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Enhancement of the therapeutic outcome of radio-immunotherapy by combination with whole-body mild hyperthermia

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Abstract

To enhance the effect of radio-immunotherapy for solid cancers, whole-body mild hyperthermia was added, and its effects on the pharmacokinetics of radiolabelled antibody, outcome of radio-immunotherapy, and radiosensitivity of the tumour were investigated. Nude mice bearing human colon cancer xenografts were heated to 40°C for 3 or 6 h. After heating, mice received intravenous (i.v.) injections of [^{131}I]-labelled anti-carcinoembryonic antigen (CEA) monoclonal antibody. Although 6-h heating did not alter the biodistribution of the radiolabelled antibody, and alone did not show any therapeutic effect on tumour growth, when combined with radio-immunotherapy, the therapeutic effect on tumour growth was significantly enhanced. Three-hour heating also significantly enhanced the effect of radio-immunotherapy. Colony formation assay showed that the radiosensitivity of the tumour was significantly enhanced after heating, which was achieved by a reduction of the hypoxic fraction of the tumour. In conclusion, the addition of whole-body mild hyperthermia significantly enhanced the therapeutic effect of radio-immunotherapy by increasing the radiosensitivity of the tumour.

Keywords: Radio-immunotherapy; Whole-body hyperthermia; Radiolabelled monoclonal antibody; Carcinoembryonic antigen; Hypoxia

1. Introduction

In spite of the progress made in radio-immunotherapy techniques, solid cancers are still hard to cure [1]. To obtain satisfactory results, radio-immunotherapy is combined with various therapeutic modalities [2–5]. In the present study, the effect of combined whole-body mild hyperthermia on the therapeutic outcome of radio-immunotherapy was investigated along with its effect on the pharmacokinetics of the radiolabelled antibody and radiosensitivity of the tumour, using nude mice bearing human colon cancer xenografts.

2. Materials and methods

2.1. Human colon cancer xenograft

Carcinoembryonic antigen (CEA)-expressing human colon cancer cells LS174T, (American Type Culture Collection, Rockville, MD, USA), were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, NY, USA) and 0.03% L-glutamine, in a 5% CO_2 environment. A single-cell suspension of 3×10^6 LS174T cells was subcutaneously (s.c.) injected into the left thighs of female BALB/c nu/nu mice. Ten to 12 days later, s.c. tumours reached the optimal size (300–500 mg) for further study.

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2.2. Monoclonal antibody and radiolabelling

The murine IgG_1 monoclonal antibody, designated as F33-104, recognises the CEA-specific proteinaceous part of the CEA molecule [6,7]. Antibodies were labelled with [^{125}I] for the biodistribution study and with [^{131}I] for the therapeutic study using the chloramine-T method [8]. Eight hundred μg of purified antibody and 224.2–264.9 MBq of [^{131}I] (Du Pont, Wilmington, DE, USA) were mixed with 8.0 μg of chloramine-T (Nacalai Tesque, Kyoto, Japan) dissolved in 0.3 M phosphate buffer. After 5 min, radiolabelled antibodies were separated from free iodine by PD-10 gel chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden). The specific activities of [^{131}I]-labelled antibodies ranged from 205.4 to 222.4 MBq/mg and the immunoreactive fractions were more than 70% for all preparations [9].

2.3. Whole-body mild hyperthermia

Whole-body hyperthermia was conducted according to the method of Burd and colleagues [10]. Mice bearing tumour xenografts were placed in isolator cages preheated to 39°C, containing food, bedding and water, and were then placed in a convection oven with preheated incoming air at 39°C. After 30 min, the temperature was raised to 40°C, and mice were kept for 3 or 6 h. The rectal temperature of some mice was monitored during heating, which showed that, within 30 min after starting 40°C-heating, the rectal temperature became more than 39°C and the mean temperature from 1 to 6 h was 39.5°C.

2.4. Biodistribution study

Just after 6-h of heating, the mice were intravenously (i.v.) injected with 37 kBq/10 μg of [^{125}I]-labelled F33-104. The control group of mice received radiolabelled antibody without heat pretreatment. The mice were killed 1 and 3 days postinjection by ether inhalation. Tumours, blood and various organs were removed and weighed and their radioactivity was counted. The percentages of injected dose per gram of tissue (%ID/g) were determined, from which tumour-to-normal tissue ratios of radioactivity were calculated. All animal experiments were carried out in accordance with the regulations regarding animal care and handling and were approved by the animal care committee in Kyoto University.

