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Abstract

To investigate the suppressive effect of human recombinant TIMP-1 (rh-TIMP-1) on tumor proliferation using an in vivo xenograft system, HT29 was suspended in 0.1 ml phosphate buffered saline (PBS) and then subcutaneously injected in the back of female mice (BALB/C nu/nu). The mice were divided into 2 groups an and the tumor diameter was measured after rh-TIMP-1 (2 mg/kg) (rh-TIMP-1 group) or PBS (control group) was administered injections according to the following schedules. Schedule 1 : Beginning 2 weeks after the subcutaneous injection of HT29, an intraperitoneal injection of rh-TIMP-1 or PBS were performed twice a day (every 12 h) for 14 consecutive days. Schedule 2 : Beginning 1 week after the subcutaneous injection of HT29, an intraperitoneal injection was performed twice a day for 14 consecutive days. Schedule 3 : Intraperitoneal injections were started simultaneously with the subcutaneous injection of HT29, and then performed twice a day for 21 consecutive days. The mice were sacrificed and the tumors extirpated for immunohistochemical investigation. In addition, gelatin zymography and a cell proliferation assay were performed. With Schedule 1, the changes in the tumor diameter in the rh-TIMP-1 group followed the same course as those in the control group, and no suppressive effect on tumor proliferation was observed. However, with Schedule 3, a remarkable suppressive effect was observed throughout the treatment period. In immunostaining, more cases negative for MMP-9 were observed in the rh-TIMP-1 group than in the control group. Cases negative for CD34 were significantly more observed in the rh-TIMP-1 group than in the control group with Schedule 3. All of the results were obtained through the suppressive effect of rh-TIMP-1 on angiogenesis.

KEYWORDS: MMP-2, MMP-9, TIMP-1, molecular targeting therapy, angiogenesis

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Original Article

Suppression Effects of Human Recombinant Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) on Tumor Proliferation Using *in Vivo* Treatment Model of Well-differentiated Colon Cancer Cell Line, HT29

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To investigate the suppressive effect of human recombinant TIMP-1 (rh-TIMP-1) on tumor proliferation using an *in vivo* xenograft system, HT29 was suspended in 0.1 ml phosphate buffered saline (PBS) and then subcutaneously injected in the back of female mice (BALB/C nu/nu). The mice were divided into 2 groups an and the tumor diameter was measured after rh-TIMP-1 (2 mg/kg) (rh-TIMP-1 group) or PBS (control group) was administered injections according to the following schedules. Schedule 1: Beginning 2 weeks after the subcutaneous injection of HT29, an intraperitoneal injection of rh-TIMP-1 or PBS were performed twice a day (every 12 h) for 14 consecutive days. Schedule 2: Beginning 1 week after the subcutaneous injection of HT29, an intraperitoneal injection was performed twice a day for 14 consecutive days. Schedule 3: Intraperitoneal injections were started simultaneously with the subcutaneous injection of HT29, and then performed twice a day for 21 consecutive days. The mice were sacrificed and the tumors extirpated for immunohistochemical investigation. In addition, gelatin zymography and a cell proliferation assay were performed. With Schedule 1, the changes in the tumor diameter in the rh-TIMP-1 group followed the same course as those in the control group, and no suppressive effect on tumor proliferation was observed. However, with Schedule 3, a remarkable suppressive effect was observed throughout the treatment period. In immunostaining, more cases negative for MMP-9 were observed in the rh-TIMP-1 group than in the control group. Cases negative for CD34 were significantly more observed in the rh-TIMP-1 group than in the control group with Schedule 3. All of the results were obtained through the suppressive effect of rh-TIMP-1 on angiogenesis.

Key words: MMP-2, MMP-9, TIMP-1, molecular targeting therapy, angiogenesis

In the processes of proliferation and invasion of cancer cells, disruption of the extracellular matrix (ECM) is essential [1]. Matrix metalloproteinases (MMPs) [2] play a central role in these processes, and are closely involved in the disruption of the basal membrane which is one of the processes of hematogenous metastasis [3]. It is thought that a part of the hematogenous metastasis process can be

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controlled by suppressing the activity of MMPs that have strong collagenolytic activity toward type IV collagen, a primary component of the basal membrane. It is thought that a part of the hematogenous metastasis process can be controlled by suppressing the activity of MMPs, which have strong collagenolytic activity toward type IV collagen, a primary component of the basal membrane. Numerous studies have investigated the suppressive effect of tissue inhibitors of metalloproteinases (TIMPs), which are specific inhibitors of MMP, on tumor proliferation and metastasis [4]. Particularly in many studies using colon cancer cell lines or tissue specimens, the quantitative balance of MMPs and TIMPs in the local tumor is reported to influence the tumor proliferation and invasion processes [5]. Some investigators have reported an inhibitory effect of TIMPs on angiogenesis [6], as well as a suppressive effect on tumor proliferation. However, in many of these studies, TIMPs were excessively expressed by introducing the TIMPs gene into hosts or tumor cells, and few reports have investigated the *in vivo* effect of recombinant TIMP administration [7]. In the present study, an *in vivo* treatment model was prepared using a human colon cancer cell line, HT29. The suppressive effect of recombinant TIMP-1, which had a strong inhibitory activity toward MMP-9, on tumor proliferation was investigated after multiple intraperitoneal injections of rh-TIMP-1.

Materials and Methods

Preparation of treatment model. A treatment model was prepared by the following methods. All animals experiments were performed according to the Guidelines for Animal Experiments of Okayama University Medical School.

(1) Preparation of cell culture and cell suspension. A monolayer culture of the cell line HT29 (ATCC, Manassas, VA, USA) derived from human colon cancer was performed in a flask with McCoy's 5A medium (Invitrogen, CA, USA) with the addition of 10% FBS (Invitrogen, Carlsbad, CA, USA), in an incubator at a 37 °C, 5% CO_2 atmosphere. The medium was completely aspirated when the cells became 90% confluent, and 0.05% trypsin/EDTA (Invitrogen, Carlsbad, CA, USA) was added. The cells were peeled off 5 min later, and suspended again in medium to prepare the cell suspension. Part of the suspension was stained with 0.2% trypan blue (Invitrogen, Carlsbad, CA, USA), and the number of cells was counted using a cell counting slide. The cells were suspended in PBS (−) so that the number of viable cells became 1×10^7/ml.

