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Expression of a Rat Ovary-independent Mammary Tumor-associated Antigen Defined by a Monoclonal Antibody, TAK-B1

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A monoclonal antibody, TAK-B1, was produced by immunization of BALB/c mice with mammary carcinoma induced in inbred Sprague-Dawley rats by treatment with 7,12-dimethylbenz[*a*]anthracene. TAK-B1 reacted with ovary-independent mammary carcinoma cells which had been transformed from ovary-dependent mammary carcinoma cells, but did not react with original mammary carcinoma cells or with cells from mammary glands exhibiting fibrocystic changes or normal mammary glands. However, TAK-B1 reacted not only with basal cells of the epidermis and epithelial cells of the bottom portion of crypts of the small intestine in adult rats, but also with basal cells of epidermis in skin and mesenchymal cells around developing hair follicles in fetuses. We therefore classify TAK-B1 as an ovary-independent rat mammary tumor-associated antigen. Immunoelectron microscopic examinations revealed that the antigen recognized by TAK-B1 was localized in the cell surface membrane of ovary-independent mammary carcinoma cells. Immunoprecipitation assay revealed that the antigen recognized by TAK-B1 was composed of M 220,000 protein and four other minor proteins.

Key words: Rat — Mammary neoplasm — Monoclonal antibody

Mammary carcinogenesis in animals and man is known to be strongly influenced by hormonal conditions. It is now generally appreciated that 20 to 30% of human breast carcinomas have estrogen receptors (ER) and progesterone receptors (PgR), and almost all tumors lacking these receptors initially fail to respond to endocrine therapy, such as surgical estrogen removal and antiestrogen drugs.[1,2] Conversion of positive ER to negative ER and non-response to therapy have been reported to occur in about one-half of the initially endocrine-responsive tumors with positive ER during endocrine therapy.[3,4] Mammary carcinomas which are unresponsive to endocrine therapy often become life-threatening. It is therefore important to clarify the characteristics of this type of mammary carcinoma. We established a monoclonal antibody, TAK-B1 which reacted with endocrine therapy-unresponsive mammary carcinomas. In the present paper, the distribution and biochemical characteristics of the antigen are reported.

MATERIALS AND METHODS

Tumors (Table I) and mammary dysplasia FT6, FT7, FT 10 and FT21 were poorly differentiated transplantable mammary adenocarcinomas without ER and PgR, and they were considered to be hormone-independent because they grew in female rats which had had ovariectomy or injections of pharmacological doses of estrogens. They had been derived from the original mammary carcinomas FT13, K25, TT509 and OK101, respectively, after implantation of the original tumors in ovariectomized inbred Sprague-Dawley (SD) rats receiving daily injections of 10 μg of 17β-estradiol (E), and had been maintained for more than 1 year. The original tumors had been induced in SD female rats by oral administration of 20 mg of 7,12-dimethylbenz[*a*]anthracene (DMBA) and were well differentiated adenocarcinomas with ER and PgR. They were considered to be hormone-dependent because they did not grow in female rats with ovariectomy or those receiving daily injections of pharmacological doses of estrogens.[5] Transplantable mammary carcinoma MT15 was established from a mammary carcinoma induced in male SD rats by multiple administration of DMBA, and was a moderately differentiated adenocarcinoma with ER and PgR. MT15 grew equally well in males, females and gonadectomized males. However, the growth of MT15 was inhibited markedly by injections of pharmacological doses of E.[6] Transplantable mammary dysplasia MD1 was induced in female SD rats by a single administration of DMBA.

