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Enhanced Efficacy of Radioimmunotherapy Combined with Systemic Chemotherapy and Local Hyperthermia in Xenograft Model

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We previously found that the efficacy of radioimmunotherapy (RIT) with ^{131}I-A7, an IgG_1 against M_r 45000 glycoprotein on colon cancer, was enhanced by local hyperthermia (HT) or chemotherapy with 5-fluorouracil (5-FU). In this study, we aimed to further enhance its efficacy by combining these three modalities. Human colon cancer xenografts (146±12 mm^3) in Balb/c *nu*/*nu* female mice were treated with 9.25 MBq ^{131}I-A7 i.v. combined with HT (43°C for 1 h) and 5-FU (30 mg/kg/day i.p. for 5 days). Tumor growth delay, $(Tq_{treated}-Tq_{control})/Tq_{control}$ where Tq is tumor quadrupling time, in mice treated with RIT+HT+5-FU was improved to 12.7 from 5.90, 7.55 and 10.1 with RIT alone, RIT+5-FU and RIT+HT, respectively. Complete response was observed in 4 out of 8 tumors with RIT+HT+5-FU and 3 out of 10 with RIT+HT. No tumor showed complete response with RIT+5-FU or RIT alone. 5-FU slightly increased myelotoxicity of RIT, but HT did not affect it. Body weight loss was not enhanced by the combination. These results indicate that the combination of three modalities is a feasible approach to enhance the antitumor efficacy of RIT without serious increase of toxicity.

Key words: Radioimmunotherapy — Local hyperthermia — Chemotherapy — 5-Fluorouracil

Efficacy of radioimmunotherapy (RIT) has been examined in various kinds of malignant tumors.[1–5] Its outcome, however, has been inadequate except for malignant lymphoma,[3, 4] mainly because of limited delivery of radiation ranging around only 0.005–0.01% of the injected dose per gram of tumor.[6] Therefore, several kinds of strategies have been examined to improve its efficacy, including the use of biological response modifiers to increase tumor targeting of labeled monoclonal antibodies (mAbs),[7–9] the use of a pretargeting system to obtain a better therapeutic ratio to normal tissues[10, 11] and combination with other therapeutic modalities such as hyperthermia (HT)[12–15] and chemotherapy.[16–21]

Our previous study demonstrated that HT enhanced the absorbed dose with ^{131}I-A7 anti-colorectal cancer mAb to colon cancer xenografts and significantly improved the therapeutic efficacy of RIT.[15] The combination of systemic chemotherapy with 5-fluorouracil (5-FU) also enhanced efficacy in the same model.[21] In addition, these studies showed that local HT did not affect myelotoxicity of RIT, and ^{131}I-A7 and 5-FU were able to be combined at near maximum tolerated doses (MTD) with only a slight increase of myelotoxicity. Intestinal toxicity of 5-FU would not be increased by ^{131}I-A7 either, as indicated by dosimetric analysis.[21] These findings suggested that the combination of these three modalities would be feasible in terms of its toxicity and may produce better antitumor efficacy than the two-modality combination of RIT with either HT or 5-FU. Therefore, the aim of this study was to investigate if the three-modality combination would be of benefit for enhancing the antitumor efficacy in colon cancer xenografts.

MATERIALS AND METHODS

Monoclonal antibody and animal model A7, an IgG_1 murine mAb that recognizes M_r 45000 tumor associated glycoprotein of colorectal cancer,[22] was a gift from former Professor Toshio Takahashi and Dr. Toshiharu Yamaguchi, First Department of Surgery, Kyoto Prefectural University of Medicine. The mAb was labeled with ^{131}I by the chloramine-T method and purified on a PD10 column (Pharmacia LKB Biotechnology, Uppsala, Sweden). To prevent autoradiolysis of ^{131}I-A7, 5 mg/ml of ascorbic acid was added as a radioprotectant.[23] The specific activity of ^{131}I-A7 was 94.7–113.2 MBq/mg and its immunoreactivity was 72.1–77.8% at infinite antigen excess, determined

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as described by Lindmo *et al.*[24] using 3×10^5 to 1×10^7 of LS180 human colon carcinoma cells. The mAbs were sterilized by passage through a filter (Millex-GV, 0.22 μm, Millipore, Bedford, MA) before injection into mice.

