Antitumor Effect of Arterial Administration of a Medium-Chain Triglyceride Solution of an Angiogenesis Inhibitor, TNP-470, in Rabbits Bearing VX-2 Carcinoma

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Using rabbits bearing VX-2 carcinoma on the inner side of the leg, we examined the antitumor activity of a medium-chain triglyceride (MCT) solution of an angiogenesis inhibitor, TNP-470 (AGM-1470, 6-O-(N-chloroacetylcarbamoyl)-fumagillol), following administration into the femoral artery feeding the tumor. The MCT solution of TNP-470 (1 and 5 mg) strongly suppressed tumor growth following a single intra-arterial (i. a.) injection 2 or 3 weeks after tumor inoculation. Moreover, remarkable regression of well-developed tumors, those 4 weeks after inoculation, was obtained by i.a. injection of the MCT solution containing 20 mg of TNP-470 without any influence on body weight. The antitumor effects were potentiated by coadministration of doxorubicin or mitomycin C (MMC) in the solution or microspheres containing MMC. In a shell-less chorioallantoic membrane (CAM) assay, angiogenesis was inhibited when a droplet of the MCT solution containing 25 μg of TNP-470 was placed on the CAM for 2 days, suggesting that the prolonged antitumor effect resulted from the inhibition of tumor neovascularization by sustained drug release from the preparation. These results indicate that i. a. injection of the MCT solution of TNP-470 is promising for treating well-developed tumors.

KEY WORDS: TNP-470; angiogenesis inhibitor; medium-chain triglyceride; intra-arterial injection; rabbit VX-2 carcinoma; CAM assay.

INTRODUCTION

TNP-470 (AGM-1470, 6-O-(N-chloroacetylcarbamoyl)-fumagillol, Fig. 1) synthesized by Takeda Chem. Ind., Ltd. (1) is a new type of anticancer drug that inhibits tumor neovascularization and blocks the supply of nutrients to tumors (2, 3). In cancer therapy, chemoembolization is a useful treatment strategy which combines chemotherapy and tumor devascularization by intra-arterial (i.a.) administration of an embolizing material. This type of treatment can achieve regional elevation of drug concentration and prolonged retention of anticancer drugs at the tumor site, and results have suggested enhanced antitumor activity with less severe systemic side effects. Furthermore, drugs dissolved in Lipiodol (LPD), a lymphographic oil, showed selective accumulation and retention at the tumor site after arterial administration due to the difference in time required for the oil to be removed from normal capillaries and the tumor vasculature (4, 5). In a previous study, we found that TNP-470 microspheres (msp) prepared using a biodegradable polymer, poly (lactic / glycolic) acid (PLGA), and a TNP-470 LPD solution which could achieve selective drug targeting and retention at the tumor site caused striking tumor regression in rabbits bearing VX-2 carcinoma after a single injection into the femoral artery running to the tumor (6, 7).

In the present study, the antitumor activity of i.a. administration of TNP-470 dissolved in medium-chain triglyceride (MCT, caprylic and capric acid triglyceride), which was found to be a preferred oil base in terms of solubility, stability and release sustainability in our formulation studies (8), was evaluated in the rabbit tumor model. The antitumor activities of TNP-470 coadministered with conventional chemotherapeutic agents, doxorubicin (ADM) and mitomycin C (MMC), and PLGA msp containing MMC were also investigated.

MATERIALS AND METHODS

TNP-470 was synthesized at Takeda Chemical Ind. (Osaka, Japan), and its structure is shown in Fig. 1. ADM, MMC and PLGA (molecular ratio of lactic and glycolic acid of 75:25, weight-average molecular weight of around 14,000) were obtained from Wako Pure Chemical Ind. (Osaka, Japan). MCT (MIGLYOL 812) was obtained from Huls A.G. (Marl, Germany), and sesame oil came from Takemoto Yushi (Aichi, Japan).

Preparation of Dosage Forms

TNP-470 was dissolved in sesame oil or MCT with mild shaking at room temperature, and the solution was sterilized by filtering (0.22 μm, Dimex filter, Millipore Japan, Tokyo, Japan). ADM or MMC was suspended in the oil solution after pulverization in an earthenware mortar. MMC was microencapsulated in PLGA by an in-water drying method. Briefly, MMC was dissolved in 50% PLGA-methylene chloride. The solution was poured into a 0.15% aqueous solution of polyvinyl alcohol under stirring with a turbine-shaped mixer. The oil/water emulsion was continuously stirred for 2 hr to evaporate the methylene chloride. The hardened msp were sized using sieves with apertures of 250 and 125 μm. The msp (125~250 μm) were rinsed with distilled water 3 times and lyophilized. The resulting msp contained 0.14 mg of MMC / 1 mg of msp. These msp (1 mg as MMC) were suspended in MCT or TNP-470 MCT solution before administration.

