Immunoperoxidase Localization of Keratin in Human Neoplasms

A Preliminary Survey

Richard Schlegel, MD, PhD, Susan Banks-Schlegel, PhD, Janet A. McLeod, BA, and Geraldine S. Pinkus, MD

The distribution of intracellular keratin was studied in a variety of human tumors using a previously described immunoperoxidase technique employing antikeratin antibodies. Squamous cell carcinomas, transitional cell tumors, and mesotheliomas exhibited strong reactivity with antikeratin antibodies. Mammary adenocarcinomas were either negative or weakly positive. In the lung, an organ which can give rise to several morphologically distinct forms of carcinoma, only the squamous cell type stained strongly for keratin; undifferentiated lung carcinomas were negative, and adenocarcinomas were either negative or weakly positive. Colonic, renal, and prostatic adenocarcinomas were negative. Sarcomas, lymphomas, and neural tumors were uniformly negative. The analysis of intracellular keratin by the immunoperoxidase technique appears helpful in establishing the epithelial nature of primary or metastatic poorly differentiated neoplasms. (Am J Pathol 1980, 101:41-50)

KERATIN PROTEINS, once regarded as differentiated cellular products of epidermis, are now recognized to have a much wider biologic distribution. Immunofluorescence studies with *in vitro* cultured cells and frozen sections of rabbit, rodent, and some human tissues have shown that many diverse types of epithelium contain keratin.[1-4] Connective tissue, nerve, brain, and muscle appear to lack these structural proteins. Recently, using an immunoperoxidase technique to localize keratin proteins, we made an extensive survey of normal human tissues and defined the specific human epithelial cells that contain keratin: the basal or reserve cells of the trachea, bronchus, breast, cervix, and prostate; the ductal cells of various ectodermal and endodermal glands; and urothelium.[5]

In the present investigation, we surveyed a variety of malignant human neoplasms for the presence of intracellular keratin. The consistent and characteristic staining reactions in many of these tumors indicate that immunohistochemical identification of intracellular keratin represents a useful adjunct in diagnostic surgical pathology.

From the Department of Pathology, Peter Bent Brigham Hospital, and Harvard Medical School, Boston, Massachusetts, and the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

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Address reprint requests to Dr. Geraldine S. Pinkus, Department of Pathology, Peter Bent Brigham Hospital, 721 Huntington Avenue, Boston, MA 02115.

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Materials and Methods

Antibody Preparation and Immunoperoxidase Technique

Rabbit antihuman keratin antiserum was prepared according to methods described previously.[6] Briefly, 600 mg of stratum corneum from the sole of the human foot was homogenized in 20 mM Tris-HCl, pH 7.4, and centrifuged 20 minutes at 17,300g. The pellet was reextracted two more times with 20 mM Tris-HCl, pH 7.4, and three times with the same buffer containing 8 M urea. Finally, the insoluble pellet was extracted with 20 mM Tris-HCl, pH 7.4, 8 M urea, and 10 mM dithiothreitol, sonicated for 15 seconds with a Bronson sonifier, and centrifuged. The supernatant, which contained multiple keratin protein species, was used as antigen. After emulsification with Freund's complete adjuvant, 1.0 mg antigen was injected intradermally into New Zealand White rabbits. One month later, a second injection containing 0.5 mg protein was given. Antiserum was collected 1 week after the second injection.

Paraffin-embedded neoplastic tissues were obtained from surgical pathology files of the Peter Bent Brigham Hospital. Fresh tissue was obtained from surgical specimens and was fixed in either Zenker's solution (4 hours at room temperature) followed by 10% buffered formalin or by 10% buffered formalin alone, then processed routinely. Both procedures gave similar results, although the staining generally appeared more intense with Zenker's fixative. Prompt fixation of tissue, regardless of fixative, proved to be an important factor for the optimal demonstration of keratin.

Paraffin sections (5 μ thick) were placed on glass slides and warmed to 45 C for 1 hour to insure adherence. The sections were deparaffinized in xylene, then placed in absolute alcohol. Immunoperoxidase studies were performed as previously described.[5] Sections were sequentially incubated for 30 minutes at room temperature with each of the following reagents: 1) methanolic hydrogen peroxide (1 volume of 3% aqueous hydrogen peroxide to 5 volumes of methanol); 2) rabbit anithuman keratin antiserum (1:20, 1:40, and 1:100 dilutions tested); 3) swine antirabbit serum IgG (1:20 dilution); 4) horseradish peroxidase–rabbit anti-horseradish peroxidase soluble complexes (1:100 dilution). Tris buffer, 0.1 M, pH 7.6, was used for all dilutions. Nonspecific background staining was minimized by incubating sections with either 5% egg albumin or normal swine serum(1:20 dilution) for 30 minutes following methanolic peroxide treatment. Following each incubation, slides were washed thoroughly with Tris–saline (1 volume Tris buffer, 0.5 M, pH 7.6, to 9 volumes normal saline), then placed in Tris buffer for 15 minutes. Antibody localization was determined on the basis of peroxidase activity, effected by a 5-minute incubation of the sections with a freshly prepared solution containing 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo; 6 mg/10 ml Tris buffer) and 0.1 ml of 3% hydrogen peroxide. This technique yields a brown reaction product. Slides were then washed with water, counterstained with hematoxylin, dehydrated, and mounted in Permount.

