Anti-tumour effects of transcatheter arterial embolisation administered in combination with thalidomide in a rabbit VX2 liver tumour model

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Objective: Using a liver tumour model we investigated whether thalidomide enhances the anti-tumour effect of transcatheter arterial embolisation (TAE).

Method: First, the viability of VX2 tumour cells co-cultured with thalidomide in a 21% and 1% O₂ atmosphere was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Second, we randomly assigned 20 rabbits bearing VX2 liver tumours to 4 groups: Group 1 (thalidomide plus TAE), Group 2 (TAE only), Group 3 (thalidomide only) and Group 4 (control). Thalidomide was orally administered for 5 days. The anti-tumour effects were assessed by the tumour proliferation rate using MRI and by immunohistochemical analysis of the area of intratumoural vessels. Analysis of variance and Tukey's honestly significant difference test were used for statistical analysis.

Results: The viability of cells grown under hypoxic and normal conditions was not significantly different, nor was there a difference among the four groups. The tumour size increased by $55.9 \pm 29.3\%$ in Group 1, $250.6 \pm 73.3\%$ in Group 2, $355.2 \pm 51.7\%$ in Group 3 and $424.7 \pm 110.7\%$ in Group 4; the difference between Group 1 and the other three groups was significant. The area of intratumoural vessels in specimens was $0.22 \pm 0.28\%$ in Group 1, $0.42 \pm 0.29\%$ in Group 2, $1.44 \pm 1.00\%$ in Group 3 and $6.00 \pm 2.17\%$ in Group 4; the difference between Group 1 and the other groups was statistically significant, as was the difference between Groups 3 and 4.

Conclusion: Thalidomide used in combination with TAE enhanced anti-tumour effects in rabbits bearing VX2 liver tumours.

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Introduction

Thalidomide is a sedative hypnotic drug developed in the 1950s by a German pharmaceutical company and placed on the market under the trade name Contergan [1, 2]. Because it induced foetus-specific malformations such as limb dysgenesis, it was taken off the market in 1961 [3]. In 1998, thalidomide was approved by the Food and Drug Administration for the treatment of multiple myeloma because it inhibited angiogenesis in carcinoma cells and because a third of patients with end-stage multiple myeloma showed improvement [4, 5]. In 2008, the Japanese Ministry of Health, Labour and Welfare endorsed the production and distribution of the drug as a medication for multiple myeloma. Thalidomide has been used in clinical trials as a treatment for advanced hepatocellular carcinoma (HCC) in several countries. In clinical trials, thalidomide monotherapy of HCC patients was tolerated and minimally effective [6–8], and in a randomised controlled trial, transcatheter arterial embolisation (TAE) combined with thalidomide therapy postponed disease

progression and prolonged the survival of HCC patients compared with TAE alone [9].

TAE is performed to treat HCC and metastatic hepatic carcinoma. At TAE, embolic agents are injected to obstruct the blood flow to the tumours, thereby inducing avascular tumour necrosis. TAE is particularly important for the treatment of HCC, which is richly supplied by blood vessels [10, 11].

We hypothesised that TAE in combination with oral thalidomide, which has an anti-angiogenic effect, might exert a greater anti-tumour effect than TAE alone. The purpose of our study was to investigate whether thalidomide enhances the anti-tumour effects of TAE in a rabbit VX2 liver tumour model.

Methods and materials

Cell line

To evaluate the anti-tumour effects of thalidomide on tumour cells, we conducted an *in vitro* experiment. Because TAE induces a hypoxic state, we cultured the cells under hypoxic conditions. VX2 tumour cells derived from rabbits were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and

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Cancer, Tohoku University. They were maintained in Dulbecco's modified Eagle medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with L-glutamine, 5% foetal bovine serum (FBS) (Gibco, New York, NY) and 1% penicillin/streptomycin (Sigma, Aldrich, St Louis, MO) [12].

Tumour proliferation assay

Cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [13, 14]. VX2 tumour cells (5×10^3 /well) were seeded in 96-well culture plates and incubated overnight at 37°C and 5% CO₂ in air (21% O₂). Then different concentrations (100, 50, 10, 1 and 0.1 mg ml⁻¹) of a 10 µl thalidomide solution were added to each well, the cells were cultured for 48 h under hypoxic (5% CO₂ and 1% O₂) or normal conditions (non-hypoxic), and 10 µl of a cell-counting solution (Nacalai Tesque) was added [15]. After 1 h of colour development under conventional conditions, absorbance was measured at 450 nm using a multiplate reader (Infinite M200, Tecan, Mannedorf, Switzerland).