Production and purification of mouse monoclonal antibody BALB/c mice were immunized by subcutaneous inoculation of 10^8 cells of a mixture of FT13, MT15 and FT6, followed by intraperitoneal boosting at 38 days after the primary inoculation. Their splenocytes were fused with the myeloma cell line Sp2/0-Ag14[7] 4 days after the intraperitoneal injection. Culture supernatants of hybridomas were screened by immunoperoxidase

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Table I. Characteristics of Rat Mammary Carcinomas

	Rat mammary carcinoma		
	FT13, K25, TT509, OK101	MT15	FT6, FT7, FT10, FT21
Induction	DMBA 20 mg×1 in famale rats	DMBA 10 mg×8 in male rats	*in vivo* transformation
Estrogen receptors	positive	positive	negative
Progesterone receptors	positive	positive	negative
Ovary dependency in transplanted rats	dependent	independent	independent
Sensitivity to pharmacological doses of estrogens	sensitive	sensitive	insensitve
Histology	papillary	medullary	medullary poorly differentiated carcinosarcoma

staining for rat mammary carcinomas as described below. Selected hybridomas were cloned by limiting dilution and propagated in ascites of pristane-primed BALB/c mice.

Monoclonal antibodies (MoAbs) in ascitic fluids were precipitated by 50% saturated ammonium sulfate and purified by Protein A-Affigel chromatography (Bio-rad, Richmond, CA). The purity of IgG was more than 95% on SDS-PAGE[8] with Coomassie blue staining. The isotype of the MoAb was determined by double immunodiffusion using rabbit anti-mouse isotype antibodies (ICN Immunobiologicals, Lisle, IL). Purified MoAb was stored in PBS-0.1% NaN_3 at 4°C until used.

Preparation of tissue sections and immunoperoxidase Tissues of adult and fetal SD rat were fixed in 10% buffered formalin and embedded in paraffin. The tissues were sectioned at 5 μm thickness. Immunoperoxidase staining was performed according to the method of Hsu *et al.*[9] with slight modifications. Deparaffinized sections were immersed in 0.5% H_2O_2-methanol for 10 min, treated with PBS containing 1% bovine serum albumin (BSA) and then incubated for 60 min at room temperature with either test hybridoma supernatants or 20 μg/ml of purified MoAb. An unrelated isotype-matched MoAb was used as a negative control in every test. After being washed in PBS, the sections were incubated for 30 min with biotinylated horse anti-mouse immunoglobulin antibody (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA). The sections were reacted with avidin DH-biotinylated horseradish peroxidase (HRPO) H complex and 3,3′-diaminobenzidine (DAB) according to the manufacturer's instructions. The sections were counterstained in 0.02% methylgreen, dehydrated through graded ethanol and mounted in Eukitt (O. Kinder, Germany) for light microscopic examination.

Immunoelectron microscopy Rat mammary carcinoma was fixed with periodate-lysine-paraformaldehyde,[10] washed with sucrose-PBS at 4°C and sectioned by using a cryostatic microtome. The sections were incubated with 1% BSA-PBS, and then with 20 μg/ml of MoAb (whole IgG), followed by reaction with HRPO-conjugated Fab portion of rabbit anti-mouse IgG antibodies. They were refixed with 1% glutaraldehyde-PBS for 10 min at 4°C, washed with PBS, and immersed for 30 min in 0.2 mg/ml DAB-1% dimethylsulfoxide-0.05 *M* Tris-HCl (pH 7.2). The sections were then developed with 0.2 mg/ml DAB-0.01% H_2O_2-0.05 *M* Tris-HCl (pH 7.2) for 10 min. After being washed, the sections were post-fixed with 2% OsO_4 in 0.1 *M* phosphate buffer, washed, hydrated with ethanol and embedded in epoxy resin. They were then stained with lead citrate and examined by electron microscopy.

Enzymatic and chemical treatment of tissue sections For analysis of the antigenic moiety recognized by TAK-B1, the sections of formalin-fixed FT6 carcinoma were pretreated with neuraminidase (10 mU/specimen) for 30 min at 37°C, 1% $NaIO_4$ in 0.1 *M* acetate buffer (pH 4.0) for 10 min at room temperature, 0.05% pronase in 0.05 *M* Tris-HCl (pH 7.2) for 20 min at room temperature or 0.1% trypsin in 0.1% $CaCl_2$-0.05 *M* Tris-HCl (pH 7.2). After being washed in PBS, the sections were stained with TAK-B1 as described above.