2.5. Radio-immunotherapy

Just after heating to 40°C for 3 to 6 h, groups of mice received an i.v. injection of 3.7 or 7.4 MBq of [^{131}I]-labelled F33-104. The protein dose was adjusted to 20 μg for each preparation by adding the unlabelled F33-104. The control group of mice received [^{131}I]-labelled F33-104 without heat pretreatment. The non-treated group of mice were injected with phosphate-buffered-saline (PBS) alone. The size of the s.c. tumour was observed thereafter, and the tripling time, that is, the number of days needed for the tumour to become 3 times as large as the starting tumour, was determined and compared among the groups.

2.6. Colony formation assay

Nude mice bearing human colon cancer xenografts were heated to 40°C for 3 h, and then received whole-body irradiation of 13 or 18 Gy using an experimental X-ray irradiator (Shimazu Co., Kyoto, Japan). Just after irradiation, tumour xenografts were excised, minced and incubated in PBS containing 0.05% trypsin and 0.02% ethylene diamine tetraacetic acid (EDTA) for 20 min at 37°C to obtain a single-cell suspension. After cell counting, appropriate numbers of cells were cultured in 60- or 100-mm dishes. After 2 weeks, cancer cell colonies were fixed and stained with Giemza's staining solution and the colony numbers in each dish were counted. Mice in the control group were irradiated without heat pretreatment and processed as described above. In every experiment, in order to obtain the plating efficiency of the non-treated tumours, single-cell suspensions were obtained from tumour-bearing mice without whole-body irradiation and heating, and colony formation assay was conducted. The surviving fractions of each group were calculated, which were normalised by the plating efficiency of non-irradiated tumours (10–20%).

To determine the hypoxic fraction of the tumour xenografts, some groups of mice were killed by cervical dislocation before irradiation. After waiting for at least 3 min to make sure that the tumours had become anoxic, mice were irradiated and then the colony formation assay was performed to determine the surviving fraction. The hypoxic fraction was calculated by dividing the surviving fraction of the tumour that was irradiated alive by that of the tumour that was irradiated more than 3 min after death.

2.7. Statistical analysis

Statistical analyses of the results were conducted using the unpaired *t*-test for the comparison of two groups and by the analysis of variance (ANOVA) with the Bonferroni–Dunn test for comparisons among three groups. All tests were double-sided, and a probability (P) value of less than 0.05 was considered significant for the *t*-test, and a P value of less than 0.0167 was considered significant for the Bonferroni–Dunn test.

3. Results

3.1. Effect of 40°C-heating on the biodistribution of the radiolabelled antibody

As summarised in Table 1, pretreatment with 6 h of 40°C-heating did not yield any significant effect on the pharmacokinetics of [^{125}I]-labelled F33-104. Tumour uptake, blood clearance and uptake to other normal organs were not changed by the addition of heat.

3.2. Effect of 40°C-heating on tumour growth

As shown in Fig. 1, 6 h of 40°C-heating alone did not show a significant growth retardation effect compared with the non-treated group.

Table 1
Biodistribution of [^{125}I]-labelled F33-104 in nude mice bearing the LS174T xenograft with and without heat pretreatment[a]

	Without heat pretreatment		With heat pretreatment	
	Day 1	Day 3	Day 1	Day 3
Blood	11.68±3.56	5.58±2.05	11.32±1.17	4.37±1.98
Liver	2.68±0.39	1.40±0.45	3.54±0.78	1.34±0.49
Kidney	3.09±0.52	1.52±0.50	3.23±0.42	1.29±0.54
Intestine	1.15±0.19	0.52±0.16	1.28±0.10	0.47±0.18
Stomach	3.00±0.78	0.94±0.21	3.23±0.71	1.21±0.31
Spleen	2.02±0.32	1.05±0.33	2.60±0.69	0.97±0.35
Lung	4.22±0.84	2.27±0.65	4.70±0.59	1.92±0.74
Muscle	0.95±0.20	0.63±0.18	0.93±0.15	0.50±0.14
Bone	1.22±0.16	0.64±0.19	1.32±0.22	0.65±0.19
Tumour	34.00±8.31	29.28±11.43	32.62±4.73	31.37±6.47

[a] Percentages of injected dose per gram of tissue (% ID/g). Mean±standard deviation (S.D.) of five mice.