Animals

All experiments were approved by our animal care and use committee and carried out according to our institution's guidelines for animal experimentation. We used 20 female Japanese white rabbits weighing 2.5–3.0 kg. They were anaesthetised with an intramuscular injection of a mixture of ketamine hydrochloride (25 mg kg⁻¹, Ketalar 50; Sankyo Yell Yakuhin Co. Ltd., Tokyo, Japan) and medetomidine hydrochloride (0.1 mg kg⁻¹, Domitor, Meiji Seika Co. Ltd., Tokyo, Japan) and pieces of VX2 tumour, 3 mm in diameter, were implanted beneath the hepatic capsule [16, 17]. Therapy was started 2 weeks later (Figure 1).

Preparation of gelatin microspheres as the embolic agent

The embolic material consisted of gelatin microspheres (GMS) prepared as reported elsewhere [18, 19]. Briefly, 10 ml of an acidic aqueous gelatin solution (10%) preheated to 37°C was added dropwise to 375 ml of olive oil (Wako Pure Chemical Industries Ltd., Osaka, Japan) and the mixture was stirred at 400 rev min⁻¹ (37°C, 10 min) to yield a water-in-oil emulsion. Stirring was continued for 30 min at 4°C and the formed microspheres were washed 3 times with acetone (Wako Pure Chemical

Industries) by centrifugation (5000 rpm, 5 min at 4°C). After air drying, the microspheres were sized by passage through sieves (100 and 200 µm apertures) and microspheres between 100- and 200 µm were collected. Non-cross-linked dry GMS were dispersed in 5 ml of an aqueous glutaraldehyde solution (7.5 mg ml⁻¹, 25%, Nacalai Tesque) at 4°C for 15 h to facilitate cross-linking. The microspheres were agitated further in 5 ml of 10 mM aqueous glycine solution (Nacalai Tesque) at 37°C for 1 h to block the residual aldehyde groups of unreacted glutaraldehyde. The resulting microspheres were finally washed three times with double-distilled water by centrifugation and freeze-dried.

In vivo therapeutic study

We randomly assigned the 20 tumour-bearing rabbits to 4 treatment groups. Group 1 was treated with thalidomide plus TAE, Group 2 with TAE alone, Group 3 with thalidomide plus an intra-arterial (ia) infusion of saline and Group 4 with an ia infusion of saline (1 mg kg⁻¹, control).

The right femoral artery was cut to allow the introduction of a 4-Fr short sheath (Clinical Supply Co., Gifu, Japan). A 4-Fr J-type catheter (Clinical Supply) was inserted into the hepatic artery, this was followed by the insertion of a microcatheter (Sniper 2 selective type; Clinical Supply) into the proper hepatic artery. GMS (100–200 µm in size, 1 mg kg⁻¹) were infused into the vessels of the TAE groups.

Thalidomide, supplied by Fujimoto Pharmaceutical Corp. (Osaka, Japan), was mixed with 8 ml of 2% carboxymethylcellulose (Nacalai Tesque) and administered orally (200 mg of thalidomide/kg) for 5 consecutive days starting on the day of TAE.

Assessment of therapeutic effects

Anti-tumour effects were assessed by comparing the pre- and post-treatment tumour volumes measured on a horizontal open MRI system (0.3 T permanent magnet; Hitachi Co. Ltd., Tokyo, Japan). To obtain these images we used spin-echo pulse sequence *T*₁ weighted images (repetition time (TR) 300 ms, echo time (TE) 14 ms, average 20, field of view 150 mm, matrix size 224 × 192, slice thickness 3 mm, interslice gap 0.5 mm). Images were interpolated to a 256 × 256 matrix on a data server and transferred to a personal computer. Liver tumour volumes were estimated using Photoshop 7.0.1 (Adobe Systems Inc., San Jose, CA). The tumours were manually

Figure 1. *In vivo* experiment. Transcatheter arterial embolisation (TAE) and thalidomide administration (by mouth) were started 2 weeks after VX2 liver tumour implantation. Thalidomide was given for five successive days. MRI scans were acquired before and 7 days after TAE. The animals were killed 7 days later.

outlined on each slice by the consensus of two radiologists and the total number of pixels was multiplied by 1.37 mm^3 . The two radiologists were blinded to the animal group from which the images were derived and to whether the images were obtained before or after treatment [17]. The relative tumour volume growth rates at 1 week after treatment (total number of pixels on day 7/total number of pixels on day 1 $\times 100$) were calculated. Values above or below 100 were recorded as an increase or decrease in the tumour size, respectively [16].