(2) Preparation of treatment model. To the bilateral back of 6–4 week-old female nude mice (BALB/c-nu/nu, body weight; about 20 g, Clea Japan, Osaka, Japan), 0.1 ml ($= 2 \times 10^6$) of HT29 cell suspension was subcutaneously injected using a 29G needle. The mice were divided into the following 2 groups an rh-TIMP-1 group of 10 mice that received intraperitoneal injections of 2 mg/kg human recombinant TIMP-1 (rh-TIMP-1, Daiichi Fine Chemical, Toyama, Japan) and a control group of 10 mice that received 0.1 ml PBS (−). The time course of the major and minor axis of the subcutaneous tumor in the back were measured in both groups. The tumor volume was obtained by using the following approximation formula:

$$\text{Tumor volume} \fallingdotseq \pi/6 \times (\text{the major axis of tumor}) \times (\text{the minor axis of tumor})^2$$

In addition, the body weight of the mice on the last dosing day in both groups was measured to evaluate the side effects of rh-TIMP-1.

(3) Treatment schedule. The intraperitoneal injections of the rh-TIMP-1 (rh-TIMP-1 group) or PBS (control group) mice were performed according to the following schedule.

Schedule 1: Beginning 15 days after the subcutaneous injection of HT29, an intraperitoneal injection of rh-TIMP-1 or PBS was performed twice a day (every 12 h) for 14 consecutive days.

Schedule 2: Beginning 8 days after the subcutaneous injection of HT29, an intraperitoneal injection of rh-TIMP-1 or PBS was performed twice a day for 14 consecutive days.

Schedule 3: An intraperitoneal injection of rh-TIMP-1 or PBS was started simultaneously with the subcutaneous injection of HT29, and performed twice a day there after for 21 consecutive days.

Investigation of MMP secretory capacity of cultured cells. To investigate the gelatinolytic ability of HT29, the MMP secretory capacity was observed through gelatin zymography. The influence of rh-TIMP-1 on gelatinolytic activity was also evalu-

ated.

(1) Sample preparation. HT29 (1×10^5) was suspended in McCoy's 5A medium (serum free) in 2 dishes for cell culture (60 mm in diameter). rh-TIMP-1 (500 ng/ml) was added to one dish only. The dishes with and without rh-TIMP-1 were incubated at a 37 °C, 5% CO_2 atmosphere for 24 h. Both the dish with rh-TIMP-1 and the dish without it were incubated at a 37 °C, 5% CO_2 atmosphere for 24 h. The medium in the dishes was completely collected, and concentrated using an Amicon Ultra centrifuged filter device (Millipore, Billerica, MA, USA). Gelatin zymography was then performed using the concentrated medium as a sample.

(2) Gelatin zymography. In gelatin zymography, the enzymatic activity is evaluated based on the gelatin resolution in gel after SDS-Poly Acrylamide Gel Electrophoresis (PAGE). This method is a superior one for evaluating MMP-2 (gelatinase A) and MMP-9 (gelatinase B). We performed gelatin zymography using a Gelatinzymo Electrophoresis Kit (Yagai, Yamagata, Japan) according to the methods proposed by Heussen *et al.* [8].

First, 10 μl of the sample was homogenized by the buffer (10 mM Tris-HCL, PH6.8, 20% glycerol, 2% SDS, 0.1 bromophenol blue), and SDS-PAGE (at 10 mA for 10 min and 20 mA for 80 min) was performed with 10% polyacryl amidegel in the addition of 0.1% gelatin. After the gel plate was shaken for 30 min twice with 2% triton-X100, it was reacted with enzyme reaction liquid (50 mM Tris-HCL, 150 mM Nacl, 10 mM $CaCl_2$, 0.02% NaN_3) at 37 °C for 36 h. After completion of the enzyme reaction, the protein in the gel plate was stained with a staining fluid (30% ethanol, 10% acetate acid, 0.3% tripan blue) and decolored with a decoloring solution (10 % acetate acid, 30% methanol in distilled water). The gel electrophoresis fragment pattern was observed to evaluate the gelatinolytic activity of HT29.

Immunohistochemical investigation.

(1) Preparation of materials. On the last dosing day of rh-TIMP-1 in Schedules 1, 2 and 3, the mice were sacrificed using 500 mg/kg pentobarbital sodium (Dainippon Pharmaceutical, Osaka, Japan), and the tumor was extirpated under clean conditions. The tumor was fixed with periodate-lysine-paraformaldehyde (PLP) fixing solution at 4 °C for 24 h, and paraffin embedding was then performed to make a tissue block. The block was sliced to a thickness of 2.5 μm, and was adhered to the MAS-coated slide to prepare sections.

(2) Immunohistochemical staining. Immunostaining of the paraffin sections was performed according to the method proposed by Sannino *et al.* [9]. Briefly, deparaffinization of the sections was performed using xylene and ethanol, and the antigen activating treatment using citric acid buffer (Target Retrieval Solution: DAKO, Glostrup, Denmark) was performed by heating in a microwave at 500W. Blocking of the endogenous peroxydase activities was then performed using 0.3% H_2O_2 solution. Generally, after blocking, tumor sections are reacted with anti-mouse monoclonal antibodies. However, since we had difficulty performing background staining due to a reaction by the second antibody with the endogenous mouse immunoglobulin, we performed the subsequent immunostaining process by applying the Polymer immunocomplex (PIC) method reported by Fukuda *et al.* [10]. First, a polymer immunocomplex was formed by mixing a primary antibody (Anti-hMMP-2 purified IgG: Daiichi Fine Chemical, Takaoka, Japan, Anti-hMMP-9 purified IgG: Santa Cruz Biotechnology, Santa Cruz, Ca, USA, Anti-CD34 purified IgG: Hycult Biotechnology, Uden, Netherlands) with a second antibody (ENVISION$^+$ for mouse: DAKO, Glostrup, Denmark). Normal mouse serum was added to the immunocomplex to block polyclonal antibodies to mouse immunoglobulin from binding to the second antibody. Subsequently, the immunocomplex was diluted 100-fold with antibody dilution (DAKO, Glostrup, Denmark). Drops of this solution were put on the tissues, and the tissues were reacted at 37 °C for 60 min. Matrix staining with 0.02% diaminobenzidine tetrahydrochloride (DAB) solution and nuclear staining with Mayer's hematoxylene were then performed. Finally, dehydration and encapsulation using ethanol and xylene were performed.