Tumor Inoculation

Experiments were carried out by the method reported previously with some modification (7). Transplantable anaplastic VX-2 carcinoma that originated from spontaneously transformed Shope papilloma was used. A female rabbit bearing VX-2 carcinoma and male rabbits (Kbl, JW) weighing around 2.5 to 3.0 kg were purchased from Funabashi Farm (Chiba, Japan) and Kitayama LABES (Kyoto, Japan),

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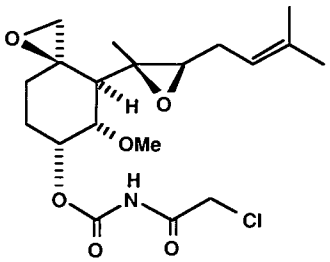

Fig. 1. Chemical structure of TNP-470.

respectively. VX-2 carcinoma was minced with scissors and sieved with a wire mesh (60 mesh), and tumor cells were suspended in Hank's solution containing 10 % rabbit serum, 120 μg/ml of penicillin and 100 μg/ml of streptomycin. Male rabbits were inoculated subcutaneously with VX-2 carcinoma cell suspension (10 %(w/v), 0.5 ml) at a position on the inside of the right leg just below the knee. The VX-2 carcinoma cell line was maintained by successive inoculation of untreated rabbits.

Antitumor Effects

Two, 3 or 4 weeks after inoculation, preparations (sesame oil solution, 1 ml; MCT solution, 0.5 ml) were injected into the femoral artery through polyethylene tubing (PE-50, Clay Adams, NJ) under pentobarbital anesthesia. After administration, the blood flow in the femoral artery was reopened by inserting the tubing upward into the artery after being shortened to a length of about 4 cm. Since total ischemia occurs for less than 3 min during the administration procedure, it should have no effect on tumor growth. Antitumor activities were evaluated using untreated control rabbits for comparison. Tumor volume was taken to be the product of the length, width and height as measured with calipers through the skin and was expressed as a ratio to the volume just before treatment (tumor volume ratio).

Shell-less Chorioallantoic Membrane (CAM) Assay

The assay was carried out by the method reported by Y. Nozaki et al. (9). Briefly, day-3 eggs were cracked, and embryos were placed on hammocks of polyethylene sheets hanging in plastic cups and then incubated at 37°C under an atmosphere of 3 % CO_2 and saturated humidity for a further 6 days. On day 9, 5 μl of oil solutions containing TNP-470 were placed on the CAM. After incubation for 2 days, responses induced by the samples were examined under a stereoscope (SMZ-10, Nikon, Tokyo, Japan).

RESULTS AND DISCUSSION

In this study, a rabbit carcinoma implanted subdermally at a position on the hind leg was utilized to evaluate antitumor activity. The ultimate goal of our experiments using rabbits and rats is the treatment of hepatic cancer (10), and although this tumor model may not be realistic for hepatic cancer, it has the advantage, especially in formulation studies, of allowing tumor size to be measured easily and continuously through the skin.

Repeated i.v. and s.c. injection of aqueous solution of TNP-470 suppressed the tumor growth in the same rabbit tumor model but did not reduce the tumor volume sufficiently, and chemoembolization with PLGA msp containing TNP-470 resulted in strong suppression of the tumor growth for about 1 week, which corresponds to the period of TNP-470 sustained release (7). In further formulation studies, we found that 1) TNP-470 is soluble in various oils and especially in MCT with a solubility of approximately 100 mg/ml at 25°C, which is an important strategic factor in the present drug targeting therapy; 2) TNP-470 is labile in aqueous solution with a degradation half life of 4~5 hr (pH = 7.0 at 37°C) and insufficiently stable in sesame oil or LPD, whereas in MCT more than 90 % of the initial TNP-470 content (10 mg/ml) is preserved even when the solution is stored at 40°C for 6 months, indicating pharmaceutical superiority of the TNP-470 MCT solution; 3) the MCT solution provided sustained release of TNP-470 for a period of more than 1 week according to first-order release kinetics in contrast to the disappearance of TNP-470 from the PLGA msp during 5 days as an *in vitro* release test (8); and 4) the MCT solution of TNP-470 achieved selective targeting to the tumor site in rats bearing Walker 256 carcinosarcoma in the liver after administration *via* the hepatic artery and the tumor-specific retention of TNP-470 for more than 2 weeks (10). MCT is used clinically as a component in a lipid emulsion formulation for parenteral nutrition, indicating satisfactory biocompatibility. In the present study, we examined the antitumor activity of this promising formulation of TNP-470 in the rabbit tumor model.

