A Novel Marker for Basal (Stem) Cells of Mammalian Stratified Squamous Epithelia and Squamous Cell Carcinomas

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ABSTRACT

We have developed a monoclonal antibody (174H.64) which selectively recognizes antigens shared by the basal cells of mammalian stratified squamous epithelium and squamous cell carcinoma (SCC). Histopathological studies of the frozen tissue sections demonstrated selective binding of this antibody to SCCs of human, bovine, canine, feline, and murine origin. Tumors of other histological types did not show reactivity with the antibody. In well-differentiated SCCs the peripheral layer of the tumor showed preferential binding of the antibody, suggesting that the antigens are associated with the proliferative compartment of the tumor. Studies on normal human tissues showed selective binding of the antibody to the basal layer of stratified squamous epithelia, thymic epithelial cells, and myoepithelial cells around breast ducts, while no antibody binding was observed for the suprabasal layers of stratified epithelia, simple epithelia, or tissues of nonepithelial origin. A similar pattern of antibody binding was also observed for bovine and murine skin with staining of the basal layer. The antigens detected by monoclonal antibody 174H.64 were characterized from cytoskeletal protein extracts of normal human keratinocytes as well as human and bovine SCC tissues by using an immunoblotting technique. The antibodies detected in normal human keratinocytes consisted of two major protein bands of approximate molecular weights of 48,000–50,000 and 57,000. In bovine SCC tumor the antigen detected was the M, 57,000 band. A murine lung SCC model was developed by fusing a mixture of HCG and Tn-HSA (200 µg each). The latter procedure was repeated on the 14th day. On the 15th day, the spleen cells from mice having high antiserum titers against HCG and Tn-HSA were fused with FOX-NY cells (HyClone Laboratories, Logan, UT). Hybrids of a mixture of HCG and Tn-HSA (200 µg each). The latter procedure was repeated on the 14th day. On the 15th day, the spleen cells from mice having high antiserum titers against HCG and Tn-HSA were fused with FOX-NY cells (HyClone Laboratories, Logan, UT). Hybrids with positive reactivity against Tn-HSA and negative reactivity with HSA were selected and recloned. The supernatants from the reclones were tested for reactivity against tumor tissues by immunohistochemical staining. A hybridoma designated 174H.64 was chosen for further studies, based on its selective reactivity with SCC tissues.

INTRODUCTION

Squamous cell carcinoma is a common histological type of cancer of the head and neck, lung, oral cavity, and cervix. Identification and characterization of molecular markers associated with SCC is an important approach to earlier diagnosis and treatment of SCCs. Functional studies on SCC markers would aid in better understanding of the biology of this type of cancer. Monoclonal antibodies are useful tools as molecular and functional probes for studying tumor-associated antigens. The majority of the Mabs raised against SCCs were produced by using SCC cell lines as the immunogen (1–8). Antigenic molecules defined by such Mabs include cell surface proteins (1–6), and cytoskeletal components (7). Most of these antibodies either show cross-reactivity with other types of cancers or their reactivity is limited to SCCs of certain organs only. A recently reported IgM antibody (Mab 17.13) is one of the few SCC-specific antibodies which react with SCCs of a variety of organs (8). The antigen recognized by Mab 17.13 has as yet not been reported. Another approach has been to develop Mabs against keratinocytes and evaluate their reactivity with SCCs (9). A Mab produced against human keratinocytes, VM-2, has been reported to bind selectively to the basal layer of the epidermis, as well as squamous and basal cell carcinomas (9, 10).

Monoclonal antibodies developed against cyto keratins have been used as probes for studying the keratin expression in normal and neoplastic stratified epithelia (11–16). Cytokeratins exhibit a differentiation-specific pattern of distribution in different layers of stratified epithelium (16). Specific keratin polypeptides have also been reported as molecular markers for SCC (17). Antikeratin Mabs have been proposed as tools for diagnosis of tumors, including SCCs (13, 18). We report here the characterization of a novel IgG1 Mab (174H.64) with high degree of selectivity toward SCCs of various origins. We describe evidence suggesting that this antibody recognizes a unique epitope on cytoskeletal proteins which may serve as a marker for the stem cell population in normal stratified epithelia and squamous cell carcinomas.

