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# Use of a Local Immunotherapy as an Adjunctive Tool for the Generation of Human Monoclonal Antibodies from Regional Lymph Nodes of Colonic Cancer Patients

Toshio Yagyu,[1] Takushi Monden,[1] Yasuhiro Tamaki,[1] Hideki Morimoto,[1] Tsutomu Takeda,[1] Tetsuro Kobayashi,[1] Takashi Shimano,[1] Hiroki Murakami[2] and Takesada Mori[1]

*[1]Second Department of Surgery, Osaka University Medical School, 1-1-50 Fukushima-ku, Osaka 553 and [2]Food Science and Technology Institute, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812*

**Human hybridomas were generated through the fusion of the human B-lymphoblastoid cell line HO-323 with the regional lymph node lymphocytes of colonic cancer patients who had received a local immunotherapy. A total of 353 hybridomas were obtained from 4 patients and 116 of these were found to secrete ≧100 ng/ml human immunoglobulin. The efficiency was remarkably high as compared with that from patients without the local immunotherapy. Further immunohistological examination showed that 5 hybridomas secreted IgM which selectively reacted with colonic cancers. The results indicate that local immunotherapy could be an adjunctive tool for the generation of highly potent human hybridomas through augmenting the host's immunity.**

Key words: Human-human hybridoma — Local immunotherapy — Colon cancer — *In vivo* activation — Tumor-associated antigen

The exploitation of human monoclonal antibodies (HuMAbs) is expected to provide us with useful means for the treatment of cancer. Although many papers have described the successful generation of HuMAbs to cancer, there have been few reports on the clinical application of HuMAbs to cancer treatment.[1)] The reason for this discrepancy is that most of the human hybridomas established so far have not consistently secreted enough immunoglobulins.[2)]

To obtain clinically effective human hybridomas, efforts have been concentrated on the preparation of competent B lymphocytes from the hosts. The methods employed for this purpose are the following: 1, *in vitro* immunization of B lymphocytes with tumor-associated antigens (TAA)[3)] or use of B lymphocytes possibly sensitized to TAA from various sources such as regional lymph nodes,[4)] spleen,[5)] and tumor-infiltrating lymphocytes (TIL)[6)]; 2, *in vitro* treatment of B lymphocytes with mitogens[7)] or *in vivo* vaccination of the hosts with adjuvants such as Bacillus Calmette-Guérin (BCG).[8)] The first method holds the promise of obtaining B lymphocytes with defined specificity, while the second seems to give rise to more hybridomas through non-specific activation. The results of recent studies on human hybridomas suggest that a combination of the two activation methods is desirable for the B lymphocytes to facilitate cell fusion.

In the present study, we used a local immunotherapy as an adjunctive tool for the generation of human hybridomas. The basis for our attempt was the concept that an effective local immunotherapy should be the best way to fulfill the requirements for activation of B lymphocytes because the immunomodulatory agents used in the treatment could be expected to evoke non-specific immune responses, and sensitization to TAA *in vivo* could also be expected because of the destruction of tumors induced by the immune reaction.[9, 10)]

To augment the immune responses, we applied a mixture of OK-432, an attenuated strain of *Streptococcus pyogenes*, and human fibrinogen as an immunogen. This immunotherapy has been described in detail elsewhere[9, 10)] and can effectively induce a state of granulomatous hypersensitivity in which tumors degenerate. Before surgery, 1 ml of a mixture of OK-432 (5 KE) (Chugai Pharmaceutical Co., Ltd., Tokyo) and human fibrinogen (80 mg) (OK-432/fbg) was administered intratumorally under endoscopy. Regional lymph nodes were obtained through surgery, and lymphocytes were isolated and resuspended in RPMI 1640 with 10% FBS. After incubation for 48 h at 37°C, the cells were fused with a human B-lymphoblastoid cell line, HO-323,[11)] as described previously.[12)] In brief, HO-323 cells and lymph node lymphocytes were fused at a 1:2 ratio and seeded into 96-well tissue culture plates filled with a medium containing hypoxanthine, aminopterin and thymidine at a density of $6 \times 10^4$ cells per well. Hybridomas secreting ≧100 ng/ml HuMAbs were selected and the isotype of the secreted immunoglobulins was determined with the enzyme-linked immunoadsorbent assay (ELISA). The reactivity to xenografts of the colon cancer cell lines, LS174T and LoVo, was tested by indirect immunohisto-