Animal studies were performed in compliance with the regulations of our institution. Balb/c *nu*/*nu* mice (female, 7–8 weeks old, 20 g) were subcutaneously xenografted with 5×10^6 LS180 cells in the thigh. Local HT to tumors in mice under i.p. pentobarbital anesthesia was conducted at 43°C for 1 h using a circulating water bath.[15] In the combined therapy, HT was conducted immediately after the mAb injection. A dose of 30 mg/kg/day of 5-FU (Wako Pure Chemical Industries, Ltd., Osaka) dissolved in 0.9% saline was i.p. injected immediately after the mAb injection on day 0 and daily thereafter for 4 days. When combined with HT, 5-FU was injected before the administration of HT. This dose of 5-FU was chosen on the basis of the report showing that this was its MTD, determined by a dose escalation study in nude mice.[25]

Observation of antitumor efficacy and toxicity Tumor volume (mm^3) was calculated as length (mm) × width $(mm)^2$ × 0.5 after the initiation of the treatment and expressed as a ratio to the volume on day 0, which was 146 ± 12 mm^3. There was no statistically significant difference in the volume among the experimental groups on day 0. The tumor growth was observed in 8 groups, including a non-treated control group, groups treated with a single modality of either HT, 5-FU or RIT, a group with the combination of 5-FU and HT, a group with the combination of RIT with 5-FU, a group with the combination RIT with HT, and a group with the combination of all three modalities of RIT, HT and 5-FU (n=5–11). To assess the antitumor efficacy of treatment, tumor quadrupling time (Tq) and tumor growth delay (TGD), $(Tq_{treated}-Tq_{control})/Tq_{control}$, were obtained.[19] The efficacy was also assessed in terms of the tumor response, which was defined as complete response (CR, tumor disappearance), partial response (PR, tumor volume reduction by ≥50% for ≥7 days) or minor response (MR, tumor reduction by <50% or for < 7 days).

To assess the toxicity of each protocol, peripheral blood cell counts were monitored. A blood sample of 4 μl was obtained from the tail vein of each mouse, and samples from mice within a group were pooled and diluted 1:10000 in phosphate-buffered saline for red blood cell counts, 1:20 in 3% acetic acid for white blood cell counts and 1:100 in 1% ammonium oxalate for platelet counts.[18] Body weight change of mice was also monitored.

Effect of HT on intratumoral distribution of A7 Radioluminograms (autoradiograms) of tumors were obtained to assess the effect of HT on the intratumoral distribution of A7. Frozen sections of 20 μm obtained 2 days after the ^{131}I-A7 injection were placed on imaging plates (BAS-SR2025, Fuji Film, Tokyo) and information was acquired with an imaging analyzer (BAS-5000, Fuji Film) as photostimulated luminescence (PSL). The resolution of images was set at 25 μm/pixel. To assess quantitatively the uniformity of radioactivity distribution in tumors, PSL/pixel was obtained in each section and the coefficient of variation (CV) (%) was calculated for the control tumors and heated tumors (n=6).

Statistical analysis Differences in tumor volume and body weight loss among the treatment groups were statistically compared by one-way analysis of variance with Fisher's protected least significant difference. Difference in tumor response was analyzed by use of the Mann-Whitney *U* test. The results of the autoradiographic study were analyzed with the unpaired *t* test. In the analyses, the level of significance was set at 5%.

RESULTS

Fig. 1 and Table I summarize the antitumor effects of treatments. HT and 5-FU had minor antitumor effects on the xenografts, but the combination of 5-FU and HT did not have an advantage over HT alone. Both 5-FU and HT

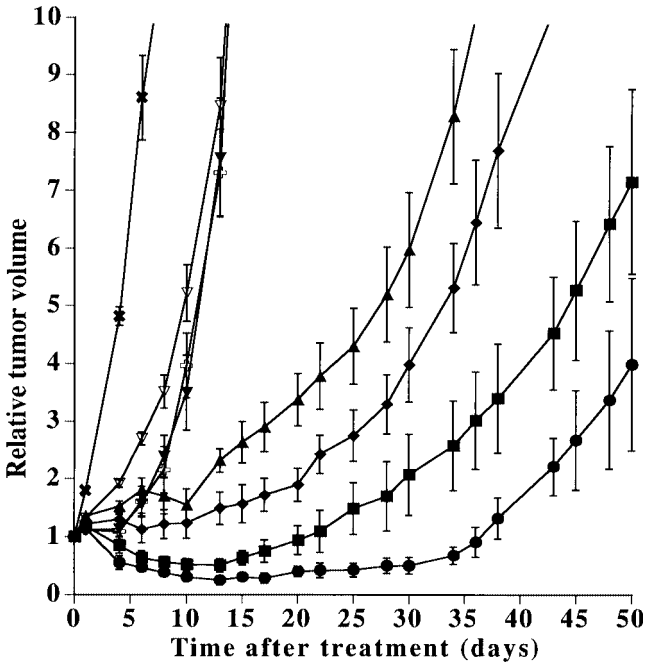

Fig. 1. Mean volume of LS180 human colon cancer xenografts in mice. Expressed as a ratio to the volume on day 0. ✖, control; ▽, 5-FU at 30 mg/kg/day i.p. for initial 5 days; ▼, hyperthermia (HT) at 43°C for 1 h on day 0; ✜, 5-FU+HT; ▲, radioimmunotherapy (RIT) with a single i.v. injection of 9.25 MBq ^{131}I-A7 on day 0; ◆, RIT+5-FU; ■, RIT+HT; ●, RIT+HT+5-FU. The treatments were initiated 8 days after the implantation of tumor cells.