(3) Staining evaluation. The immunostained tissues were evaluated according to the following criteria.

MMP-2, -9: Five arbitrary visual fields of preparation were microscopically visualized at 200-fold magnification. When the number of visual fields in which color development was observed in not less

than 10% of the cancer cells was 3 or more in one preparation, the staining result was evaluated as "positive". When the number of visual fields in which color development was observed in not less than 10% of the cancer cells was 2 or less in one preparation, the staining result was evaluated as "negative".

CD34: The preparation was microscopically visualized at a 20-fold magnification to find the high-density areas. These areas were observed again at 200-fold magnification, and the number of stained lesions inside the lumen were counted. The same observations were performed at 5 arbitrary visual fields, and the mean values were recorded. These values were regarded as the microvessel count (MVC), and compared between the rh-TIMP-1 and Control Groups.

Cell proliferation assay. HT29 of the 1×10^5/well was placed in a 96-well cell culture plate, and incubated at a 37 °C, 5% CO_2 atmosphere for 72 h in the presence (500, 250, 100, 50 and 10 ng/ml) and absence (0 ng/ml) of rh-TIMP-1. Subsequently, 20 μl of Cell Titer 96 Aqueous One Solution Reagent (Promega, Madison, MI, USA), which is a reagent containing tetrazolium compound

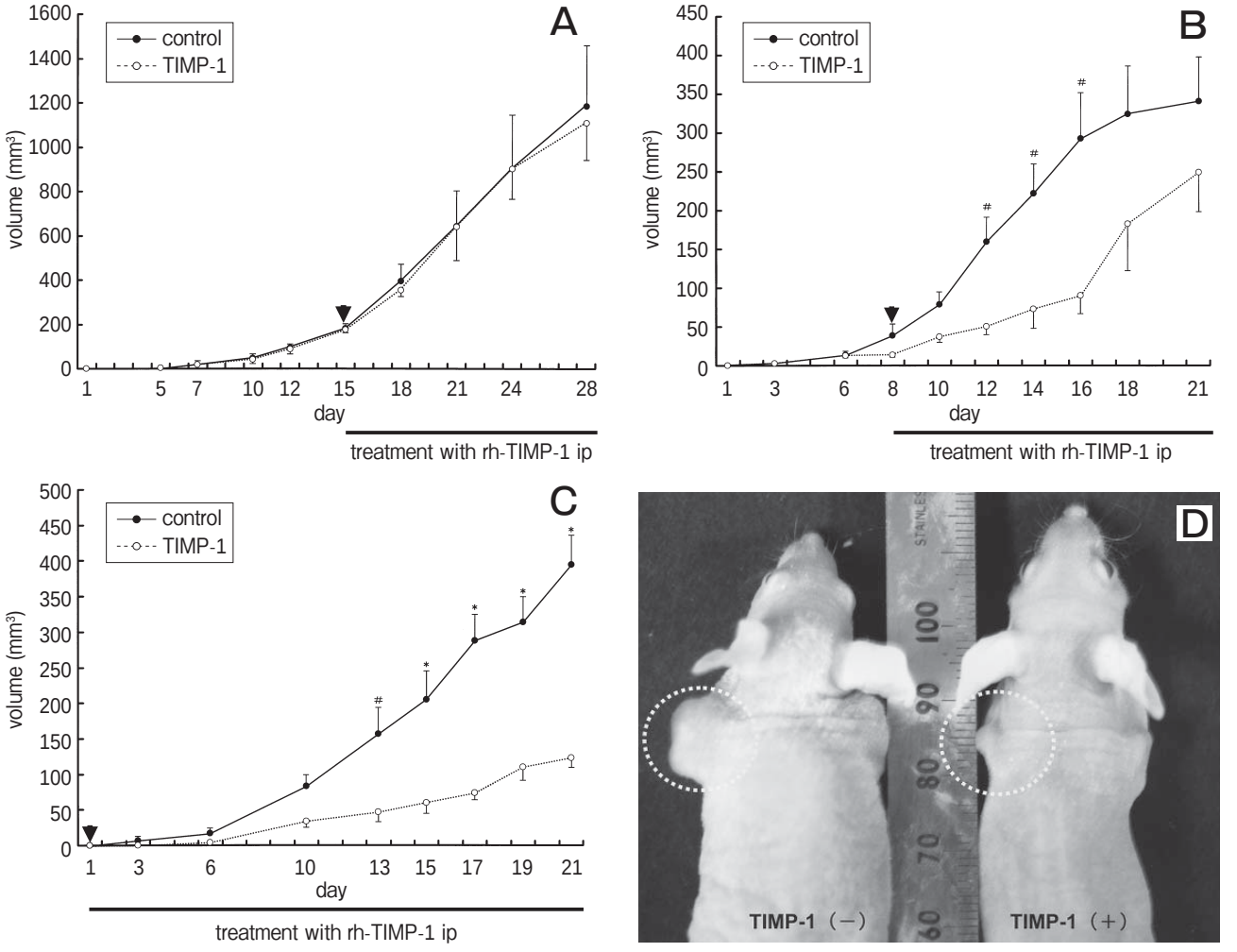

Fig. 1 Subsequent differences in tumor diameter among various treatment schedules with rh-TIMP-1 were investigated. (**A**) With Schedule 1, there was no difference in tumor diameter between [the] rh-TIMP-1 and control groups. (**B**) With Schedule 2, the effect of rh-TIMP-1 was transient. (**C**) However, with Schedule 3, a significant difference in tumor diameter between the rh-TIMP-1 and control groups was continuously observed (#: $p < 0.05$, *: $p < 0.01$ vs. control, ▼: day when the intraperitoneal administration of rh-TIMP-1 was started). (**D**) The conditions of the subcutaneous tumor 21 days after the start of Schedule 3 treatment (white circle) are shown.

(MTS), was added to each well. After incubation at 37 °C, 5% CO_2 for 2 h, the absorbance at 490 nm was measured using a micro plate reader.

Statistical analysis. All statistical analyses were performed using the chi-square test or Student-*t* test. *P* values of less than 0.05 were considered statistically significant.