Table I. Generation of Human Hybridoma with Lymphocytes from Regional Lymph Nodes of Colorectal Cancer Patients

| Ex | Days after OK/fbg injection | Hybridoma /wells | Antibody producer[a] | Cancer-reactive hybridoma[b] |
|---|---|---|---|---|
| 1 | 1 | 80/480 (16.7%) | 26/80 (32.5%) | 5/26 (19.2%) |
| 2 | 1 | 107/480 (22.2%) | 35/107 (32.7%) | 10/35 (28.6%) |
| 3 | 3 | 53/480 (11.0%) | 20/53 (37.7%) | 6/20 (30.0%) |
| 4 | 7 | 113/480 (23.5%) | 35/113 (30.9%) | 10/35 (28.6%) |
| 5 | —[c] | 92/360 (25.6%) | 4/92 (4.3%) | 0/4 |
| 6 | — | 12/360 (3.3%) | 0/12 | 0/0 |
| 7 | — | 105/360 (29.2%) | 10/105 (9.5%) | 1/10 (10.0%) |
| 8 | — | 66/240 (27.5%) | 6/66 (9.1%) | 0/6 |

*a*) Production of ≧100 ng/ml human immunoglobulin as measured by ELISA.
*b*) Immunohistochemical reaction against xenografted adenocarcinomas, LS174T and LoVo.
*c*) Without local immunotherapy.

Table II. Reactivity of Human MAbs to Colon Cancer and Normal Colonic Epithelium

| MAb | Colon cancer (positive/test) | Normal colonic epithelium (positive/test) |
|---|---|---|
| YJ37[a] | 7/19 | 0/15 |
| YJ41[b] | 5/9 | 0/8 |
| YJ21[b] | 2/8 | 0/7 |
| YJ11[b] | 5/7 | 0/7 |
| YJ22[b] | 5/9 | 0/8 |
| YJ42[b] | 6/6 | 4/8 |
| YJ24[b] | 3/4 | 2/4 |
| YJ12[b] | 4/9 | 3/7 |
| YJ23[b] | 5/9 | 4/7 |

*a*), *b*) Determined on microwave-fixed specimens by direct immunoperoxidase assay (*a*) or by indirect immunoperoxidase assay (*b*).

chemistry using the avidin-biotin peroxidase complex method. Hybridomas which showed strong reactivity to cancer cell lines were cloned twice by limiting dilution.

Through the fusions performed with lymphocytes from four patients who had received local immunotherapy one to seven days before operation, a total of 353 hybridomas were obtained. Although the yield of hybridomas in the patients who had received local immunotherapy was similar to that in the patients without this treatment, the ratio of the generation of hybridomas secreting a high level of HuMAbs was remarkably high in the patients with local immunotherapy. The isotype of the HuMAbs was determined to be IgM. Furthermore, immunohistochemical analysis showed that 20% or more of the hybridomas secreting HuMAbs showed strong reactivity to colon cancer xenografts. In contrast, cancer-reactive hybridomas could hardly be obtained from the patients without immunopotentiation (Table I).

We performed further immunohistochemical analysis of nine hybridomas which strongly reacted with xenografts. To test the specificity of HuMAbs, surgically resected colonic cancer tissues including adjacent normal epithelia were fixed through rapid microwave fixation, reportedly useful for the immobilization of various antigenic substances, and the tissues were then embedded in paraffin.[13] The results of immunostaining of the sections with the HuMAbs are shown in Table II. All HuMAbs tested stained positively for colonic cancer tissues, and five out of nine hybridomas were shown to secrete HuMAbs that selectively reacted to cancer tissues but not to adjacent normal epithelia. Apical staining of cancer cells indicated that the antigenic substances recognized by these HuMAbs were associated with the cell surface (Fig. 1a), and subsequent analysis with a membrane immunofluorescence technique clearly showed that the HuMAbs stained the cell membrane (Fig. 1c). We performed further direct immunohistochemical staining on various human tissue sections with biotinylated YJ37, one of the monoclonal antibodies presented in Table II. The biotinylation was done to prevent non-specific reaction caused by endogenous immunoglobulin in the tissue sections. The results showed that HuMAb YJ37 selectively reacted to about half of the cases of colonic cancer, gastric cancer, endometrial cancer and colonic adenoma (data not shown).