Table I. Tumor Responses in LS180 Human Colon Cancer Xenografts

Treatment	Tumor number	Tq[a)]	TGD	Relative volume on day 30	Response			
					NR	MR	PR	CR
No treatment	11	3.66±0.42	—	—	11	0	0	0
5-FU	5	9.09±1.11	1.48	—	5	0	0	0
HT	5	10.7±0.97	1.92	—	2	3	0	0
HT+5-FU	5	10.3±0.82	1.83	—	2	3	0	0
RIT	9	25.3±3.39	5.90	5.98±1.17	6	2	1	0
RIT+5-FU	10	31.3±2.13	7.55	3.99±0.76	3	5	2	0
RIT+HT	10	40.7[b)]	10.1	2.08±0.78	2	1	4	3
RIT+5-FU+HT	8	50.0[b)]	12.7	0.50±0.15	0	1	3	4

Tq, tumor quadrupling time (days); TGD, tumor growth delay as defined in the text; CR, complete response (disappearance of tumor); PR, partial response (volume reduction by ≥50% for ≥7 days); MR, minor response (volume reduction by <50% or for <7 days); NR (no regression); 5-FU, 30 mg/kg/day of 5-FU for 5 days; HT, hyperthermia at 43°C for 1 h; RIT, 9.25 MBq of ^{131}I-A7.

a) Mean±SEM.

b) Obtained from the mean tumor growth curve because some tumors in these groups showed complete regression.

c) $P<0.05$.

enhanced the RIT efficacy. The combination of HT was more effective than that of 5-FU as indicated by TGD, relative tumor volume on day 30 and tumor response rate. The efficacy of the three-modality combination was further improved as compared with that of the two-modality treatment of RIT and HT.

Change of peripheral blood cell counts in the RIT groups is shown in Fig. 2. The combination of RIT with 5-FU induced a more prominent depression of cell counts than the single-modal RIT, but the combination with HT did not. The three-modality combination caused similar toxicity to the two-modality combination of RIT and 5-FU. Cell counts recovered similarly in all groups.

Maximum body weight loss in the treated groups is summarized in Table II. RIT caused greater body weight loss than non-RIT treatments. There was no statistically significant difference in body weight loss between the combination RIT and the single-modality RIT. Body weight recovered similarly in all groups in 3–4 weeks.

Radioluminograms of the tumors showed that the CV of PSL/pixel in the heated tumors, 11.3±0.98%, was significantly lower than that in the control tumors, 18.6±0.97%, indicating that a more uniform intratumoral distribution of A7 was obtained by HT.

DISCUSSION

This study has demonstrated the better efficacy of the three-modality combination than the two-modality combination with HT or 5-FU. We previously found a 2.25-fold increase of tumor absorbed dose with ^{131}I-A7 by local HT, and suggested that this improvement was due to the hyperthermic effect on tumor vasculature—increase of blood flow and vascular permeability—as well as on antigen expression, which was 2.66-fold enhanced 2 days after heating.[15)] The increased blood flow to the tumor may also be of benefit in improving the oxygenation of tumor tissues and thus enhancing radiation-induced toxicity with ^{131}I-A7. In addition, this study revealed a more uniform intratumoral distribution of ^{131}I-A7 in the heated tumors than in the control tumors, which would be of great benefit in enhancing the RIT efficacy. The better penetration of A7 into tumors was probably caused by the reduction of interstitial fluid pressure in heated tumors, as reported by Leunig *et al*.[26)] Furthermore, HT may produce a cytotoxic effect on radioresistant hypoxic cells and a synergistic effect by inhibiting the repair of radiation damage.[27)] In addition to these improvements caused by HT, an additive interaction between 5-FU and ^{131}I-A7 can be expected in the three-modality combination.[21)] In patients with high risk of developing metastases, the three-modality combination must be of more benefit than the two-modality RIT with either HT or 5-FU, because the RIT efficacy on lesions accessible to HT can be enhanced mainly by the synergism of RIT and HT and partly by the additive interaction of 5-FU, and that on other lesions can be improved by the additive effect of 5-FU.

