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A Monoclonal Antibody against Human Colon Cancers

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Kotanagi, H., Takahashi, T., Masuko, T., Hashimoto, Y. and Koyama, K. *A Monoclonal Antibody against Human Colon Cancers.* Tohoku J. exp. Med., 1986, **148** (4), 353-360 —— A monoclonal antibody was prepared by hybridizing mouse myeloma cells with spleen cells from the mouse which was immunized with human colon cancer transplanted in nude mice. The reactivity of the monoclonal antibody, named A7, was tested by immunoperoxidase method. A7 reacted strongly with human adenocarcinoma cell lines and carcinoembryonic antigen (CEA). In surgical specimens, A7 reacted with 10 cancer tissues and 2 normal colon mucosa from 19 colorectal cancer patients. A7 did not react with other cancers. It was thought that A7 reacted with colon- or colon cancer-specific CEA. The reactivity of A7 with colorectal cancers was markedly reduced by preoperative irradiation. ———— monoclonal antibody; human colorectal cancers; carcinoembryonic antigen

Many monoclonal antibodies against human colorectal cancers have been prepared by cell fusion technique, using established cell lines as immunogen (Koprowski et al. 1979; Smedley et al. 1983; Kaszubowski et al. 1984). However, cell lines might change their antigenicity during continuous cell cultures (Irie et al. 1974; Dexter et al. 1979; Laboisse et al. 1981). Human cancer transplanted in nude mice is considered to maintain similar antigenicity to original cancer in patient (Visfeldt et al. 1972; Sordat et al. 1974; Shimosato et al. 1976). Accordingly, human colon cancer transplanted in nude mice was used as immunogen in this study.

Materials and Methods

Human cells and tissues. As an antigen for monoclonal antibody, human colon cancer Colon-6 was obtained from 64 year old patient and was maintained in nu/nu, BALB/c nude mice in our laboratory. For immunohistochemical analysis, cultured human cell lines as shown in Table 1, and surgically resected tissues were used in this series. In surgically resected colorectal cancer tissues, 7 of 19 tissues received irradiation at the dose of 3,000 rad

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9 to 15 days before operation.

Preparation of hybridomas. The isolated Colon-6 tissue was minced and treated with 0.25% trypsin solution for 30 min at 37°C to obtain free cell suspension. The well-washed cells were used for immunization immediately after preparation. A female BALB/c mouse was immunized with s.c. and i.p. injections, each time with 1.5×10^6 Colon-6 cells suspended in 0.8 ml of phosphate buffered saline (PBS) at two-week intervals. The mouse was further immunized by an i.v. injection of 0.7×10^6 cells, and was sacrificed 3 days after immunization. Following depletion of erythrocytes, spleen cells (1.0×10^8 cells) from the immunized mouse were fused with the mouse myeloma P3.×63. Ag8. 653 cells (2.0×10^7 cells) in 50% polyethylene glycol 1540 solution for two min at 37°C. The cells were suspended in RPMI 1640 medium containing 10% fetal calf serum and distributed into Costar 96-well plates. After a 16 hr incubation, a half volume of the culture medium in each well was replaced by the medium containing hypoxanthine-aminopterin-thymidine. The medium changes were repeated over 10–14 days at 2-day intervals until hybridoma colonies were obtained. Hybridomas were subsequently cloned by limiting dilution in 96-well culture plates with thymocytes (1.0×10^6 cells/well) of BALB/c mouse as feeder cells. The cloned hybridoma cells were expanded by cultures in Costar flasks. The supernatants of cloned hybridoma cultures were used as the source of monoclonal antibodies.

Assays for reactivity of monoclonal antibodies. Hybridoma culture supernatants with no purification were used as antibody solution for the assays.