Cell culture TF6 tumor was removed aseptically, washed with PBS to remove blood and necrotic tissues and then dispersed with 0.25% trypsin and 0.1% EDTA in PBS. The dispersed cells were passed through a stainless steel mesh (62.5 μm) to remove undigested tissues, washed

twice, placed in 10 cm dishes and cultured in DMEM (Nissui Seiyaku Co. Ltd, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, North Ryde, Australia) and kanamycin (100 μg/ml, DMEM growth medium). The cultured cells were composed of pang-stone-shaped tumor cells and fibroblasts. A tumor cell line (KU-1) was established by repeating the cloning procedures with a paper disc method.

Radioimmunoprecipitation and SDS-PAGE analysis KU-1 cells were surface-labeled with ^{125}I by incubating a confluent monolayer of cells (2×10^6/10 cm dish) with 1 ml of reaction mixture containing 200 μg of lactoperoxidase, 0.01% H_2O_2 and 0.25 mCi of $Na^{125}I$. After incubation for 15 min at 22°C, the cells were washed 3 times with 5 ml of PBS containing 0.02% NaN_3 and 2 m*M* KI, and lysed with 1 ml of lysing buffer (20 m*M* Tris-HCl, pH 8.0, containing 100 m*M* NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.02% NaN_3 and 2 m*M* phenylmethylsulfonyl fluoride) for 10 min at 0°C. The lysate was centrifuged at 100,000*g* for 30 min at 4°C. The supernatant was used immediately for immunoprecipitation.

Exponentially growing cells (5×10^5/35 mm dish) were metabolically labeled by incubation with 0.1 ml of methionine-free MEM supplemented with 10% dialyzed FCS and 30 mCi of [^{35}S]methionine (1160 Ci/mmol; ICN Radiochemicals, Irvine, CA) for 30 min at 37°C and chased with DMEM growth medium supplemented with 0.2 m*M* of unlabeled methionine. The labeled monolayer cells were washed with PBS and lysed in 0.25 ml of lysing buffer. The cell lysate was centrifuged as described above and used immediately for immunoprecipitation. For indirect immunoprecipitation,[11] 10 μl of a 10% suspension of protein A-Affigel beads (Bio-Rad) was incubated at 4°C for 1 h with 20 μg of rabbit anti-mouse IgG (Cappel, West Chester, PA) in 0.2 ml of TNB buffer. The beads were washed twice with TNB buffer, incubated for 1 h at 4°C with 40 μl of 1 mg/ml MoAb in TNB buffer and washed twice with TNB buffer. The beads were then incubated overnight at 4°C with a 50 μl aliquot of radiolabeled cell extract which had been preabsorbed with rabbit anti-mouse IgG-conjugated protein A-Affigel. The reacted beads were washed 6 times with TN buffer and boiled in Laemmli's buffer.[8] The eluate was analyzed by SDS-PAGE on slab gels and autoradiographed for ^{125}I-labeled samples or fluorographed for ^{35}S-labeled samples.[12]

RESULTS

Isolation of TAK-B1 MoAb Six hundred hybridoma culture supernatants were screened by immunoperoxidase staining for FT13, MT15 and FT6 mammary carcinomas. Among them, six hybridomas exhibited positive staining for at least one of the three mammary carcinoma tissues. One of the 6 hybridomas reacted only with FT6 and not with FT13, MT15 or normal mammary glands. This hybridoma was cloned, designated TAK-B1 and propagated in ascites. The isotype of TAK-B1 MoAb was identified as IgG1 by double immunodiffusion.