Control sections of normal human skin were processed at dilutions of antikeratin antiserum, identical with those used for test tissues. Only intracellular, cytoplasmic staining was interpreted as a positive reaction. The specificity of all positive reactions was corroborated by neutralization studies using immune serum that had been adsorbed with keratin [5] and parallel studies with preimmune rabbit serum. Strong and weak reactivity of tumors with antikeratin antibodies was defined as positive staining at antibody dilutions of 1:100 and 1:20, respectively.

Horseradish peroxidase–rabbit antihorseradish peroxidase soluble complexes and swine antirabbit serum IgG were purchased from Dakopatts A/S of Copenhagen, Denmark (U.S. agent, Accurate Chemical and Scientific Co., Hicksville, NY).

Electron-Microscopic Examination

For ultrastructural studies, tissue was minced into 1-mm blocks immediately following excision and fixed for 4 hours at room temperature in a solution containing 2% paraformaldehyde and 2.5% glutaraldehyde in cacodylate buffer. Blocks were then washed

with cacodylate buffer, postfixed in osmium tetroxide, dehydrated, and embedded in Epon. Sections were examined in a Philips 201 or JEOL JEM 100S electron microscope. The presence of intracellular tonofilaments and desmosome-associated tonofilaments was interpreted as evidence of squamous or epidermoid differentiation.

Results

Table 1 summarizes the results of immunoperoxidase studies for intracellular keratin proteins in a variety of human neoplasms.

Keratin proteins are identifiable in many types of human epithelial tumors but are present in greatest quantity in squamous cell carcinomas, regardless of their organ derivation. Thus, squamous cell carcinomas of the skin (Figure 1A), lung, esophagus, and salivary gland display strong, intracellular staining with keratin antibodies. Although the staining intensity correlates with the degree of tumor cell differentiation, even poorly differentiated and "sarcoma-like" variants of squamous cell carcinoma (Figure 1B) react with antikeratin antibodies. Electron-microscopic examination of the tumor in Figure 1B demonstrated desmosome-associated tonofilament bundles and verified the epithelial nature of the neoplasm.

Strong staining for keratin is also found in the nonsquamous, basal cell carcinoma of epidermis (Figure 1C), in urothelial carcinomas, and in mesotheliomas (Figure 1D). The demonstration of prominent quantities of intracellular keratin in epithelial mesothelioma cells is consistent with previous ultrastructural studies that revealed cytoplasmic tonofilament bundles in these cells.[6] Transitional cell carcinomas, derived from bladder urothelium, also contain cytoplasmic tonofilaments and large amounts of intracellular keratin (Figure 1E). Focal regions of squamous differentiation are characterized by increased cellular staining.

Adenocarcinomas have a quantitatively different staining pattern. For example, in all cases of colonic adenocarcinoma examined, there was no specific cytoplasmic staining by antikeratin antibodies. Adenocarcinomas of the prostate do not react with antikeratin antibodies despite the presence of keratin proteins in the basal cells of normal prostatic glands.[5] In the benign breast tumor, fibroadenoma, it is the myoepithelial cells which react with antikeratin antibodies. Similar staining of myoepithelium has been observed in normal breast tissue.[5] Both lung and breast tumor displayed variable staining with antikeratin antibodies. Breast tumor reactivity ranged from negative to weakly positive and lung tumors from negative to strongly positive, dependent upon tumor cell type.

Malignant tumors of connective tissue, lymphoid tissue (Figure 1F), and male germinal epithelium are negative for keratin proteins, as are tumors derived from brain or peripheral nerve. The differential staining pattern for keratin proteins in these various human neoplasms is summarized in Table 2.