Immunoperoxidase methods. Cells (2×10^3 cells/well) from culture cell lines were incubated in Falcon microtest plates, for 24–48 hr at 37°C. The plates were washed with PBS and incubated with hybridoma culture supernatants for 45 min at 37°C. After a wash with PBS, cells were incubated for an additional 45 min with a solution of peroxidase-conjugated rabbit anti-mouse immunoglobulin (Zymed), washed, and developed with a solution containing 0.05% 2,2′-azino-di-(3-ethylbenzothiazolin)-6-sulfonic acid (ABTS, Sigma) and 0.01% hydrogen peroxide in 0.05% citrate buffer, pH 4.0, for 20 min at room temperature. The development of a green color was evaluated macroscopically and graded as negative (−), weak positive (±) and positive (+). Cryosections of 9 μm thickness were prepared from the surgical specimens and were stained within 72 hr after operation by the method of Finan et al. (1982). After preincubation with 10% rabbit serum in PBS, sections were overlayed with hybridoma culture supernatants for 45 min at room temperature. A second 45 min incubation was performed with peroxidase-conjugated rabbit anti-mouse immunoglobulin. After a wash, sections were developed by incubation for 15 min with PBS containing 0.05% diaminobenzidine (Sigma) and 0.2% hydrogen peroxide. The sections were counterstained with hematoxylin, mounted and examined at ×100 magnification. The sections, which were including more than 10% of cells stained brownly by diaminobenzidine were evaluated positive (+), and which were including less than 10% of cells were evaluated negative (−). Reactivity of the monoclonal antibodies with highly purified CEA preparation which was isolated from a metastatic liver tumor from human colon cancer (donated by Dr. Y. Matsuoka, School of Medicine, Fukuoka University, Fukuoka) was examined by following procedure. Fifty μl of CEA solution which contained 0.1 mg of CEA per 1 ml of PBS, was added to each of a Costar polyvinyl chloride plate and was kept overnight at 4°C. After a wash with PBS, 1% bovine serum albumin (Sigma) in PBS was added and the plates were incubated for 2hr at 4°C to cover the remaining non-specific binding sites of the wells. The wells were washed again, and were incubated with antibody solution for 1 hr, and additional 1 hr with peroxidase-conjugated rabbit anti-mouse immunoglobulin. The activity of peroxidase in each well was assayed by incubating with ABTS solution for 20 min. The development of a green color was judged as positive (+).

Radioimmunoassay (RIA). Reactivity of the monoclonal antibodies with erythrocytes and lymphocytes from healthy volunteers were examined by RIA. Test cells (1×10^3 cells) were successively treated with monoclonal antibody solution, rabbit anti-mouse

IgG and IgM serum, and ^{125}I-labeled staphylococcus aureus Protein A (100,000 cpm/sample). All these treatments were completed within 1 hr at 4°C. The cell-bound radioactivities (cpm) were counted in a gamma scintilation counter. Control cells were treated with fresh culture medium instead of monoclonal antibody. When cpm of the cells treated with monoclonal antibody were less than two folds of that of control, the monoclonal antibody was judged as negative for reacting with erythrocytes and lymphocytes.

Results

Reactivity of monoclonal antibodies with cultured human cell lines and CEA preparation. In the initial screening test, 306 growing hybridomas were found to produce antibodies reactive to colon cancer cell line SW 1116. One of the hybridomas which secreted antibodies, unreactive to erythrocytes, lymphocytes and normal fibroblast, were selected and cloned. The monoclonal antibody, named A7, was classified IgG_1 with *k*-light chain as judged by a double immunodiffusion test with standard rabbit anti-mouse immunoglobulin sera. The reactivity of the monoclonal antibody with established human cell lines and purified CEA preparation was tested by immunoperoxidase method (Table 1). Monoclonal antibody A7 reacted strongly with CEA preparation and adenocarcinoma cell lines, such as colon, stomach and breast cancer cell. A7 reacted weakly with a few squamous cell carcinoma cell lines. A7 did not react with

Table 1. *Reactivity of monoclonal antibody A7 with human cell lines and CEA by immunoperoxidase method*