Immunohistochemical analysis of rat mammary disorders and normal rat mammary tissues The results of immunoperoxidase staining of tissue sections of rats with mammary disorders and normal rat mammary tissues

Table II. Immunohistochemical Staining of Rat Mammary Disorders and Normal Mammary Tissues with TAK-B1

Tissues	No. of positives/ no. of tissues tested	% of cells stained	Staining location
Mammary carcinoma			
FT13 series	0/20[a]		
K25 series	0/3[a]		
TT509 series	0/2[a]		
OK101 series	0/3[a]		
MT15 series	2/23[a]	20–40	Membrane
FT6 series	14/17[a]	20–90	Membrane
FT7 series	12/52[a]	0.1–10	Membrane
FT10 series	24/67[a]	0.1–40	Membrane
FT21 series	4/16[a]	0.1–5	Membrane
Mammary dysplasia	0/6		
Normal mammary gland	0/4		

a) Number of generations of tumors tested.

Table III. Immunohistochemical Staining of Normal Tissues of Adult Rats with TAK-B1

Group	Tissues	Positives/ tissues tested	Staining location
1	Epidermis	4/4	Membrane of cell layers
	Hair follicles	4/4	Membrane of outer root sheath cells
	Tongue	3/3	Membrane of basal cells
	Duodenum	3/3	Membrane of epithelial cells
	Jejunum	3/3	in the bottom of crypts
	Ileum	4/4	
2	Colon	4/4	Golgi area of all epithelial cells
	Pancreas	4/4	Golgi area of acinar cells
3	Submandibular gland	2/2	Cytoplasm of serous gland cells
	Sublingual gland	4/4	Cytoplasm of serous gland cells
	Stomach	4/4	Cytoplasm of chief cells
4	Adrenal gland	0/2	
	Pituitary gland	0/1	
	Thyroid gland	0/1	
	Liver	0/4	
	Spleen	0/4	
	Kidney	0/4	
	Urinary bladder	0/1	
	Ovary	0/1	
	Testis	0/1	
	Uterus	0/2	
	Mammary gland	0/4	
	Heart	0/3	
	Lung	0/4	
	Skeletal muscle	0/3	
	Bone marrow	0/1	
	Cerebrum	0/3	
	Cerebellum	0/3	
	Medulla oblongata	0/2	
	Spinal cord	0/1	
	Thymus	0/1	

Table IV. Immunohistochemical Staining for the Skin and Mammary Gland of Fetus and Newborn Rats

	Age (days)	Epidermis		Hair follicles	Mesenchymal cells around hair follicles	Mammary gland
		epidermal cells	basal cells			
Fetus	17–19	(+)	(−)	(−)	(+)	(−)
	20–21	(+)	(+)	(+)	(+)	(−)
Newborn	2	(−)	(+)	(+)	(+)	(−)
	11	(−)	(+)	(+)	(−)	(−)

with TAK-B1 antigen are summarized in Tables II, III and IV. TAK-B1 antigen was detectable in ovary-independent FT6, FT7, FT10 and FT21 tumors (Fig. 1), whereas no staining was observed in any generation of ovary-dependent FT13, K25, TT509 and OK101 tumors. TAK B1 antibody was reactive with MT15 tumors of only 2 out of 23 generations. In positively stained carcinomas, the immunostaining was localized in the cell