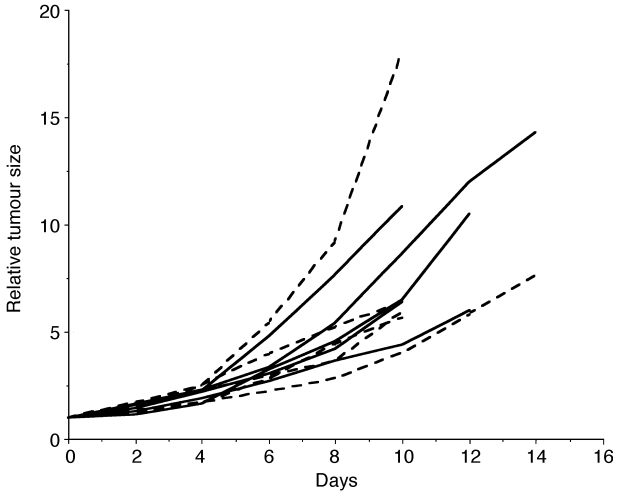

Fig. 1. Effect of 40°C-heating on tumour growth. After 40°C-heating for 6 h, the sizes of the subcutaneous (s.c.) tumours of five mice were measured and compared with those of non-treated mice ($n=5$ for each group). Change in the relative tumour size of each mouse is plotted against time (— , heated; - - - -, non-heated).

3.3. Effect of heat pretreatment on the outcome of radio-immunotherapy

Heat pretreatment significantly enhanced the effect of radio-immunotherapy. When 6 h of heating to 40°C was applied before the administration of 7.4 MBq of the [^{131}I]-labelled antibody, tripling time increased from 20.0±2.0 days without heat pretreatment to 26.0±3.5 days with heat pretreatment (Fig. 2a, $P=0.0108$). When combined with 3.7 MBq of antibody, tripling time increased from 9.9±2.0 to 17.3±4.2 days (Fig. 2b, $P=0.006$), respectively. The addition of heat pretreatment enhanced the therapeutic effect of 3.7 MBq of antibody to almost the same level as that of 7.4 MBq of antibody.

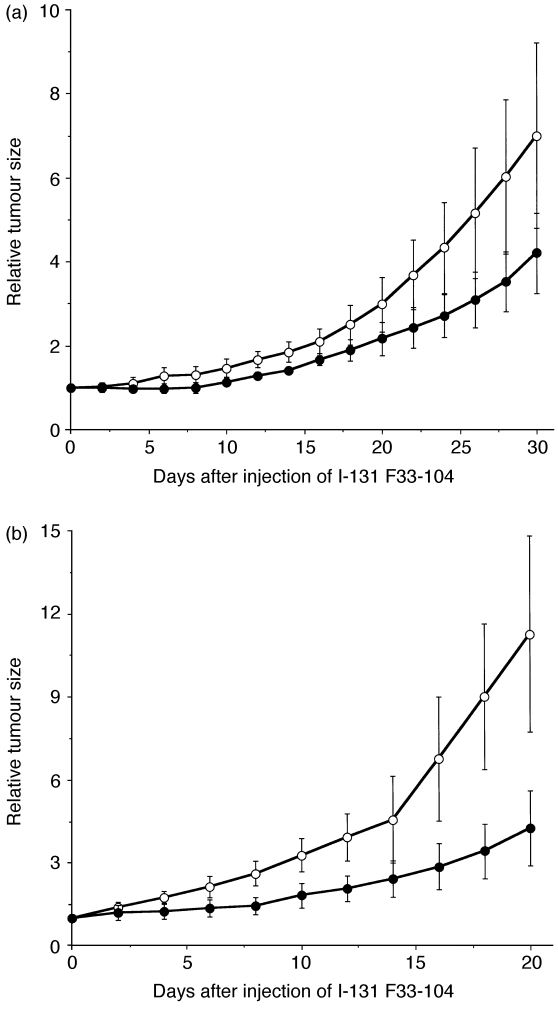

Fig. 2. Effect of heat pretreatment on the outcome of radio-immunotherapy. Just after 40°C-heating for 6 h, mice received an intravenous (i.v.) injection of 7.4 (a, $n=5$ for heated and non-heated group) or 3.7 MBq (b, $n=7$ for heated, $n=8$ for non-heated group) of [^{131}I]-labelled F33-104. Sizes of the subcutaneous (s.c.) xenografts were measured and compared with those of the non-heated mice. Changes in relative tumour size (mean±standard devation (S.D.) is plotted against time (●, heated; ○, non-heated).

Although 6 h of heating showed a significant enhancing effect of radioimmunotherapy, some (23%) of the mice could not tolerate the heat treatment and died. In order to make the whole-body hyperthermia tolerable for most of the mice, the duration of heating was reduced to 3 h, and the effect on radio-immunotherapy was investigated. As illustrated in Fig. 3, 3 h of heating also significantly enhanced the effect of radio-immunotherapy compared to the non-heated group ($P=0.0038$ for 3 h, $P=0.0005$ for 6 h heating). Although there was a tendency for the 6 h of heating to enhance the effect of radio-immunotherapy more than the 3 h of heating did, there was not a significant difference between the two groups ($P=0.2236$). With this 3 h of heating protocol, more than 95% of the mice tolerated the heating procedure.