Table 1—Immunohistochemical Detection of Keratin Proteins in Various Human Neoplasms

Tissue	Antibody reactivity
Skin	
Basal cell carcinoma (6)	Strongly positive
Squamous cell carcinoma (4)	Strongly positive
Nevus (7)	Negative
Lung	
Squamous cell carcinoma (2)	Strongly positive
Adenocarcinoma (3)	Negative to weakly positive
Undifferentiated (2)	Negative
Carcinoid (1)	Negative
Pleura	
Mesothelioma (2)	Strongly positive
Peritoneum	
Mesothelioma (1)	Strongly positive
Breast	
Adenocarcinoma, ductal (10)	Negative to weakly positive
lobular (1)	Weakly positive
Fibroadenoma (2)	Weakly positive
Cystosarcoma phyllodes (1)	Negative
Colon	
Adenocarcinoma (7)	Negative
Bladder	
Transitional Cell Carcinoma (2)	Strongly positive
Kidney	
Renal cell carcinoma, clear cell (2)	Negative
Oral mucosa, nasopharynx, salivary gland	
Squamous Cell Carcinoma (5)	Strongly positive
Pancreas	
Islet cell tumor (1)	Negative
Adenocarcinoma, ductal (2)	Positive
Lymph nodes	
Non-Hodgkin's lymphoma (3)	Negative
Hodgkin's disease (1)	Negative
Testis	
Embryonal cell carcinoma (1)	Negative
Seminoma (1)	Negative
Ovary	
Brenner cell tumor (1)	Weakly positive
Cervix	
Squamous cell carcinoma (2)	Strongly positive
Prostate	
Adenocarcinoma (2)	Negative
Leiomyosarcoma (1)	Negative

Table 1—Continued

Tissue	Antibody reactivity
Esophagus	
Adenoid cystic carcinoma (1)	Positive
Connective tissue	
Fibrous histiocytoma, benign and malignant (2)	Negative
Fibrosarcoma (1)	Negative
Leiomyosarcoma (1)	Negative
Rhabdomyosarcoma (1)	Negative
Lipoma (1)	Negative
Brain	
Astrocytoma (1)	Negative
Meningioma (1)	Negative
Nerve	
Ganglioneuroma (1)	Negative
Schwannoma (1)	Negative
Neuroma (1)	Negative
Liver	
Hepatoma (1)	Negative

Discussion

In general, the staining characteristics of malignant human tumors correlate well with the tissues from which they are derived. Tumors originating from keratin-positive tissue (eg, squamous epithelium, mesothelium, and urothelium) continue to express this differentiated product, although poorly differentiated tumors may demonstrate a decreased staining intensity. Tumors arising from tissue known to lack keratin (eg, brain, nerve, lymphoid, hematopoietic, and connective tissue) are uniformly negative.

Our results reveal that primary lung neoplasms are not easily classified by keratin content. Although tumors which are comprised of moderately to well differentiated squamous epithelium (ie, squamous cell carcinomas) contain prominent intracellular keratin (consistent with their possible origin from the bronchial reserve or basal cells), adenocarcinomas are also noted to stain variably with keratin antibodies (Table 2). These findings are consistent with recent reports that most human lung tumors (approximately 50%) apparently represent a mixture of squamous and adenomatous differentiation.[7] Pure squamous cell carcinoma and pure adenocarcinoma occur much less frequently. Thus, morphologically as well as by keratin content, many lung tumors represent a proliferation of malignant cells with divergent differentiation. The highly malignant undifferentiated small cell tumors of the lung have a controversial cellular origin,[8] and a thorough comparison by light microscopy, electron microscopy, and keratin analysis of these pulmonary neoplasms is warranted.

Table 2—Staining Pattern for Keratin Proteins in Human Neoplasms

A.	Keratin-positive tumors
	1. Strong
	Squamous and basal cell carcinoma
	Mesothelioma
	Transitional cell carcinoma
	2. Weak
	Adenocarcinoma of breast, lung, and pancreas
B.	Keratin-negative tumors
	Adenocarcinoma of colon, prostate, kidney, and sometimes breast and lung
	Sarcomas
	Lymphomas
	Neoplasms of central or peripheral nervous system

The finding that some breast adenocarcinomas stain with antikeratin antibodies is not entirely unexpected. We have shown that the myoepithelial cells of normal human breast tissue [5] and of benign fibroadenomas (Table 1) stain with the antikeratin antibody. Similar findings have been reported in the myoepithelial cells in normal rabbit and rat breast tissue.[1,4] Thus, malignant transformation of the basal myoepithelial cells could presumably produce a keratin-positive adenocarcinoma. In contrast, tumors differentiating toward ductal-type cells would be anticipated to be keratin-deficient. It is not known whether there is a prognostic difference in the biological behavior of keratin-positive versus keratin-negative breast tumors. However, attempts have been made to identify the myoepithelial nature of certain breast carcinomas by using a histochemical technique to visualize membrane ATPase activity. Although early reports claimed a more benign clinical course of "myoepithelial" tumors,[9] subsequent studies suggested that ATPase activity was not a reliable method for identifying myoepithelial cells.[10] The latter study noted that breast tumors identified by ATPase activity often lacked the characteristic myofilaments seen in myoepithelium. Tonofilaments were apparently not observed. It would be informative to correlate membrane ATPase activity and intracellular keratin content in various breast carcinomas as a means of validating the myoepithelial differentiation of certain breast tumors.