Results

Changes of tumor volume and body weight with each treatment schedule. When HT29 was subcutaneously injected to the treatment model, a subcutaneous tumor visible to the naked eye formed in approximately 1 week. The tumor was observed in all the mice receiving the HT29 subcutaneous injection. With Schedule 1, there was no difference in tumor diameter between the rh-TIMP-1 and control groups during the entire treatment period. With Schedule 2, a significant difference ($p < 0.05$) between the rh-TIMP-1 and control groups was observed during the initial 10 days of rh-TIMP administration, but subsequently the tumor diameters in the rh-TIMP-1 group approximated those in the control group. With Schedule 3, a significant suppression of tumor proliferation ($p < 0.01$) was continuously observed (Fig. 1A–1D). With all schedules, there were no significant differences in body weight at the last dosing day between the control and rh-TIMP-1 groups (data not shown).

MMP secretory capacity. In gelatin zymography, ProMMP-9, MMP-9 and MMP-2 were observed as clear unstained belts at 92KDa, 82KDa and 62KDa, respectively. In the monoculture of HT29, the secretion of MMP-2 and MMP-9 was confirmed. In addition, HT29 was monoculture in the rh-TIMP-1 added medium to investigate the secretory capacity of MMPs, but no changes in the gelatinolysis pattern were observed. The concentrations of rh-TIMP-1 were changed from 10 to 500 ng/ml, but the patterns did not change, indicating no concentration dependence (data not shown). This indicated that the rh-TIMP-1 administration had no influence on the secretory capacity of MMP-2 or MMP-9 of the tumor cells (Fig. 2).

Localization and staining properties of MMPs evaluated by immunohistochemical staining. MMPs were stained mainly in the interstitium, although a small portion of them were stained in the nuclei. MMP-2 was strongly stained in the lumen of the microvessels and the surrounding region (Fig. 3A).

With Schedule 1, positive stainings for both MMP-2 and MMP-9 were observed in all mice of the rh-TIMP-1 and control groups. With Schedule 2, positive stainings for MMP-2 were observed in all mice, and negative stainings for MMP-9 were observed in approximately 20% of the mice. With Schedule 3, positive stainings for MMP-2 were observed in all mice, and negative stainings for MMP-9 were observed in approximately 55% of the mice (Fig. 3B).

Evaluation of microvessels formation by immunohistochemical staining. Immunohistochemical staining using CD34, which was a specific marker of vascular endothelial cells, was performed to evaluate angiogenesis (Fig. 4). Based on the result, MVC was measured. With Schedule 1 or 2, no significant difference in MVC was observed between the rh-TIMP-1 and control groups. With Schedule 3, a significant suppression of angiogenesis was observed in the rh-TIMP-1 group ($p < 0.01$ vs control) (Fig. 5).

Influence of rh-TIMP-1 on cell proliferation capacity. A cell proliferation assay was performed to investigate the influence of rh-TIMP-1 on

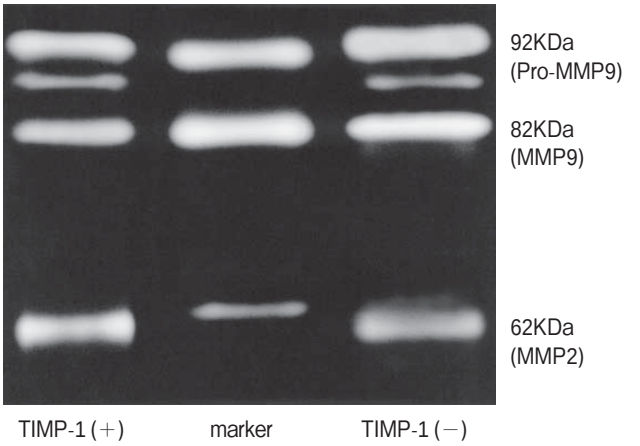

Fig. 2 The influence of rh-TIMP-1 administration on gelatinolytic activity of HT29 was investigated by gelatin zymography. The activity is indicated as an achromatic white band. There was no difference in the activity between the control (TIMP-1 (−)) and rh-TIMP-1 (TIMP-1 (+)) groups.

tumor cells. The reaction time of rh-TIMP-1 was changed from 24 to 72 h under the concentration of rh-TIMP-1, 10 ng/ml, but the proliferation capacity of tumor cells did not change, indicating no influence of the reaction time (Fig. 6). The concentrations of rh-TIMP-1 were changed from 50 to 500 ng/ml, but the proliferation capacity did not change, indicating no concentration dependence (data not shown).

Discussion

Several complicated steps are necessary for tumor cells to separate from the primary lesion, be implanted in distant organs, and form new lesions. The tumor cells must: 1) proliferate, 2) be infiltrated from the primary lesion, 3) invade the vessels, 4) reach the capillary bed of distant organs, 5)