MATERIALS AND METHODS

Immunization and Hybridoma Production. RBF/Dn mice were immunized by two consecutive i.p. injections, 1 week apart, of a mixture of human chorionic gonadotrophin (50 µg) and a synthetic carbohydrate antigen Tn-HSA (50 µg) (19) in complete Freund's adjuvant. On the 12th day the animals were given another i.p. injection of a mixture of HCG (400 µg) and Tn-HSA (400 µg) in PBS. On the 13th day the mice were further immunized by an i.v. and an i.p. injection, each consisting of a mixture of HCG and Tn-HSA (200 µg each). The latter procedure was repeated on the 14th day. On the 15th day, the spleen cells from mice having high antiserum titers against HCG and Tn-HSA were fused with FOX-NY cells (HyClone Laboratories, Logan, UT). Hybrids with positive reactivity against Tn-HSA and negative reactivity with HSA were selected and recloned. The supernatants from the reclones were tested for reactivity against tumor tissues by immunohistochemical staining. A hybridoma designated 174H.64 was chosen for further studies, based on its selective reactivity with SCC tissues.

Isotyping. Mab 174H.64 was isotyped with a Mouse Type Isotyping Kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada) by an enzyme immunoassay method. A microtitre plate was coated with the antibody by an overnight incubation at 4°C of the culture supernatant of the hybridoma cells, and reacted with goat anti-rabbit IgG (Bio-Rad Laboratories, Mississauga, Ontario, Canada) by an enzyme immunoassay method. The antibody was then detected with a microtitre plate by using a microtitre plate (Bio-Rad Laboratories, Mississauga, Ontario, Canada) by an enzyme immunoassay method. The antibody was then detected with a microtitre plate (Bio-Rad Laboratories, Mississauga, Ontario, Canada) by an enzyme immunoassay method.
After each of the above steps the plate was washed with PBS. Finally, the microtiter wells were incubated with horseradish peroxidase substrate solution for 30 min at room temperature and the optical density was read by an enzyme immunoassay reader.

Purification of 174H-64, Radiolabeling, and Biotinylation. Ascitic fluids were produced by injecting hybridoma 174H-64 cells (2 x 10^6) i.p. into pristane-primed BALB/c mice. The antibody was purified by a two-step procedure involving ammonium sulfate (50%) precipitation and protein A Sepharose column chromatography (20). The purified antibody fraction contained a single protein band on cellulose acetate electrophoresis and was immunoreactive on SCC frozen sections as shown by immunoperoxidase staining.

The antibody was radioiodinated following the Iodo-Gen method (21). Purified Mab 174H-64 (50 µg) in PBS (50 µL) was mixed with "no carrier added" Na^125I (500 µCi; Edmonton Radiopharmaceutical Centre, Edmonton, Alberta, Canada) in PBS (10 µL) and was transferred into a borosilicate glass tube coated with Iodo-Gen (2 µg; Pierce Chemical Co., Rockford, IL) at the bottom. The reaction was allowed to proceed at room temperature for 2 min and the solution was then transferred into another tube containing 0.05% KI in PBS (140 µL) and allowed to equilibrate for 2 min. The antibody was separated from inorganic iodide by using a Bio-Gel P-100 spun column (Bio-Rad).

Mab 174H.64 was biotinylated according to a reported procedure (22). A solution of Mab 174H.64 (2 mg) in PBS (400 µL) was mixed with 0.2 M sodium bicarbonate solution containing 0.15 M potassium chloride, pH 8.1. To this a solution of sulfosuccinimidyl-6-(biotinamido)hexanoate (125 µg, Pierce Chemical Co.) in PBS (25 µL) was added and incubated at room temperature for 20 min. The reaction was stopped by addition of 1 M ammonium chloride solution, pH 6.0 (50 µL), and the solution was extensively dialyzed against PBS.

Cell Lines. All the SCC cell lines were obtained from American Type Culture Collection, Rockville, MD, and were grown in RPMI 1640 medium (Gibco, Canada, Inc., Burlington, Ontario, Canada) containing 10% fetal calf serum. Normal human epidermal keratinocytes were obtained from Clonetics Corp., San Diego, CA, and were cultured in serum-free keratinocyte growth medium (Clonetics Corp.).