Not all adenocarcinomas contain intracellular keratin. Of the 7 colon adenocarcinomas examined, for example, all were consistently negative. In addition, prostatic adenocarcinomas also appear to contain little or no keratin protein. It is interesting that although there are striking similarities in the histologic arrangement and keratin content of prostatic and endocervical glands, the prostate has a predisposition to develop tumors

resembling the keratin-deficient glandular cells, whereas the endocervical glands more frequently develop keratin-positive tumors mimicking the reserve or basal cells.

Normal human brain tissue, including the ependymal epithelial cells (unpublished results), lacks detectable keratin protein. Detection of intracellular keratin in a brain tumor, therefore, indicates the metastatic epithelial nature of the neoplasm.

The staining pattern for keratin proteins in various human neoplasms is summarized in Table 2. the presence of keratin proteins in a metastatic tumor indicates an epithelial origin of the tumor. Strong reactivity with keratin antibody provides good evidence for an origin from squamous, urothelial, or mesothelial epithelium. A negative reaction with keratin antibodies is much less specific and can indicate an origin from brain, nerve, connective, or lymphoid tissue, or from one of the variety of glandular epithelial tumors.

The keratin antibody used in this study was prepared against whole keratins purified from human callus, consisting of 8 keratin protein species as determined by polyacrylamide gel electrophoresis.[5] Refinement of the keratin staining technique awaits the isolation of antibodies specific for these individual keratin species. It is possible that epidermis and other epithelium may vary in the spectrum of synthesized keratin proteins, depending upon their commitment to squamous cell differentiation.

Determination of intracellular keratin by the immunoperoxidase technique is more rapid and sensitive than electron microscopic examination [5] and is readily applicable to diagnostic surgical pathology. This method affords a permanent preparation with excellent cytologic detail and represents a valuable adjunct in the evaluation and diagnosis of primary and metastatic human tumors.

References

1. Franke WW, Schmid E, Osborn M, Weber K: Different intermediate-sized filaments distinguished by immunofluorescence microscopy. Proc Natl Acad Sci USA 1978, 75:5034–5038
2. Franke WW, Weber K, Osborn M, Schmid E, Freudenstein C: Antibody to prekeratin: Decoration of tonofilament-like arrays in various cells of epithelial character. Exp Cell Res 1978, 116:429–445
3. Sun TT, Green H: Immunofluorescent staining of keratin fibers in cultured cells. Cell 1978, 14:469–476
4. Sun T-T, Shih C, Green H: Keratin cytoskeletons in epithelial cells of internal organs. Proc Natl Acad Sci USA 1979, 76:2813–2817
5. Schlegel R, Banks-Schlegel S, Pinkus GS: Immunohistochemical localization of keratin in normal human tissues. Lab Invest 1980, 42:91–96
6. Suzuki Y, Churg J, Kannerstein M: Ultrastructure of human malignant diffuse mesothelioma. Am J Pathol 1976, 85:241–262

7. McDowell EM, McLaughlin JS, Merenyi DK, Kieffer RF, Harris CC, Trump BF: The respiratory epithelium: V. Histogenesis of lung carcinomas in the human. J Natl Cancer Inst 1978, 61:587–606
8. Yesner R: Spectrum of lung cancer and ectopic hormones. Pathol Annu 1978, 13:217–240
9. Murad TM: A proposed histochemical and electron microscopic classification of human breast cancer according to cell of origin. Cancer 1971, 27:288–299
10. Bencosme SA, Raymond MJ, Ross, RC, Mobbs B, Tsutsumi V, Ortiz H, Gonzalez R, Segura E: A histochemical and ultrastructural study of human breast carcinomas with a view of their classification by cell of origin. Exp Mol Pathol 1979, 31:236–247