Immunohistochemical staining

The livers were removed immediately after the acquisition of MRI scans and fixed in 10% formalin. Formalin-fixed tissues were embedded in paraffin and cut into $5 \mu\text{m}$ slices along the maximum cut surface of the tumour specimen. We estimated the number of intratumour vessels based on the immunohistochemical expression of CD31 (Dako Japan), a marker for endothelial cells [20–22]. The ratio of areas positive for CD31 was determined at 100-fold magnification at 4 sites per specimen; mean values were calculated with Image J software.

Statistical analysis

The tumour proliferation rate obtained by MTT assay in the *in vitro* study and by the CD31-positive area ratios in the *in vivo* study was compared using analysis of variance and Tukey's honestly significant difference test. Differences of $p < 0.05$ were considered statistically significant.

Results

In vitro viability of VX2 cells

MTT assay showed that cell viability was as high under hypoxic as under normal conditions (Table 1). There were no significant differences among the cells exposed to five different concentrations of thalidomide. Our *in vitro* experiments indicated that the VX2 cells were tolerant to the hypoxic environment and that, at the concentrations used, thalidomide alone exerted no anti-tumour effects.

In vivo therapeutic study

The tumour size increased by $55.9 \pm 29.3\%$ in Group 1, $250.6 \pm 73.3\%$ in Group 2, $355.2 \pm 51.7\%$ in Group 3 and $424.7 \pm 110.7\%$ in Group 4, indicating that TAE plus thalidomide (Group 1) suppressed tumour growth

significantly compared with the other three treatment groups ($p < 0.05$). The difference between Group 2 (TAE only) and Group 3 (thalidomide alone) was also significant ($p < 0.05$). There was no significant difference between Groups 3 and 4 (control) ($p = 0.45$) (Figure 2).

The ratio of areas positive for CD31 staining was $0.22 \pm 0.28\%$ in Group 1, $0.42 \pm 0.29\%$ in Group 2, $1.44 \pm 1.00\%$ in Group 3, and $6.00 \pm 2.17\%$ in Group 4. It was significantly lower in Group 1 than Group 3 ($p < 0.05$) but not significantly lower than in Group 2 ($p = 0.31$). There was a significant difference between Groups 3 and 4 (Figure 3).

Discussion

Our study showed that inhibition of the growth of VX2 liver tumours was significantly greater in rabbits treated with TAE plus thalidomide (Group 1) than in rabbits treated by TAE alone (Group 2), thalidomide alone (Group 3) or saline (Group 4, control). Immunohistochemical study also indicated that Group 1 manifested fewer intratumoural vessels than the other groups. However, MTT assay revealed that VX2 cells were not affected by a hypoxic environment or by thalidomide.

We carried out *in vitro* experiments to assess whether thalidomide exerted anti-tumour effects. We performed these experiments under hypoxic conditions because we planned to carry out *in vivo* experiments in which tumour-bearing rabbits were subjected to both TAE and thalidomide treatment. First, we examined the effect of thalidomide under normal cell culture conditions (37°C , $5\% \text{ CO}_2$ in air) to determine whether it alone exerted any anti-tumour effects manifesting as growth inhibition. We found that even under hypoxic conditions, thalidomide exerted no anti-tumour effects. We recognise that it is difficult to evaluate the anti-angiogenesis effect of thalidomide in cultured VX2 tumour cells and that these cells would not be injured even under hypoxic conditions simulating a TAE-induced environment. We are also aware that the environment surrounding a tumour *in vivo* is not identical to the hypoxic conditions created in our experiments.

Our results support earlier findings that thalidomide alone exerted no anti-tumour effects [23, 24]. On the other hand, in combination with other drugs including cisplatin [25], dexamethasone [26] and fludarabine [27] it reportedly demonstrated enhanced anti-tumour effects. Lee et al [28] found that thalidomide enhanced the effects of irradiation. To our knowledge, the effect of concomitantly administered thalidomide on TAE-induced anti-tumour effects has not been reported.