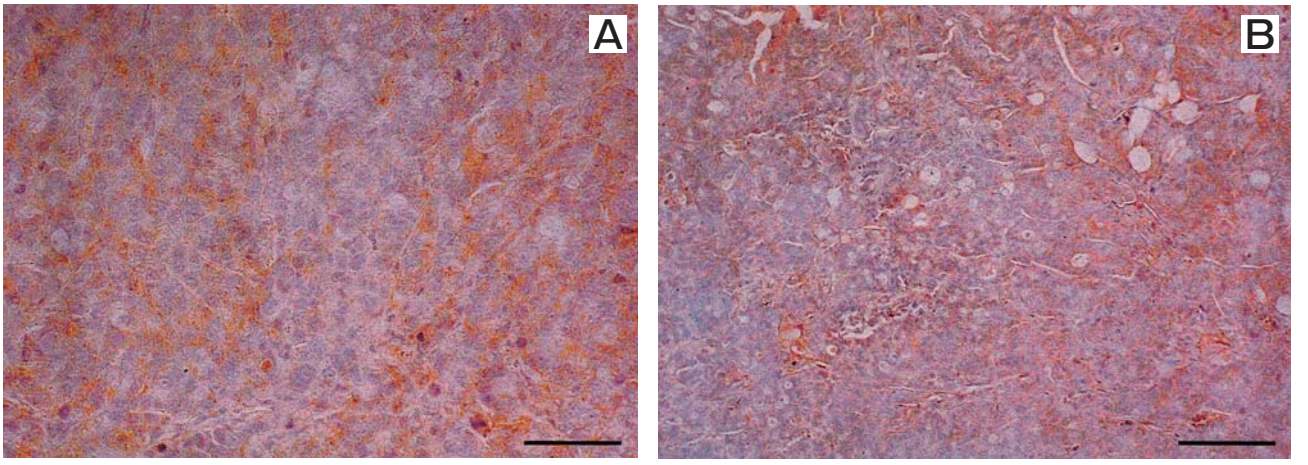

Fig. 3 The influence of rh-TIMP-1 administration on MMPs production was immunohistochemically investigated. MMPs were stained mainly in the interstitium, and a small portion of them were stained in the nuclei. Regarding MMP-2, strong stains were observed in the microvessels lumen and its surroundings. When staining properties with each treatment schedule were compared among the rh-TIMP-1 and control groups (n = 10 each), positive stainings for both MMP-2 and MMP-9 were observed in all mice in both groups with Schedule 1. With Schedules 2 and 3, positive stainings for MMP-2 were observed in all mice, and negative stainings for MMP-9 were observed in 20% and 40% of mice, respectively (**A**: MMP-2 positive case, × 200. **B**: MMP-9 positive case, × 100). Bars in figures indicate 25 μm.

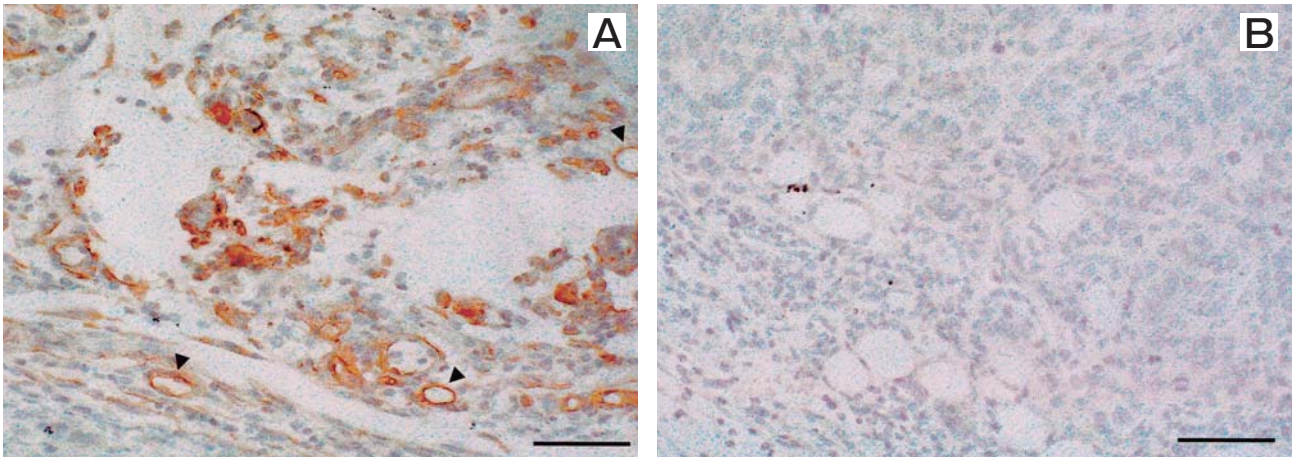

Fig. 4 Immunohistochemical staining using CD34, which was a specific marker of vascular endothelial cells, was performed to evaluate microvessels formation. **A**, In the control group, there were stained endothelial cells (arrow head) and a wide range of microvessels formation (× 200) with schedule 3; **B**, Stained endothelial cells were rarely observed in the rh-TIMP-1 group (× 200) with schedule 3. Bars in figures indicate 25 μm.

adhere to the endocapillary cells, 6) escape from the vessels, and 7) infiltrate distant organs. These processes include the three steps of adhesion, degradation and movement (3-step theory of Liotta [11]). Among these steps, the degradation (infiltration) of Type IV collagen, which is a constituent of the basal membrane, must be controlled to inhibit the infiltration and metastasis of tumor cells, and many fundamental experiments to accomplish this have been performed.

To infiltrate the surrounding tissues and metastasize to the distant tissues and organs through the capillary vessels and lymph ducts, the tumor cells should locally degrade ECM first [1]. In addition, the process of ECM degradation is necessary for the angiogenesis that is essential for tumor proliferation. ECM degradation through MMP is required for the migration and proliferation of endocapillary cells that is necessary for angiogenesis [2]. There is a close correlation between the constituents of ECM and tumor cells. The interaction of tumor cells with ECM protein at a receptor level on the cell surface induces the production and secretion of proteinase (metalloproteinases), which makes it possible for tumor cells to decompose ECM constituents and infiltrate to the basal membrane. The degradation products of ECM (*e.g.*, a fragment of fibronectin) emphasize the gene expression induction of proteases, which results in the formation of the positive feedback loop that accelerates the infiltration and proliferation of tumor cells. Furthermore, the isolation of growth factors accompanied by ECM degradation promotes the proliferation of tumor cells [12]. Proteinases which are thought to play a central role in this ECM degradation are MMPs, and Type IV collagenase (gelatinase A = MMP-2, gelatinase B = MMP-9) in particular, which specifically degrades Type IV collagen has a close correlation with the infiltration and metastasis of tumors. Each MMP is secreted as an inactive pro-form, when gene expression is induced by cytokines or growth factors. The pro-form becomes an active form when propeptide is removed through the action of proteinases. These MMPs are controlled by specific inhibitors, TIMPs [4]. The blood TIMP concentrations in healthy subjects can be quantitated by a Sandwich immunoassay using antibodies. The blood TIMP-1 and TIMP-2 concentrations have been measured at about 170 ng/ml [13] and 56 ng/ml [14], respectively, and are present in body fluids other than

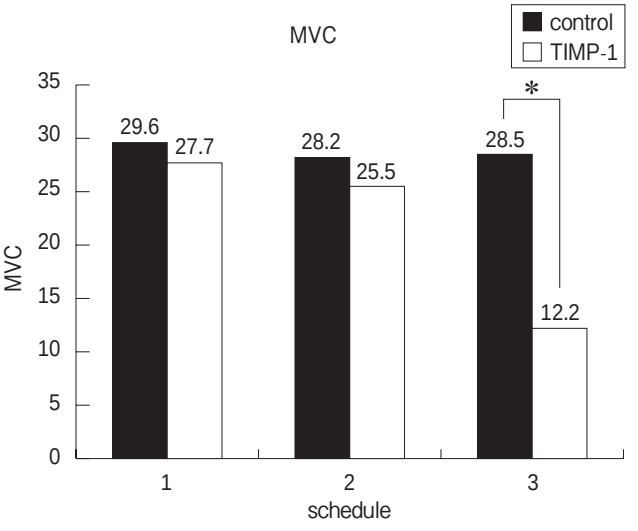

Fig. 5 MVC by treatment schedules was measured in the rh-TIMP-1 and control groups. With Schedule 3, the MVC levels were significantly lower in the rh-TIMP-1 group than in the control group. Based on this result, it was confirmed that TIMP-1 had an effect on microvessels formation, and that the timing of TIMP-1 administration greatly enhanced this effect (*: < 0.01 vs. control).