Target cells			Reactivity of A7
Cell origin	Cell line	CEA synthesis*	
Colon cancer	SW1116	+	+
Colon cancer	SW1083	+	+
Stomach cancer	GAS-1†	+	+
Breast cancer	MCF-7	−	+
Kidney cancer	SK-RC-9	−	+
Esophagus cancer	TE-2	+	±
Skin cancer	HSC-1	−	±
Lung cancer	SK-MES-1	−	−
Cervix cancer	ME-180	+	−
Bladder cancer	T-24	−	−
Bladder cancer	KU-1	−	−
Astrocytoma	SK-MG-1	−	−
Fetal fibroblast	HEL	−	−
Carcinoembryonic antigen (CEA)‡			+

* CEA level in the used medium of confluent culture was measured by a ABBOTT's CEA-EIA kit.
† established in our laboratory from stomach cancer maintained in nude mice.
‡ originated from a metastatic lesion of colon cancer.

TABLE 2. *Reactivity of monoclonal antibody A7 with human fresh cells and tissues by immunoperoxidase method*

Target cells and tissues	Reactivity of A7
Normal	
Erythrocytes	0/10*
Peripheral lymphocytes	0/10
Colon mucosa	2/19
Ileum mucosa	0/ 3
Stomach mucosa	0/ 8
Malignant	
Colorectal cancer	10/19
Stomach cancer	0/ 7
Breast cancer	0/ 2
Stomach sarcoma†	0/ 1
Colon-6‡	1/ 1

* number of positive cases/number of cases tested.
† malignant lymphoma of the stomach.
‡ colon cancer maintained in nude mice.

cervix, bladder cancer nor astrocytoma cell line.

Reactivity of the monoclonal antibody with fresh human cells and tissues. Reactivity of A7 with erythrocytes and peripheral lymphocytes was examined by RIA. A7 did not react with erythrocytes nor lymphocytes from 10 volunteers. Reactivity of A7 with surgically resected normal and malignant tissues was examined by immunoperoxidase method, after preparing those cryosections (Table 2). A7 reacted with 2 normal colon mucosa, 10 colorectal cancers from 19 patients, and with Colon-6 cancer maintained in nude mice. A7 did not react with 3 normal ileum mucosa nor 8 stomach mucosa.

Reactivity of A7 with human colorectal cancers. Reactivity of A7 with 19 colorectal cancers was indicated in Table 3. A7 reacted with 10 colorectal cancers by immunoperoxidase method. In 8 of these 10 reacted cancers, only cancer cells were selectively stained, but in remaining 2 cancers, normal mucosa were stained slightly. There was no cases in which only normal mucosa was stained. With regared to the relationship between the reactivity of A7 and irradiation, A7 reacted with 9 of 12 not-irradiated cancers, but with only 1 of 7 irradiated cancers. As to the serum CEA level of patients, it was higher than normal limit (4 ng/ml) in 14 patients. A7 reacted with 8 of these 14 cancers, and reacted with 2 of 5 cancers whose serum CEA levels were within normal limit. Reactivity of A7 with colorectal cancers had no relation to locations or histologic figures of cancers.

TABLE 3. *Reactivity of monoclonal antibody A7 with colorectal cancer patients*

Case	Age	Sex	Cancer location	Cell type	Serum CEA level* (ng/ml)	Peoperative irradiation (3,000 rad)	Reactivity	
							Cancer tissue	Normal mucosa
1	71	F	R†	Well	28.1	−	+	−
2	59	M	R	Moderate	2130.0	−	+	−
3	63	M	R	Mucinous	5.1	−	+	−
4	68	F	S	Moderate	21.0	−	+	−
5	72	M	S	Moderate	2.2	−	+	−
6	54	F	D	Moderate	6.0	−	+	+
7	67	F	D	Moderate	4.6	−	+	−
8	35	M	A	Well	18.3	−	+	−
9	71	F	A	Moderate	3.7	−	+	+
10	40	F	A	Moderate	5.3	−	−	−
11	48	M	S	Well	3.4	−	−	−
12	37	M	S	Moderate	3.0	−	−	−
13	58	F	R	Well	17.0	+	−	−
14	76	M	R	Well	6.0	+	−	−
15	59	F	R	Well	67.0	+	−	−
16	58	F	R	Moderate	3.0	+	−	−
17	70	M	R	Moderate	11.1	+	−	−
18	71	F	R	Moderate	16.2	+	+	−
19	42	F	S	Moderate	52.8	+	−	−

* level of serum CEA was measured just before operation by using a ABBOTT's CEA-EIA kit.

