may be helpful to use in situ hybridization techniques with relevant probes of the extracellular portion of the DCC gene to see whether at the messenger level the same pattern is seen, i.e., no staining of colon.

We have shown that at least one isoform of the DCC protein seems to be expressed on reticuloendothelial cells of several lymphoid organs and is likely, therefore, to play some role in the regulation of the immune system. Because of the inability to demonstrate the DCC protein in the colon with five anti-DCC antibodies, and the occurrence of alternative splicing, the DCC protein described in colonic mucosa is likely to have some substantial differences from the one detected in the immune system. We propose that at least two major forms of DCC gene product should be recognized: one as reported by Hedrick et al. (7), expressed in normal colon, and one as reported in the present study, expressed mainly in the reticuloendothelial system.

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