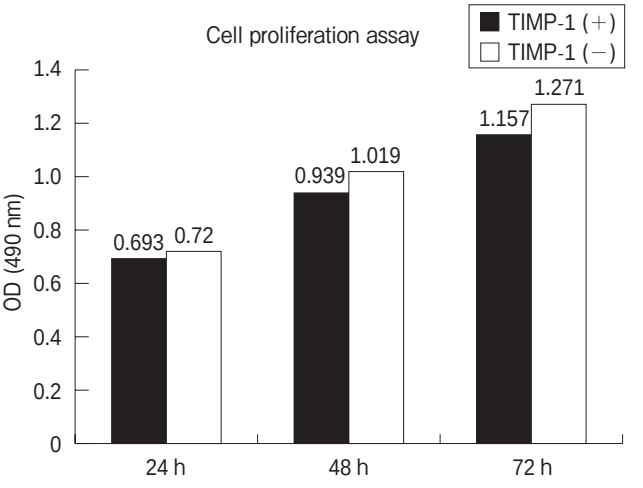

Fig. 6 The influence of rh-TIMP-1 (10 ng/ml) on the cell proliferation of HT29 was investigated *in vitro*. There was no remarkable difference in cell proliferation between the control (TIMP-1 (−)) and rh-TIMP-1 (TIMP-1 (+)) groups for 24–72 h after rh-TIMP-1 administration. The influence of rh-TIMP-1 with changing concentrations was investigated, but no differences between the 2 groups was observed (data not shown).

blood. In addition, they are reported to be secreted in normal cells including fibroblasts [17], endothelial cells [18] and vascular smooth-muscle cells [19]. Four subtypes of TIMPs [20–23] have been known, and each controls the activity of MMPs. TIMP-1 specifically binds to progelatinase B (pro MMP-9), which is a pro-form of gelatinase B (MMP-9), and strongly inhibits the transformation to gelatinase B [24]. The influence of TIMP-1 on tumor cells has been confirmed by various studies. In an *in vitro* experiment using the reconstructed basal membrane of matrigel, it was reported that the infiltration ability of metastatic tumor cells is inhibited by TIMPs [24]. In an *in vivo* experimental system, Khokha *et al.* [25] reported that tumor formation is observed when the antisence TIMP-1 gene is introduced to nonneoplastic Swiss 3T3 cells and the clone that inhibits the TIMP-1 gene expression is selectively implanted in nude mice. However, some investigators have confirmed the effect of TIMPs by introducing the TIMP gene to the metastatic tumor cell strain and compellingly producing TIMPs. When the TIMP-1 gene was introduced to the mouse melanoma cell line, B16-F10, by Khokha *et al.* [7], and to the human unspecialized gastric cancer stain by Tsuchiya *et al.* [26], the metastatic capability of these cells was reported to clearly decrease. It was reported that the repeated administration of TIPM-1 to animals after the implantation of the metastatic tumor cell line can inhibit metastasis *in vivo* [27]. In contrast, Yamauchi *et al.* [28] performed an experiment to inhibit hepatic metastasis using the gastric cancer cell line, and concluded that the intraperitoneal injection of rh-TIMP-1 is less efficacious than introduction of the TIMP-1 gene. They suggest the reason for this is that the blood half-life of rh-TIMP-1 is short and an effective concentration cannot be maintained without repeated administration. Furthermore, the suppressive effect of TIMP-1 on microvessels formation, as well as on tumor proliferation, has been reported. Johnson *et al.* [6] and Ikenaka *et al.* [29] investigated the suppressive effect of TIMP-1 on tumor proliferation *in vivo* and *in vitro*, and suggested that this effect is ascribed to the suppression of angiogenesis by TIMP-1.

In the present study, we investigated the suppressive effect of TIMP-1, which is a specific inhibitor of MMP-9, and found, through observation of tumor diameters over time and evaluation of immunohistochemical staining, that TIMP-1 had a sufficient suppressive effect on tumor proliferation. When TIMP-1 was administered from the very early stage of tumor formation, as with Schedule 3, the suppressive effect on angiogenesis, in addition to tumor proliferation, could be confirmed. The results of a cell proliferation assay showed no cytotoxic effect of TIMP-1 on HT29. Therefore, the suppressive effect on tumor proliferation might result from the suppression of angiogenesis by TIMP-1. However, the suppressive effect of TIMP-1 on tumor proliferation was hardly observed when the treatment was initiated after the tumor had grown to some extent, as with Schedule 1. This suggested that the suppressive effect of TIMP-1 on angiogenesis was not produced when the microvessels were formed on some level in the regions around tissues. In the present study, in consideration of the blood half-life of rh-TIMP-1, intraperitoneal injection was performed twice a day (every 12 h). When the tumor diameters were compared, the suppressive effect of tumor proliferation was observed clearly with Schedule 3, and partially with Schedule 2. We did not perform the experiment using methods other than regularly prescribed medication, but the everyday medication was still thought to be the most effective administration method. When rh-TIMP-1 is used in clinical practice, the administration methods, manufacturing cost and side effects should be considered.