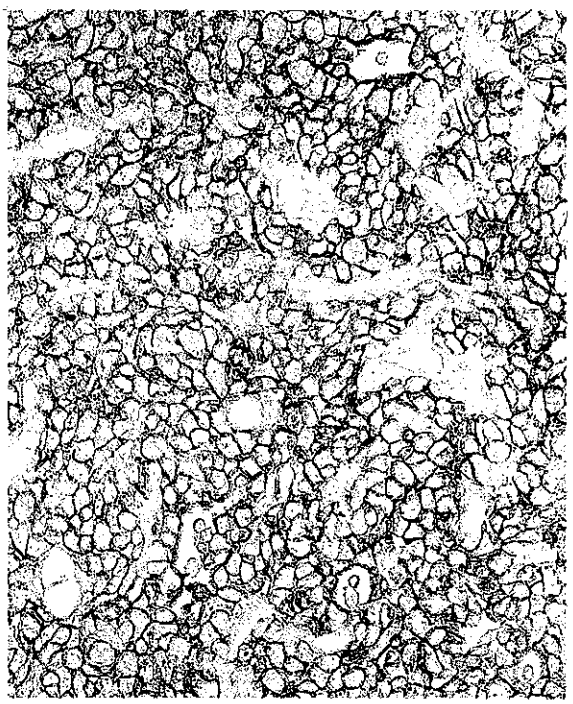

Fig. 1. Immunoperoxidase staining of ovary-independent mammary carcinoma, FT6. Note the membrane localization of staining in positive cells. ×350.

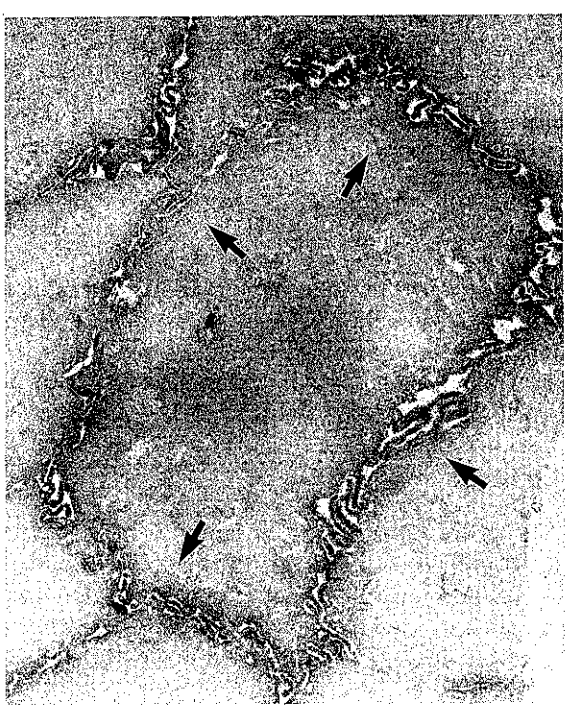

Fig. 2. Immunoelectron microscopic analysis of FT6 with TAK-B1. Microvillous portions of surface membrane (←) were positively stained. ×6500.

surface membrane. This finding was confirmed by immunoelectron microscopic examinations in which TAK-B1 antigens were found to be localized in the microvillous portions of the surface membranes of FT6 tumors (Fig. 2). On the other hand, no detectable staining was observed with TAK-B1 in normal mammary glands or DMBA-induced mammary dysplasia.

Immunohistochemical analysis of normal tissues of adult rats A total of 85 tissues from 26 different organs of adult SD rats were examined for TAK-B1 antigen. As shown in Table III, the tissues were categorized into 4 groups according to the pattern of TAK-B1 staining. Group 1 includes the epidermis, hair follicles, tongue and small intestine. TAK-B1 antigen was expressed in the cell surface membranes of basal cells of the epidermis near the outlet of hair, epithelial cells of the outer root sheath of hair follicles, basal cells above the secondary papillae of the tongue and epithelial cells in the bottom portion of crypts in the small intestine (Fig. 3). Group 2 includes the colon and pancreas, where the positive staining was localized in the Golgi area of the cells. Group 3 includes salivary glands, where TAK-B1 antigen was expressed in the cytoplasm of serous cells, and the stomach, where it was expressed in the cytoplasm of chief cells. Group 4 includes normal mammary glands and other tissues with no expression of TAK-B1 antigen.

Immunohistochemical staining of mammary glands and skin in rat fetuses and newborns The results of immunohistochemical examination of expression of TAK-B1 in fetal mammary glands and skin tissues are summarized in Table IV. No staining was observed in the

Fig. 3. Immunoperoxidase staining of the intestine of adult rats. Epithelial cells (←) in the bottom portion of crypts were positive. ×350.