3.4. Effect of heat pretreatment on the radiosensitivity and hypoxic fraction of the tumour

As illustrated in Table 2, the colony formation assay showed that, when mice were irradiated alive just after heating for 3 h at 40°C, the surviving fraction of cancer cells was markedly decreased for both irradiation doses (0.0646 ± 0.00217 without heat versus 0.00181 ± 0.00043 with heat for 13 Gy, $P<0.0001$; 0.00196 ± 0.00119 without heat versus 0.00021 ± 0.00009 with heat for 18 Gy, $P=0.0061$), indicating that the heat pretreatment increased the radiosensitivity of the tumour. When mice were irradiated after having been dead for at least 3 min to make the tumour totally anoxic, there were no significant differences in the surviving fractions between the heated and non-heated mice for both dose levels ($P=0.5461$ for 13 Gy and $P=0.2175$ for 18 Gy). The hypoxic fractions calculated from these data were 47.10–47.71% for non-heated tumours and were decreased to 6.50–11.51% after heat pretreatment.

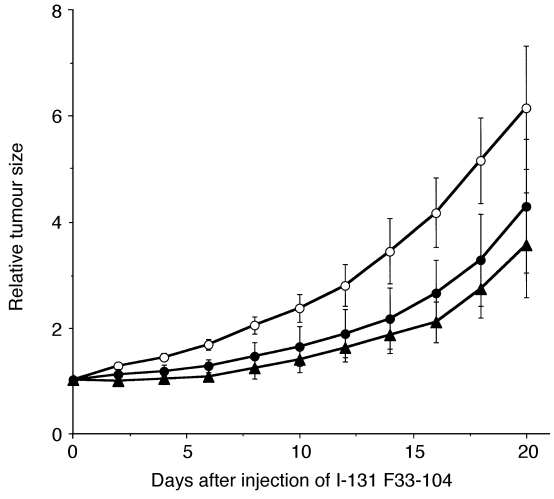

Fig. 3. Effect of heating time on the outcome of radio-immunotherapy. Just after 40°C-heating for 0, 3 and 6 h, mice received intravenous (i.v.) injection of 3.7 MBq of [^{131}I]-labelled F33-104. Sizes of the subcutaneous (s.c.) xenografts were measured and compared. Changes in relative tumour size (mean±standard deviation (S.D.) is plotted against time (○: 0 h, $n=6$; ●, 3 h, $n=8$; ▲; 6 h, $n=6$).

4. Discussion

Radio-immunotherapy of solid cancers has met with only limited success [1]. In order to enhance the effect of radio-immunotherapy, various therapeutic modalities have been combined, such as chemotherapy, cytokines, radiosensitisers and hyperthermia [2–5,11–13].

Hyperthermia has been used to enhance the effect of chemotherapy and radiotherapy and is now applied in clinical therapeutics [14,15]. In most cases, hyperthermic procedures are conducted to obtain a tumour core temperature of 42–43°C. Heat is mostly applied locally to the tumour site for approximately 1 h. With this procedure, however, heatable tumours are limited to relatively superficially-located tumours with a measurable size, and deeply seated tumours, multiple tumours, lung lesions, etc., are difficult to heat successfully. In the present study, we selected whole-body hyperthermia as the heating method and a heating temperature of 40°C. In whole-body hyperthermia, the body temperature, not the local temperature of the tumour, is increased and the breathing air is also heated. Therefore, it is applicable to tumours of any location and size and multiple tumours can also be heated. Since the targets of radio-immunotherapy are mostly multiple widespread lesions of varying sizes and locations, whole-body hyperthermia is suitable as a complementary therapeutic modality for radio-immunotherapy.

By combining hyperthermia and radio-immunotherapy, we expected various advantages, as follows: (1) Hyperthermic treatment may favourably alter the pharmacokinetics of the antibody or modify the expression of the tumour-associated antigen, resulting in the

Table 2
Results of colony formation assay

		Surviving fraction (mean±S.D.[a])	Hypoxic fraction (%)
13 Gy	Heated/alive[b]	0.00181 ± 0.00043*	11.51
	Heated/dead[c]	0.01575 ± 0.00839†	
	Control/alive	0.00646 ± 0.00217	47.71
	Control/dead	0.01354 ± 0.00289	
18 Gy	Heated/alive	0.00021 ± 0.00009‡	6.50
	Heated/dead	0.00323 ± 0.00141§	
	Control/alive	0.00196 ± 0.00119	47.10
	Control/dead	0.00417 ± 0.00184	

S.D., standard deviation. *$P<0.0001$ compared with control/alive; †$P=0.5461$ compared with control/dead; ‡$P=0.0061$ compared with control/alive; §$P=0.2175$ compared with control/dead.