When systemic administration is performed, as shown in our study, repeated administration of a large amount of TIMP-1 is required to maintain an effective blood concentration, and the cost, under present circumstances, can be enormous. Furthermore, in systemic administration, TIMP-1 acts on regions other than the local tumor and given this, side effects are unavoidable. There have been previous attempts to use TIMPs in clinical practice based on the results of basic experiments in the US, but it was concluded to be difficult to apply TIMPs in a clinical setting [30] due to difficulty in accomplishing the large-scale manufacturing of TIMPs as well as problems with the instability and multifunctionality [20] of TIMPs. These problems might be resolved when large-scale, low-cost manufacturing of TIMPs can be realized by the improvement of recombinant DNA techniques. If improvement of the drug deliv-

ery system (DDS) leads to localization of TIMPs on the tumor itself, the dosage can be reduced and side effects suppressed. Liu *et al.* [31] suggested that a complex composed of an antitumor agent and antitumor antibody is effective in animals, and Yamaguchi *et al.* [32] indicated that a complex composed of a human colon cancer monoclonal antibody and a Neocarzinostatin is useful in clinical study. If these methods can be applied, a good treatment effect from the locally administration of rh-TIMP-1 can be expected. On the other hand, other than systemic administration, TIMP-1 is applied to the treatment by the method of introduction to local tumor and overexpression of TIMP-1 gene. The suppressive effect of this method on tumor proliferation and metastasis has been confirmed in many basic experiments [28, 32–35]. In Japan, the clinical applications of gene therapy using virus vectors in lung [36] and renal [37] cancer has been approved and practically performed. This indicates that it is not technically difficult to introduce the TIMP-1 gene to living bodies. The method of injecting rh-TIMP-1 directly to the region adjacent to the tumor (intratumoral injection) should also be considered, and its effect should be confirmed *in vivo* in the future.

In the present study, it could be confirmed that the effects of rh-TIMP-1 greatly differed depending on the timing of administration. The effect of rh-TIMP-1 on tumor contraction could not be confirmed. However, a slowing down of the proliferation rate was observed more in the rh-TIMP-1 group than in the control group. To maximize the effect of rh-TIMP-1, it might be necessary to start administrating it starting when the tumor is minute in size. Therefore, in clinical practice, the treatment effect of rh-TIMP-1 could be maximized if it is used after shrinkage of the primary focus is obtained by surgical resection, or by successful chemotherapy and radiotherapy. Furthermore, the results of our study reproduced the tumor dormant state [38–39], in which the tumor tissues were not shrunk, but their proliferation was suppressed. Takahashi *et al.* [40] reported no correlation between the tumor shrinkage effect of chemotherapy and the life-prolonging period, and tumor shrinkage is not necessary for extension of the survival time. They suggested that the longer a tumor remains dormant, the longer the survival time will be. In addition, Nemunaitis *et al.* [41] performed a clinical study using Marimastat which is one of the synthetic MMP inhibitors, and reported that tumor shrinkage is not indispensable for the prolongation of survival time and that significant prolongation is observed in patients whose tumor proliferation has been suppressed. We expect MMP inhibitors to inhibit, even indirectly, the proliferation and metastasis of tumor cells, and to maintain the state of coexistence of tumor and recipient (so called tumor dormancy) as long as possible even though the tumor tissues have not shrunk. If quality of life can be improved and survival time can be prolonged in tumorbearing patients, MMP inhibitors including rh-TIMP-1 might have great clinical significance. The results of our study showed that rh-TIMP-1 was an agent with the potential to be applied in clinical settings as an MMP inhibitor. However, there were several problems with rh-TIMP-1 that need to be solved in further studies.

References

1. Liotta LA, Steeg PS and Stetler-Stevenson WG: Cancer metastasis and angiogenesis imbalance of positive and negative regulation. Cell (1991) 64: 327–336.
2. Liotta LA: Tumor invasion and metastases-role of the extracellular matrix. Cancer Res (1986) 46: 1–7.
3. MacDougall JR and Matrisian LM: Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. Cancer Metastasis Rev (1995) 14: 351–362.
4. Docherty AJP, Lyons A, Smith BJ, Wright EM, Stephens PE, Harris TJ, Murphy G and Reynolds JJ: equence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating Activity. Nature (1985) 318: 66–69.
5. Alvarez OA, Carmicheal DF and De Clerck YA: Inhibition of collagenolytic activity and metastasis of tumor cells by a recombinant human tissue inhibitor of metalloproteinases. J Nhat Cancer Inst (1990) 82: 589–595.
6. Johnson MD, Kim RH, Chesler L, Tsao-Wu G, Bauck N and Polverini PJ: Inhibitor of collagenolytic activity and metastasis of tumor cells by a recombinant human tissue inhibitor of metalloproteinases. J Cell Physiol (1994) 160: 194–202.
7. Khokha R: Suppression of the tumorigenic and metastatic abilities of murine B16-F10 melanoma cells in vivo by the overexpression of the tissue inhibitor of metalloproteinases-1. J Natl Cancer Inst (1994) 86: 299–304.
8. Heussen C and Dowdle EB: Electrophoretic analysis of plasminogen activator in polyacrylamide gels containing sodium dodecyl sulphate and copolmerised substrates. Anal Biochem (1980) 102: 196–202.
9. Sannino P and Shousha S: Demonstration of oestrogen receptors in paraffin wax sections of breast carcinoma using the monoclonal antibody 1D5 and microwave oven processing. J Clin Pathol (1994) 47: 90–92.
10. Fukuda T, Tani Y, Kobayashi T: A new western blotting method