Fig. 4. Immunoperoxidase staining of hair follicles at the 20th day of gestation. Mesenchymal cells around hair follicles were strongly stained. ×350.

rudiment of mammary glands from the 16th to the 21st day of gestation. On the other hand, positive staining was observed in basal cells of the epidermis and in mesenchymal cells around hair follicles. On the 17th day of gestation when the rudiment of hair follicles began to appear, TAK-B1 antigen appeared in a few layers of mesenchymal cells around the rudiments of hair follicles. The mesenchymal cells were most strongly stained on the 20th day of gestation (Fig. 4), and became negative for TAK-B1 by the 11th day after birth. Epithelial cells of developing hair follicles were slightly stained before birth. On the second day after birth, the surface membranes of the outer root sheath cells of hair follicles were prominently stained.

Effect of enzymatic and chemical treatments of sections In order to characterize the epitope of TAK-B1 antigen, sections of FT6 tumor were pretreated with pronase, trypsin, neuraminidase and $NaIO_4$. The treatments with pronase and trypsin destroyed the antigenicity, whereas neuraminidase and $NaIO_4$ treatments had no effect on immunostaining with TAK-B1. This indicates that the antigenic moiety for TAK-B1 is protein and not carbohydrate in nature.

Biochemical analyses of TAK-B1 antigens Fig. 5 depicts the result of indirect immunoprecipitation of radiolabeled extracts of KU-1 cells and subsequent analyses by SDS-PAGE. Under both reducing and nonreducing conditions, TAK-B1 immunoprecipitated from surface-iodinated cell extracts a major component with a molecular weight of approximately 220K and four other minor bands with molecular weights of approximately 240K,

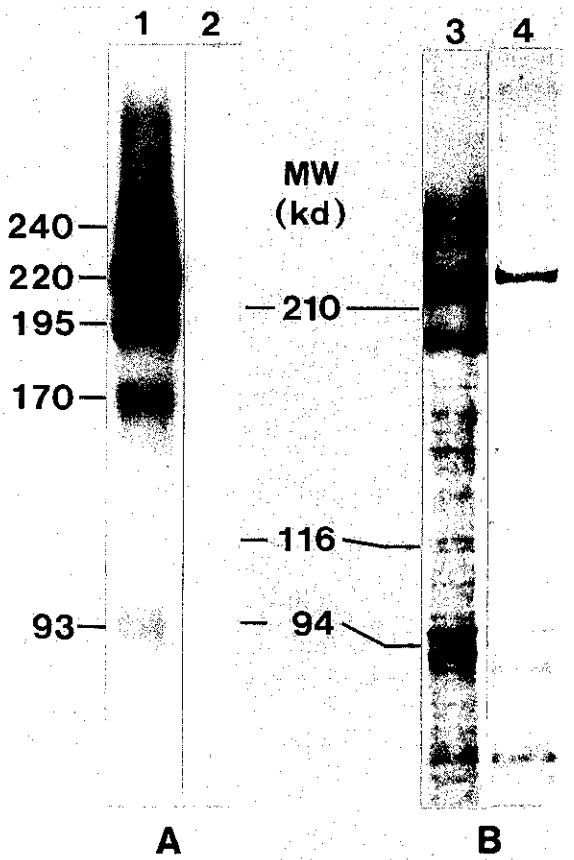

Fig. 5. SDS-PAGE analysis of immunoprecipitates obtained by reacting monoclonal antibody immunosorbents with detergent extracts of KU-1 cells labeled with either ^{125}I (A) or [^{35}S]-methionine (B). Lanes 1 and 3, TAK-B1; lanes 2 and 4, KV-A1 (negative control). Migration of molecular weight standards is shown in the center.

195K, 170K and 93K (Fig. 5A). Similar immunoprecipitation patterns were obtained from [^{35}S]methionine-labeled extracts under both reducing and nonreducing conditions. The same major component and four other minor bands were visualized (Fig. 5B).