[a] $n=4$ for each group

[b] Mice were irradiated alive.

[c] Mice were irradiated at least 3 min after cervical dislocation.

enhancement of tumour uptake of radiolabelled antibody and making the intratumoral distribution of the antibody more homogeneous [12,13]; (2) hyperthermic treatment, especially high-temperature hyperthermia, has a therapeutic effect and can retard tumour growth [12]; (3) hyperthermic treatment may change the radiosensitivity of the tumour [16,17]. In the present investigation, the first and the second points were not the case. Six-hour heat pretreatment to 40°C yielded no significant effects on the tumour uptake of radiolabelled antibody. In contrast to the report by Burd and colleagues, who described that 6 h of heating at 40°C alone showed a significant growth delay effect using severe combined immunodeficient (SCID) mouse- or Balb/c mouse-tumour models [10,18], the present study showed no significant growth delay effect by mild hyperthermia treatment alone, although the reason is unclear.

In spite of these findings, however, when combined with radio-immunotherapy, it did significantly enhance the therapeutic outcome of the radio-immunotherapy. The colony formation assay showed that the third point was relevant in this study. The surviving fraction was significantly reduced after mild hyperthermia, and this seems to be the major mechanism of the enhancement of the effect of radio-immunotherapy. Our study also showed that a reduction in the hypoxic fraction was the main reason for the increased radiosensitivity achieved by mild hyperthermia. Masunaga and colleagues have reported previously using a mouse tumour model, that 40°C heating for only 60 min reduced the hypoxic fraction of the tumour, especially the quiescent cell populations [17] and this was also probably the case in this study.

Although Burd and colleagues applying 6–8 h of 40°C heating described no side-effects [10,18], 23% of the treated mice in this investigation could not tolerate the 6 h of heating and died. Toxicity of whole-body hyperthermia has been reported in a canine model and in a patient study, but in both studies, the temperature was raised to 41.8–42°C [19,20]. The exact reason for this side-effect is uncertain, but it is possible that some hot areas are formed in the cages kept in the oven due to the inefficient mixing of the hot incoming air. Fortunately, the later experiments conducted with 3 h of heating were tolerable to most mice and also significantly enhanced the therapeutic effect.

The enhancement effect of radio-immunotherapy by combined whole-body hyperthermia was not satisfactory. We could only get growth delay and shrinkage of the tumour was not observed. This was partly because of the use of relatively large established tumours, but also necessitates us to further enhance the therapeutic effect. In the present investigation, heat was applied before the antibody administration. We originally hypothesised that the effect of mild hyperthermia should persist for several days, as reported by Burd and colleagues [10], and thought that the order of heat application and antibody administration would not result in any differences in the therapeutic outcome. However, since the enhancing effect was not sufficient, the procedure should be optimised. One way is to apply heat after antibody injection, which might enhance the antibody delivery to the tumour. Since the tumour accumulation of radiolabelled IgG requires a long time and the effect of heating on the vessels may be transient, heating before antibody injection is not enough to enhance the tumour accumulation, and heat application after antibody injection may enhance targeting of the tumour with antibody. In addition, in order to exert the radiation effect, antibody labelled with [^{131}I], which has relatively long physical half-life, should stay at the tumour site for a long period of time. In this regard, repeated heating may be able to maintain the enhanced radiosensitivity of the tumour for a longer period of time and can further enhance the therapeutic effect. Addition of other therapeutic modalities that may benefit from hyperthermic treatment, such as chemotherapy will also be important. Since whole-body hyperthermia was reported to cause apoptosis and lymphocyte recruitment [10], use of cytokines, etc., to enhance these processes will also be beneficial.

It is worth noting that Thrall and colleagues reported that the addition of whole-body hyperthermia had altered the metastatic process and shortened the time to metastasis of canine sarcomas [21]. Thus, to prevent this, the addition of systemic therapeutic modality is important, and radioimmunotherapy seems to be suitable for this purpose.

In conclusion, the present investigation showed that the addition of whole-body mild hyperthermia could significantly enhance the therapeutic effect of radio-immunotherapy. This enhancement was not because of a modulation of the pharmacokinetics of the radiolabelled antibody, but due to an enhancement of the radiosensitivity of the tumour induced by decreasing the hypoxic fraction.

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