using polymer immunocomplexes: Detection of Tsc1 and Tsc2 expression in various cultured cell lines. Anal Biochem (2000) 285: 274–276.
11. Liotta LA, Rao CN and Wewer UM: Biochemical interactions of tumor cells with the basement membrane. Annu Rev Biochem (1986) 55: 1037–1057.
12. Juliano RL and Haskile S: Signaling transduction from the extracellular Matrix. J Cell Biol (1993) 120: 577–585.
13. Kodama S: Rapid one-step sandwitch enzyme immunoassay for human matrix metalloproteinases. An application for rheumatoid arthritis serum and plasma. J Immuno Methods (1990) 127: 108.
14. Fujimoto N: A one-step sandwitch enzyme immunoassay for tissue inhibitor of metalloproteinases-2 using monoclonal antibodies. Clin Chem Acta (1993) 220: 31–45.
15. Cawston TE, Galloway WA, Mercer E, Murphy G and Reynolds JJ: Purification of rabbit bone inhibitor of collagenase. Biochem J (1981) 195: 159–165.
16. Bunning RA, Murphy G, Kumar S, Phillips P and Reynolds JJ: Metalloproteinases inhibitors from bovine cartilage and body fluids. Eur J Biochem (1984) 139: 75–80.
17. Stricklin GP and Welgus HG: Human skin fibroblast collagenase inhibitor. Purification and biochemical characterization. II. Expression of collagenase and stromelysin activities is regulated by endogenous inhibitors. J Biol Chem (1983) 258: 12252–12258.
18. Herron GS, Banda MJ, Clark EJ, Gavrilovic J and Werb Z: Secretion of metalloproteinases by stimulated capillary endothelial cells. J Biol Chem (1986) 261: 2814–2818.
19. DeClerck YA: Purification and characterization of a collagenase inhibitor produced by bovine vascular smooth muscle cells. Arch Biochem Biophys (1988) 265: 28–37.
20. Hayakawa T, Yamashita K, Tanzawa K, Ushijima E and Iwata K: Growth-promoting activity of tissue inhibitor of metalloproteinases-1 (TIMP-1) for a wide range of cells. A possible new growth factor in sarum. FEBS Lett (1992) 298: 29–32.
21. Stetler-Stevenson WG, Krutzsch HC and Liotta LA: Tissue inhibitor of metalloproteinases (TIMP-2): a new member of the metalloproteinases inhibitor family. J Biol Chem (1989) 264: 17374–17378.
22. Leco KJ, Khokha R, Pavloff N, Hawkes SP and Edwards DR: Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distincyive pattern of expression in mouse cells and tissues. J Biol Chem (1990) 269: 9352–9360.
23. Greene J, Wang M, Liu YE, Raymond LA, Rosen C and Shi YE: Molecular cloning and caracterization of human tissue inhibitor of metalloproteinases 4. J Biol Chem (1996) 271: 30375–30380.
24. Denhardt DT, Feng B, Edwards DR, Cocuzzi ET and Malynnkar UM: Tissue inhibitor of metalloproteinases (TIMP, aka, EPA): Structure, control of expression and biological functions. Pharmacol Ther (1993) 59: 329–341.
25. Khokha R, Waterhouse P, Yagel S, Lala PK, Overall CM, Norton G and Denhardt DT: Antisence RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science (1989) 243: 947–950.
26. Tsuchiya Y, Sato H, Okada Y, Mai M, Sasaki T and Seiki M: Tissue inhibitor of metalloproteinases-1 is a negative regulator of the metastatic ability of a human gastric cancer cell line, KKLS, in the chick embryo. Cancer Res (1993) 53: 1397–1402.
27. Stetler-Stevenson WG, Aznavoorian S and Liotta LA: Tumor cell interaction with the extracellular matrix during invasion and metastasis; in Annual Review of Cell Biology, Palade GE, Alberts BM and Spudich JA eds, 9 th Ed, Annual Review Inc Palo Alto, California (1993) pp 541.
28. Yamauchi K, Ogata Y, Nagase H and Shirouzu K: Inhibition of liver metastasis from orthotopically implanted colon cancer in nude mice by transfection of the TIMP-1 gene in KM12SM cells. Surg Today (2001) 31: 791–798.
29. Ikenaka Y, Yoshiji H, Kuriyama S, Yoshii J, Noguchi R, Tsujinoue H, Yanase K, Namisaki T, Imazu H, Masaki T and Fukui H: Tissue inhibitor of metalloproteinases-1 (TIMP-1) inhibits tumor growth and angiogenesis in the TIMP-1 transgenic mouse model. Int J Cancer (2003) 105: 340–346.
30. DeClerck YA and Imren S: Proteases inhibitor: role and potential therapeutic use in human cancer. Eur J Cancer (1994) 30: 2170–2180.
31. Liu C, Tadayoni BM, Bourret LA, Mattocks KW, Derr SM, Widdison WC, Kedersha NL, Ariniello PD, Goldmacher VS, Lambert JM, Blattler WA and Chari RV: Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. Proc Natl Acad Sci USA (1996) 93: 8618–8623.
32. Yamaguchi T, Tsurumi H, Kitamura K, Otsuji E, Kotani T, Miyagaki T and Takahashi T: Production, binding and cytotoxicity of human/mouse chimeric monoclonal antibody-neocarzinostatin conjugate. Jpn J Cancer Res (1993) 84: 1190–1194.
33. Martin DC, Ruther U, Sanchez-Sweatman OH, Orr FW and Khokha R: Inhibition of SV40 T antigen- induced hepatocellular carcinoma in TIMP-1 transgenic mice. Oncogene (1996) 13: 569–576.
34. Watanabe M, Takahashi Y, Ohta T, Mai M, Sasaki T and Seiki M: Inhibition of metastasis in human gastric cancer cells transfected with tissue inhibitor of metalloproteinasa 1 gene in nude mice. Cancer (1996) 77: 1676–1680.
35. Kruger A, Sanchez-Sweatman OH, Martin DC, Fata JE, Ho AT, Orr FW, Ruther U and Khokha R: Host TIMP-1 overexpression confers resistance to experimental brain metastasis of a fibrosarcoma cell line. Oncogene (1998) 16: 2419–2423.
36. Kagawa S, Fujiwara T and Tanaka N: Clinical study of adenoviral mediated p53 gene therapy for non-small lung cancer in Japan. Jpn J Cancer Chemter (2003) 30: 193–197 (in Japanese).
37. Kawai K, Tani K and Yamashita N: Advanced renal cell carcinoma treated with granulocyte-macrophage colony-stimmulating factor gene therapy: a clinical course of the first Japanese experience. Int J Urol (2002) 9: 462–466.
38. Weinhold KJ, Goldstin LT and Wheelock EF: Tumor dormant states established with L5178Y lymphoma cells in immunized syngeneic murine host. Nature (1977) 270: 59–61.
39. Uhr JW, Scheuerman RH, Street NE and Vitetta ES: Cancer dormancy; Opportunities for new therapeutic approaches. Nat Med (1997) 3: 505–509.
40. Takahashi Y and Nishioka K: Survival without tumor shrinkage: Re-evaluation of survival gain by cytostatic effect of chemotherapy. J Natl Cancer Inst (1995) 87: 1262–1263.
41. Nemunaitis J, Poole C, Primrose J, Sosemurgy A, Malfetano J, Brown P, Berrington A, Cornish A, Lynch K, Rasmussen H, Kerr D, Cox D and Millar A: Combined analysis of studies of the effect of the matrix metalloproteinases inhibitor marimastat on serum tumor markers in advanced cancer: Selection of a biological active and tolerable dose for long-term studies. Clin Cancer Res (1998) 4: 1101–1109.