The interaction between HT and 5-FU might also have occurred because HT may enhance the delivery of 5-FU to tumors by its effect on tumor blood flow and may positively modulate the cellular uptake of 5-FU.[27)] However, as shown in Fig. 1 and Table I, the combination of HT and 5-FU had no advantage over the single-modal HT, suggesting that the interaction between these two modalities would not make a significant contribution in the three-modality combination in this model. This must be partly

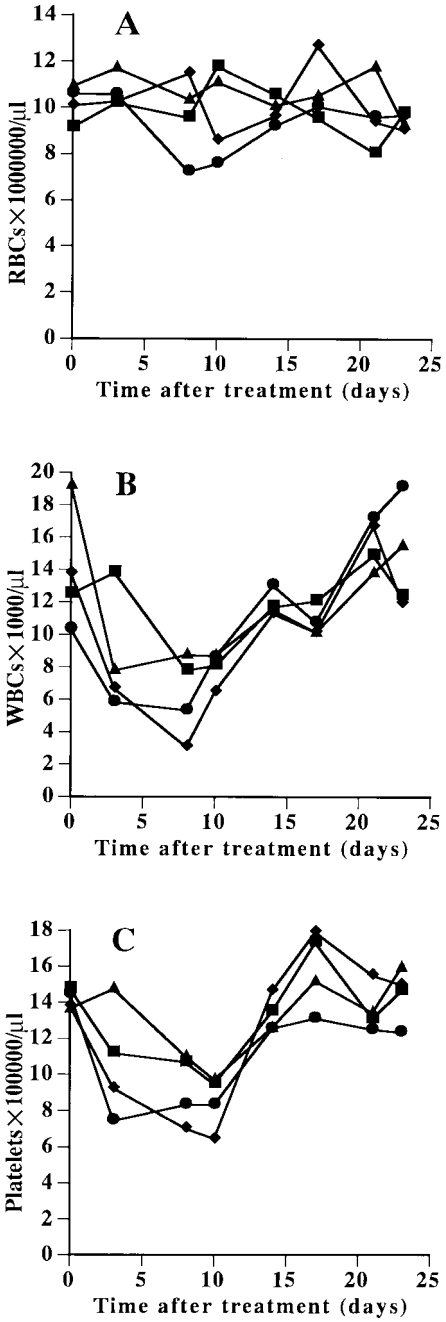

Fig. 2. Red blood cell (A), white blood cell (B) and platelet (C) counts in mice treated with RIT (9.25 MBq of ^{131}I-A7) (▲), RIT +5-FU (30 mg/kg/day for initial 5 days) (◆), RIT+HT (43°C for 1 h) (■) and RIT+HT+5-FU (●).

due to the stasis of tumor vessels occurring after the end of HT.[28] With the treatment protocol used in this study, HT may increase the delivery of 5-FU to tumors at the first administration on day 0, but vascular stasis may rather prevent it on days 1–4. Furthermore, small molecules such as 5-FU are rapidly cleared from tumors in spite of the increased initial delivery by the enhanced perfusion and permeability of tumor vasculatures.[29–31] On the other hand, HT improved the targeting of ^{131}I-A7, a large molecule, by the phenomenon of so-called "enhanced permeability and retention (EPR)."[29–31]

Table II. Maximum Body Weight Loss

Treatment	% weight loss[a]
5-FU	12.8±2.3
HT	10.6±1.2
HT+5-FU	10.5±1.4
RIT	19.3±1.1[b]
RIT+5-FU	19.2±1.3[b]
RIT+HT	18.5±1.0[b]
RIT+5-FU+HT	20.1±2.0[b]

a) Mean±SEM.
b) $P<0.05$ vs. HT, 5-FU and HT+5-FU.
No significant difference among RIT groups or among non-RIT groups.
Abbreviations are the same as those in Table I.

Bone marrow suppression is a major toxicity of RIT. We found in previous studies that the combination of 5-FU slightly increased the myelotoxicity of RIT at its MTD dose, but cell counts recovered similarly to that in the single-modal RIT[21] and local HT did not affect the toxicity.[15] From these observations, we expected that myelotoxicity would not be a limitation in the combined use of these three modalities, and this was confirmed here.

Intestinal toxicity is one of the major side effects of 5-FU. Intestinal absorbed dose with ^{131}I-A7 at MTD was estimated, in the previous studies, to be less than 1 Gy, which was not affected by local HT to the tumor-bearing leg or by the administration of 5-FU.[15, 21] Local HT did not affect systemic circulation and, therefore, would not increase the distribution of 5-FU to the intestine.[15] These findings indicate that intestinal toxicity should not be an obstacle to combining these three modalities in this animal model. However in a clinical setting, HT may be applied to the pelvis, peritoneum and liver[14] which are likely to be involved by metastatic disease of colon cancer. In this situation, HT may alter the distribution of mAb and 5-FU to the intestine, which may increase the risk of intestinal toxicity in the combination regimen.

In conclusion, the feasibility of multimodality RIT with HT and 5-FU, hyperthermic radioimmunochemotherapy, was demonstrated. The therapeutic efficacy on tumors accessible to HT can be enhanced by the interaction of these three modalities and that on other tumors can be improved by the additive effect of 5-FU.

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