DISCUSSION

The results of the present study revealed that the TAK-B1 antigen was expressed in the cell surface membrane of hormone-independent mammary carcinomas which had been derived from hormone-dependent mammary carcinomas. The TAK-B1 antigen was not, however, expressed in the original hormone-dependent mammary carcinomas, mammary glands with fibrocystic changes or normal mammary glands. This antigen is therefore thought to be expressed in the progression of

mammary carcinomas. Histochemical examination revealed that the TAK-B1 antigen was expressed in some normal tissues which were not thought to be sex hormone-target tissues. The distribution of the TAK-B1 antigen in normal tissues is quite restricted (Tables III and IV). As in hormone-independent mammary carcinoma cells, TAK-B1 antigen was expressed in the cell surface of basal cells of skin, outer root sheath cells of hair follicles and epithelial cells in the bottom portion of crypts of the small intestine in postneonatal rats and in the cell surface of epidermal basal cells and hair follicular cells in the differentiating stage of rat fetus. These normal cells are generally known to be actively proliferative in postneonatal rats.[13] These facts suggest that TAK-B1 antigen might be associated with sex hormone-independent cell proliferation and that ovary-independent mammary carcinomas might obtain this character from abnormal expression of the TAK-B1 antigen. Several investigators have reported MoAbs directed to mammary tumor-associated antigens in humans, rats and mice.[14–17] A comparison of the molecular weights and the distribution in normal tissues shows TAK-B1 MoAb to be distinct from those MoAbs.

The distribution of the TAK-B1 antigen in fetuses and adult rats seems partly similar to that of epidermal growth factor (EGF) receptors according to previous studies.[18–20] However, EGF receptors are known to be expressed in normal mammary glands,[20] whereas TAK-B1 is not. Green *et al.* reported that EGF receptors were consistently present in a layer of basal skin cells and in the outer root sheath cells of developing hair follicles between 17–20 days of gestation.[19] We observed TAK-B1 antigen in basal skin cells at 20–21 days of gestation. However, TAK-B1 antigen was only slightly expressed in the epithelial cells of hair follicle rudiments, but was strongly expressed in mesenchymal cells around the developing hair follicles. It is noteworthy that the TAK-B1 antigen is not expressed in fetal mammary glands and surrounding mesenchymal cells, particularly since the rudiment of both mammary glands and hair follicles originates from the ectoderm and appears as solid epidermal proliferation with dense underlying mesenchymal cells at the 13th day of gestation in the former and at the 17th day in the latter.[19,21] It thus seems that the TAK-B1 antigen may not involve exactly the same EGF receptor, and it may be involved in the induction of hair follicles by mesenchymal cells and play an important role in dermal/epidermal interactions required for the development of hair follicles.[22]

In addition to disparities of cell-type localization within specific tissues, we also found various localizations of TAK-B1 antigen within cells, as has also been reported for EGF receptors.[20] The localization of the TAK-B1 antigen in the cytoplasm may reflect internalization of the antigen or may represent newly biosynthesized molecules. Further studies are necessary.

Examination by immunoprecipitation and autoradiography revealed that the TAK-B1 antigen precipitated the major 220K protein and four minor proteins with a molecular weight of 240K, 195K, 170K, and 93K from the cell lysates of UK-1, which was derived from ovary-independent adenocarcinoma, FT6. One interpretation of this result is that TAK-B1 reacts only with the major 220K protein and that the other 4 components are closely associated with 220K protein so that these components are coprecipitated with TAK-B1/220K protein complex in immunoprecipitation. Alternatively, TAK-B1 may bind to each of the 5 protein which may share a common cross-reactive epitope.

The treatment of FT6 tissue sections with trypsin and pronase destroyed the antigenicity of TAK-B1 antigen, whereas treatment with $NaIO_4$ and neuraminidase did not. This indicates that TAK-B1 antigen binds to protein, although further biochemical analyses will be necessary to define the properties of the TAK-B1 antigen.

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