Studies on molecular target therapies have been reported. Growth factors and related receptors are often

Table 1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (absorbance at 450 nm (standard deviation))

Thalidomide concentration (mg ml ⁻¹)	Control	100	50	10	1	0.1
21% O ₂ (normal)	0.88 (0.31)	0.91 (0.17)	0.95 (0.03)	0.80 (0.06)	0.94 (0.03)	0.87 (0.12)
1% O ₂ (hypoxic)	0.78 (0.32)	1.03 (0.19)	0.81 (0.18)	0.94 (0.22)	1.2 (0.06)	1.2 (0.05)

Cell viability under hypoxic conditions was as high as under normal oxygen conditions. There was no significant difference.

Figure 2. The tumour proliferation rate was calculated based on the tumour size on MRI scans. * $p < 0.05$.

overexpressed in HCC and clinical trials indicated that growth factor receptors and their related signalling pathways play important roles in HCC. The anti-tumour effects of sorafenib, an oral multikinase inhibitor that targets Raf kinase, and receptor tyrosine kinases have been assessed in advanced HCC patients. In a large randomised Phase III study [29] of patients with HCC, median survival and the time to radiological progression were nearly 3 months longer for patients treated with sorafenib than for those given a placebo. Other targeted agents that have been evaluated in Phase II clinical trials for the treatment of HCC include tyrosine kinase inhibitors of the epidermal growth factor receptor (erlotinib), a humanised monoclonal antibody against anti-vascular endothelial growth factor (bevacizumab) and a multitargeted tyrosine kinase inhibitor (sunitinib). Thomas et al [30] showed that in patients with advanced HCC the combination of bevacizumab and erlotinib exerted

Figure 3. Tumour vascularisation is shown as the ratio of areas positive for CD31 in the VX2 liver tumours. * $p < 0.05$.

significant, clinically meaningful anti-tumour activity. Zhu et al [31] documented that sunitinib exhibited modest anti-tumour activity in advanced HCC.

We developed GMS, whose size and dissolution time can be manipulated, as an embolic agent [18]. In detailed studies of the velocity of the degradation of GMS and the degree of tissue ischaemia attributable to their presence we found that GMS exerted embolising effects identical to those of conventional embolic agents such as gelform and sponge gel or of new embolic agents such as SAP microspheres and embospheres [32–34]. Elsewhere we documented that GMS enhanced anti-tumour effects if used in combination with the new anti-cancer agents tirapazamine and flavopiridol [17].

Assessment of the tumour volumes showed that TAE using GMS in combination with thalidomide exerted significantly greater suppressive effects on tumour growth than any other treatment. However, pathological study of the tumour specimens revealed no difference in the number of intratumour vessels between tumours from rabbits treated by TAE plus thalidomide (Group 1) and those subjected to TAE alone (Group 2). When GMS or other embolic agents are used concomitantly they may occlude the blood flow and inhibit vascular proliferation in the central and peripheral parts of the tumour differently. Therefore, studies are under way in our laboratory in which we evaluate the different tumour parts separately to gain further insight into the number of intratumour vessels at different sites.

Although there was no significant difference in tumour growth suppression between Group 3 (thalidomide alone) and Group 4 (control), there was a significant difference between the area ratios of intratumour vessels. These results may reflect the point in time at which we assessed the anti-tumour effects, *i.e.* 7 days after the first thalidomide administration. This interval may be too short for a correct identification of changes in the tumour volume. Histologically, the suppression of vascular proliferation, which has a high potential for inducing necrosis, might have been detectable. The study of contrast-enhanced MRI or CT scans and histological examination of living tumours may have detected significant differences. Because thalidomide, besides its anti-angiogenesis effects, has an inhibitory effect on tumour necrosis factor (TNF)- α , a factor partially responsible for cachexia in cancer, its administration may help to improve the quality of life of patients with advanced cancer. In the current study we demonstrated the additive effect of thalidomide on the efficacy of TAE in rabbits with VX2 liver tumours.

Conclusions

Thalidomide, used in combination with TAE, enhanced anti-tumour effects in a rabbit VX2 liver tumour model.

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