† Abbreviation: R=rectum, S=sigmoid colon, D=descending colon, A=ascending colon.

DISCUSSION

To obtain the monoclonal antibody against human colorectal cancers by a cell fusion technique, human colon cancer maintained in nude mice was used as immunogen in this study. This immunogen could be used repeatedly, and was thought to have characteristics more similar to original human cancer than cell lines. It was indeed recognized by the facts that much monoclonal antibodies reactive with human erythrocytes, lymphocytes, normal fibroblast were obtained in this early screening test. Therefore, for the production of monoclonal antibody against human cancers, cancer transplanted in nude mice was thought to be much useful.

Monoclonal antibody A7 reacted strongly with adenocarcinoma cell lines and CEA, but A7 did not react with 3 stomach cancers whose serum CEA levels were higher than normal limit. Despite of positive reaction with cultured cell lines of stomach and breast cancers, A7 did not react with surgically resected-

adenocarcinomas except colon cancers. Restricted to the reactivity with surgical specimens, A7 reacted only with colon mucosa and colon cancers. Though 2 of 19 normal colon mucosa were stained by immunoperoxidase method, their staining intensities were much faint and local. It suggests that the A7-recognizing antigen was much more included in cancer tissues than in normal mucosa of the colon. Therefore, A7 might recognize colon- or colon cancer-specific CEA.

CEA was first described by Gold and Freedman (1965) as a cancer-specific fetal antigen in adenocarcinoma of the human digestive tract. Although the cancer specificity is, at present, rather controversial, CEA is considered to be one of cancer-related antigens, and its clinical significance is being widely accepted. The fact that CEA is a large molecule (Kupchik et al. 1973), is heterogenous (Gold et al. 1973), and possesses several different carbohydrates (Banjo et al. 1974) raises the possibility that multiple antigenic determinants may be present on the molecule. Matsuoka et al. (1975) showed a unique determinant in the CEA molecule which was detectable by specially prepared guinea pig anti-CEA antisera in almost all CEA preparations from tumor tissues, and designated it tentatively as cancer determinant. Kuroki et al. (1981) reported that CEA molecule possessed cancer specific antigenic determinants and the antigenic determinants cross-reactive with antigens which are extractable from feces of normal adults. Furthermore, Mori et al. (1975) reported the colon cancer-specific CEA by using conventional, polyclonal antibodies. Grunert et al. (1983) reported the difference of the antigenic determinants of CEAs which were derived from colon, lung and breast cancer, by using a set of monoclonal antibodies which were prepared against perchloric acid extracts of colon cancer. These reports suggest that CEA possesses cancer specific and organ specific antigens, besides common antigens found in normal adult feces. Though the reactivity of A7 with some cell lines did not corresspond to their CEA synthesis, this may be explained by the reasons that these cell lines have the A7-recognizing epitope which is not corss-reactive with the conventional CEA or have the epitope which is reactive with conventional CEA but is different from A7-recognizing epitope. Based on published data and our findings, it was considered that A7 was a first monocolonal antibody reactive with colon- or colon cancer-specific CEA.

Reactivity of A7 with colorectal cancers was markedly reduced by preoperative irradiation. It may be explained by the reason that cancer cells were destructed by irradiation, or by the reason that cancer cells changed their antigenicities without their destruction. Microscopic examination of irradiated cancer tissues revealed that more than a half of cells were living in these tissues. Accordingly, the reduction of reactivity of A7 with irradiated cancers was thought that cancer cells changed their antigenicities by irradiation. Some investigators (Ferrands et al. 1982; Smedley et al. 1983) reported cases that radiolabeled monoclonal antibody was not detected in the patients subjected to irradiation therapy. But, there was no report on the reactivity of monoclonal antibody with

directly resected materials from irradiated patients. The relationship between the reactivity of A7 with colorectal cancers and irradiation should be further examined and will be described in an another report.

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