Isolation of Mouse Epidermal Keratinocytes. Epidermal keratinocytes were isolated from neonatal (1-day-old) ICR mice according to a reported procedure (23). Briefly, mice were decapitated, skin was removed and washed in Hank's balanced salt solution. The skin was then placed dermal side down in Hank's balanced salt solution containing trypsin (0.025%-EDTA (0.02%) and incubated at 37°C for 1 h. The trypsin-EDTA solution was aspirated and replaced with RPMI-1640 medium containing 10% fetal calf serum and gentamicin (50 units/ml). Normal human epidermal keratinocytes were obtained from Clonetics Corp., San Diego, CA, and were cultured in serum-free keratinocyte growth medium (Clonetics Corp.).

RESULTS

Immunization and Hybridoma Production. Mice were immunized with HCG and Tn-HSA with the intention of developing hybridomas specific for these antigens. After repeated immunizations, spleen cells from mice showing high titers of antisera against these antigens were fused with mouse myeloma cell line FOX-NY. Hybridomas were initially selected on the basis of the reactivity of their culture supernatant against the above antigens. Culture supernatants of one of these hybridomas showed positive reactivity with Tn-HSA and with human SCC tissues in immunoperoxidase staining of frozen sections. This hybridoma was further recloned and the supernatants were tested for their reactivity with Tn-HSA and with human SCCs. A reclone designated 174H.H.64 showed strong and selective reactivity with human SCCs, although it did not show any reactivity with Tn-HSA. Ascitic fluid obtained by injecting the above hybridoma into mice was used as the source of Mab 174H.64 in further studies. The antibody isotype was IgG1.

Reactivity of Mab 174H.64 with Tumor Tissues. Frozen sections of various normal and tumor tissues of mammalian origin were tested for reactivity with Mab 174H.64 by immunoperoxidase staining with the ABC method. An isotype matched control antibody (MOPC-21) was used as a negative control. The results of these studies are summarized in Table 1. Mab 174H.64 showed selective binding with all human SCCs tested irrespective of the organ of origin, and did not show detectable binding to a variety of other histological types of human tumors. Fig. 1A shows the pattern of Mab 174H.64 binding to well-differentiated SCC tissues with preferential binding of the an-
E, F, and G). No reactivity was observed for simple epithelium, and myoepithelial cells around breast ducts (Fig. 1, epithelial tumors of dogs, basal cell tumors showed homoge
bovine and feline SCCs. Fig. IB shows the staining of a well-
reactivity. The reactivity of the antibody was also observed for
nomas and tumors of mesenchymal origin did not show any
(Table 1). Canine squamous and basal cell carcinoma tissues
were also studied for their reactivity against Mab 174H.64
stratified squamous epithelium was also demonstrated on bo
nized Mab 174H.64 against one histological type of tumor,
reactivity of the antibody with thymic epithelium could also
be demonstrated in mice, cows, and dogs.

Reactivity of Mab 174H.64 with Cell Lines. Several human
SCC cell lines (SCL-1, SIHA, ME 180, C-33A) were tested for
their reactivity with the antibody by cell smear immunoperoxidase staining and were found to be negative. In contrast, a
murine metastatic lung SCC cell line (KLN-205) was reactive with the antibody.

Characterization of Antigens Detected by Mab 174H.64. The antigens detected by Mab 174H.64 were characterized from bovine and human SCC tissues as well as normal human keratinocytes by using an immunoblotting technique. A human adenocarcinoma tumor was used as a negative control sample. The immunoblots of the SDS extracts of bovine SCC, human SCC, and human keratinocytes showed selective binding of 125I-labeled Mab 174H.64 with specific protein bands (Lanes 5–7), while the immunoblots of the corresponding NP-40 extracts (Lanes 1–3) did not show any binding with the antibody (Fig. 2). The NP-40 and SDS extracts of adenocarcinoma (Lanes 4 and 8) showed no binding with 125I-labeled Mab 174H.64. The immunoreactive protein bands in the keratinocyte SDS extract were of approximate molecular weight of 48,000–50,000 (possibly a doublet) and M, 57,000. The bovine and human SCC SDS extracts showed only one prominent band each with the approximate molecular weight of 48,000–50,000 for the former and M, 57,000 for the latter. A negative control antibody (155H.7) failed to bind with any of the above tumor extracts.