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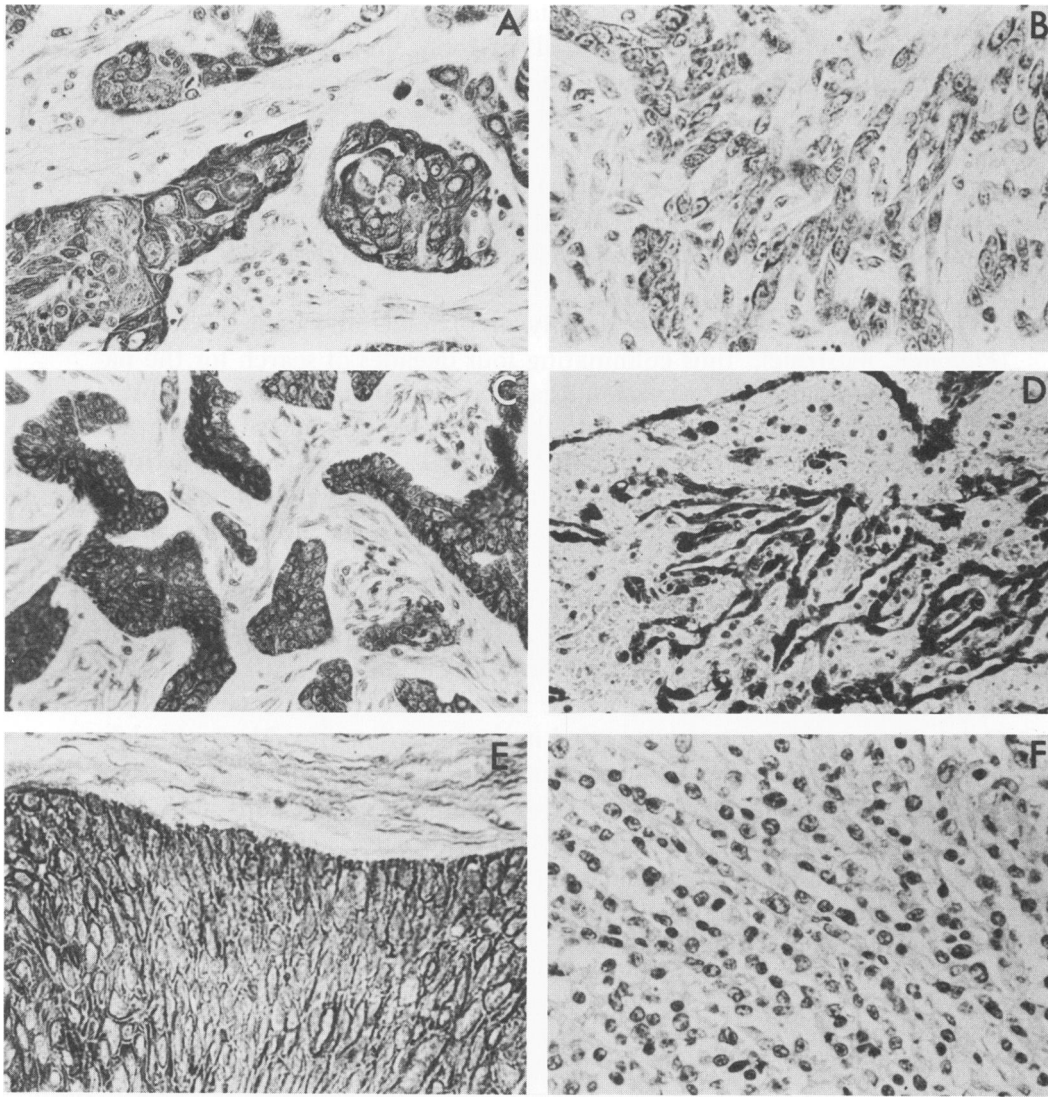

Figure 1—Immunoperoxidase localization of intracellular keratin in human neoplasms. **A**—Squamous cell carcinoma of skin. Strong staining for intracellular keratin is observed in tumor cells (*black*). (×250) **B**—Pseudosarcomatous areas of squamous cell carcinoma of the nasopharynx. Spindle-shaped tumor cells reveal weak to moderately strong staining for keratin. Intervening stromal cells are negative. (×250) **C**—Basal cell carcinoma of skin. Nests of tumor cells exhibit strong cytoplasmic staining for keratin (*black*). (×100) **D**—Mesothelioma of pleura. Keratin protein (*black*) is identified in mesothelial cells of surface lining and those forming tubules. (×100) **E**—Transitional cell carcinoma of bladder. Tumor cells reveal strong staining for intracellular keratin (*black*). (×250) **F**—Non-Hodgkin's lymphoma of lymph node. Lymphoid cells are negative for keratin proteins. (×250) All tissues were counterstained with hematoxylin.