This is the first report demonstrating that local immunotherapy could be an adjunctive tool for generation of human hybridomas which secrete cancer-reactive HuMAbs. The intratumoral injection of OK-432/fbg seems to have contributed to both the sensitization and the differentiation of B lymphocytes because OK-432 has

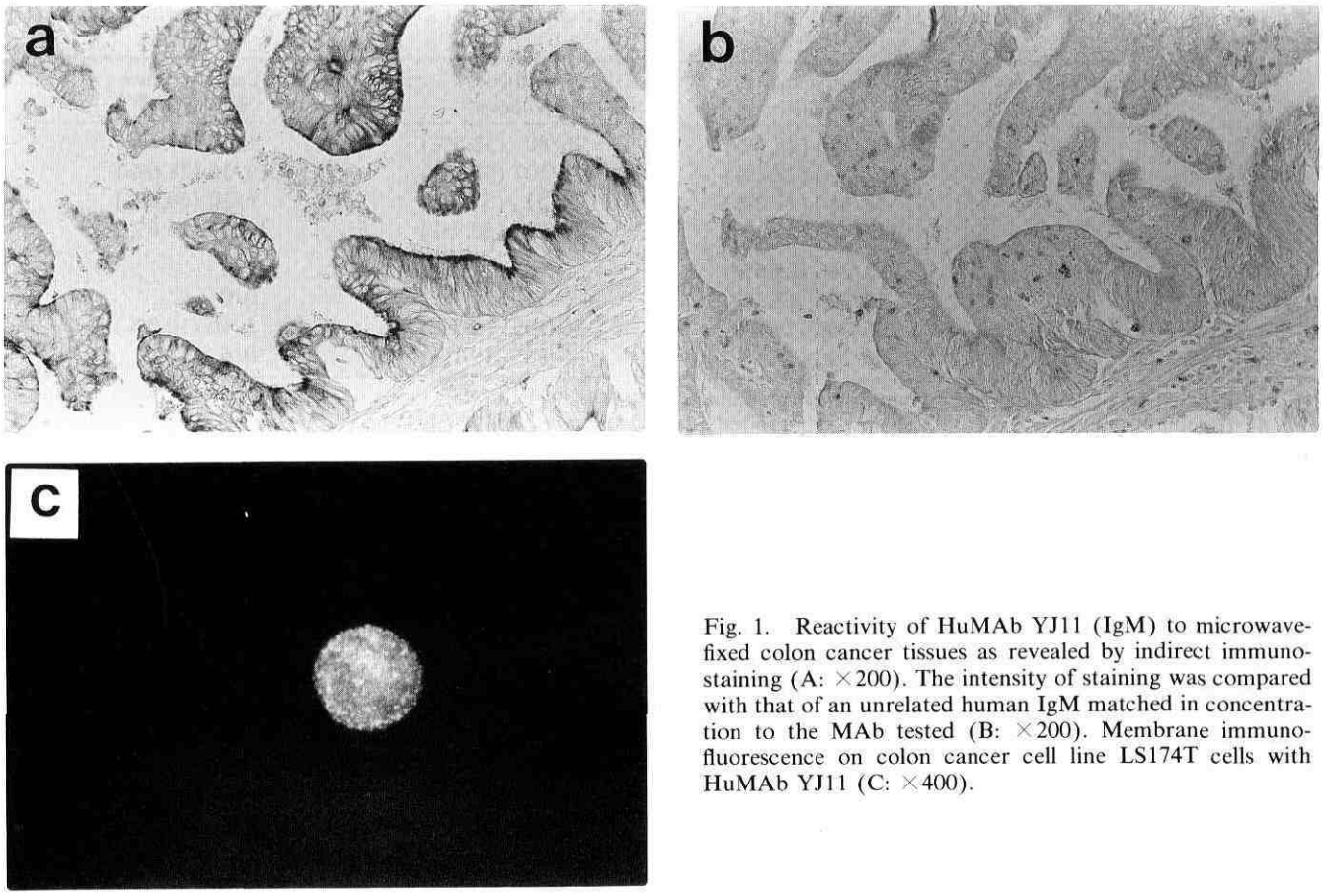


Fig. 1. Reactivity of HuMAb YJ11 (IgM) to microwave-fixed colon cancer tissues as revealed by indirect immunostaining (A: ×200). The intensity of staining was compared with that of an unrelated human IgM matched in concentration to the MAb tested (B: ×200). Membrane immunofluorescence on colon cancer cell line LS174T cells with HuMAb YJ11 (C: ×400).

been reported to induce the production of tumor necrosis factor (TNF)[13] and interleukins[14] by mononuclear cells, and the simultaneously injected fibrinogen has been shown to augment the immunomodulatory activity of OK-432.[9,10] The human hybridomas established in this way have been successfully cultivated for more than one year without a decrease in their ability to secrete HuMAbs.

Although the antigens recognized by the HuMAbs have not yet been identified, immunohistochemical analysis showed that the HuMAbs did not react to substances such as OK-432 and fibrinogen used in our local immunotherapy. Further biochemical analysis of the epitope recognized by the HuMAbs is expected to provide us with valuable information on tumor biology.

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