DISCUSSION

During our effort to develop Mabs against HCG and Tn antigen, we have serendipitously isolated a hybridoma specific for SCC. The origin of the hybridoma 174H.64 is not clearly understood. Since this antibody fails to react with HCG and Tn-HSA, it is unlikely to be related to the antigens used for immunization. The reactivity of this murine Mab against the basal cell population of the mouse skin suggests that its origin may be related to an autoreactive B-cell clone present in the mouse used for the immunization. Such autoantibodies against cytoskeletal components have been observed in laboratory animals (29). The characterization of the antigens detected by this antibody as cytoskeletal components is consistent with this hypothesis.

The results presented in Table 1 show the highly specific reaction of the Mab toward one histological type of tumor, SCC, irrespective of the organ of origin. Further, the SCC-specific reactivity of this Mab is not limited to human cancers as demonstrated by the immunohistological studies on sponta-
Fig. 1. Immunoperoxidase staining of various normal and tumor tissues of mammalian origin by using Mab 174H.64: (A) human anorectal SCC; (B) bovine SCC; (C) canine papilloma; (D) canine perianal adenoma; (E) stratified squamous epithelium of normal human cervix; (F) normal human thymus; (G) normal human mammary duct; (H) normal mouse keratinocytes; (I) normal human keratinocytes; and (J) murine lung SCC (KLN-205 cells).
neously arising tumors of cows, dogs, and cats. In well-differentiated SCC tumors, the peripheral layer of the tumor showed preferential binding with the Mab (Fig. 1A). Increased peripheral layer staining with this Mab was also observed for a spontaneous metastatic lung SCC tumor obtained from a cow (Fig. 1B). A mouse SCC tumor metastatic to the lung (KLN-205 mouse SCC) also demonstrated a similar pattern with selective binding of the antibody to the peripheral layer of the tumor (Fig. 1J). Well-differentiated SCCs are characterized by highly proliferative cells at the periphery of the tumor with the keratinized differentiated cells toward the center (30). Previous studies on the KLN-205 mouse SCC tumor model have demonstrated that the tumor periphery is composed of highly proliferative primitive cells which are the progenitors of the more differentiated cells in the central portion of the tumor (31). Therefore, the increased peripheral layer staining of SCC tumors with this Mab suggests that the antigen detected is associated with the proliferative compartment of the tumor. Among the benign tumors of dogs, the staining by the Mab was also limited to cells having basal characteristics (Fig. 1, C and D).

The only normal tissues showing reactivity with this antibody are the basal layer of the stratified squamous epithelium, thymic epithelium, and the myoepithelial cells of breast ducts (37). Cytokeratin 14 and 5 have also been reported to be present in epidermal keratinocytes (34, 35), thymic epithelial cells (36), and myoepithelial cells of breast ducts (37). Cytokeratin 14 and 5 have also been reported to be markers for neoplasms originating from stratified epithelium and are not detected in tumors of simple epithelium (17).

The high specificity of Mab 174H.64 toward SCCs of various origins suggests that it could be used as a reagent for unambiguous diagnosis of squamous tumors by immunohistochemical staining. This could be of special value in cases where histological typing of tumors by conventional staining methods are not satisfactory, as in some cases of adenosquamous carcinomas where it may be difficult to determine whether undifferentiated areas are of squamous or glandular origin. We are currently evaluating the potential of radiolabeled Mab 174H.64 as a reagent for noninvasive diagnosis of SCCs by using the murine lung SCC model described in this paper. Our preliminary results on the biodistribution of this antibody showed greater uptake of 125I-labeled Mab 174H.64 in the tumor-bearing lung compared to the normal lung, whereas the uptake of a control radiolabeled antibody (125I-labeled MOPC-21) in the tumor-bearing lung was not significantly different from that in the normal lung.5 These preliminary results suggest that the epitope detected by Mab 174H.64 is expressed also on the cell surface of the KLN-205 cells. Such cell surface domains have been reported for some cytoskeletal proteins (40, 41). Mab 174H.64 may be detecting novel cytoskeletal proteins expressed only in the stem cell populations of normal and neoplastic stratified squamous epithelium.

ACKNOWLEDGMENTS

We are grateful to Dr. M. R. Suresh for helpful suggestions and to T. Tan, B. Widtman, L. MacQueen, and M. Meeker for technical assistance.

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