Figure 2 shows the antitumor effect on tumors 2 weeks

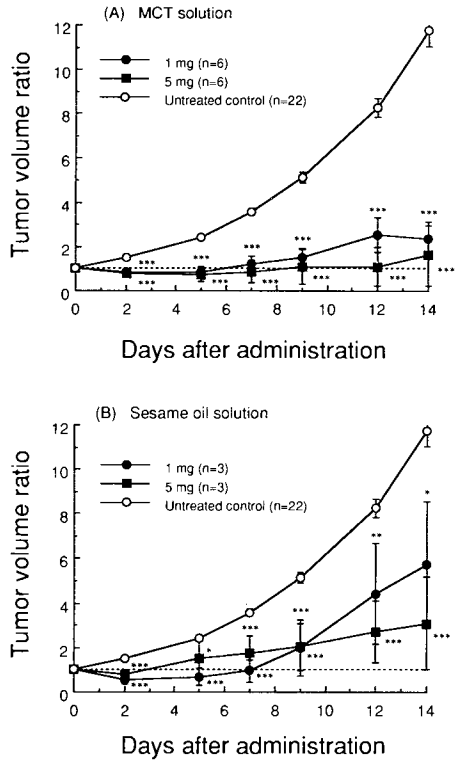

Fig. 2. Antitumor activity of TNP-470 MCT (A) and sesame oil (B) solutions against week-2 VX-2 carcinoma in rabbits after i.a. injection at different doses. Each point represents the mean ± S.E. Significantly different from the corresponding point for the untreated control by Student's *t*-test (*;$P<0.05$, **;$P<0.01$, ***;$P<0.001$).

after inoculation (week-2 tumors) following a single i.a. injection of MCT solutions or sesame oil solutions (for comparison) containing 1 and 5 mg of TNP-470. Untreated tumors grew extensively, and volume increased 11.8 times during the following 2-week period. On the other hand, dose-dependent suppression of the tumor growth was observed after administration of TNP-470 oil solutions. MCT solutions tended to cause more persistent tumor regression than the sesame oil solutions after i.a. administration. One possible cause for this is the difference in the period of sustained release of TNP-470 from the two formulations as observed in an *in vitro* release test: the release half life of TNP-470 from a sesame oil solution was 0.53 day^{-1}, while that from an MCT solution was 2.0 day^{-1} (8). Considering that repeated i.v. or s.c. administration of a TNP-470 aqueous solution (total dose; 5~25 mg) could not suppress the tumor growth sufficiently (7), the effect of i.a. administration of oil formulations, especially the MCT solution, is noteworthy. In a previous study, antitumor activities of an LPD solution of TNP-470 and PLGA msp encapsulating TNP-470 with a diameter of 53 ~ 125 μm were investigated in the same *in vivo* system. The antitumor activity of MCT solution obtained in the present study was comparable with that of LPD solution reported. On the contrary, PLGA msp containing TNP-470 suppressed the tumor growth with slight regression for initial 9 days after i.a. administration, but followed by lively regrowth of tumors (6, 7).

We also examined the antitumor activity of i.a. injection of TNP-470 MCT solutions using well-developed tumors, that is, tumors 3 and 4 weeks after inoculation (week-3 and week-4 tumors, respectively). Figure 3 shows the effects of a plain MCT or an MCT solution containing TNP-470 on week-3 tumors. Untreated tumors grew extensively, and volume increased 7.4 times during the following 3-week period. The tumors in animals receiving a plain MCT also grew extensively after transient initial suppression probably due to partial embolization of the tumor microvasculature by the oil droplets for a short time following administration as was observed in a rat hepatic tumor model (10). On the other hand, the tumors in animals receiving the 5 mg of TNP-470 dissolved in MCT were persistently suppressed with regression for the entire 3 weeks. Since plain MCT caused little tumor suppression past that observed initially, the retention

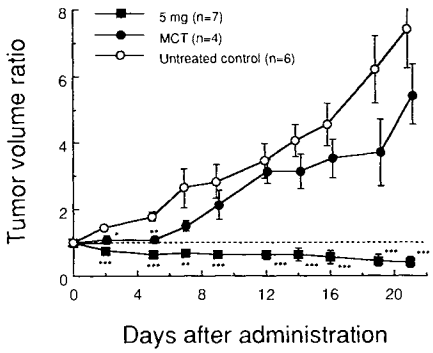

Fig. 3. Antitumor activity of an MCT solution containing TNP-470 (5 mg) and plain MCT against week-3 VX-2 carcinoma in rabbits after i.a. injection. Each point represents the mean ± S.E. Significantly different from the corresponding point for the untreated control by Student's *t*-test (*;P<0.05, **;P<0.01, ***;P<0.001).

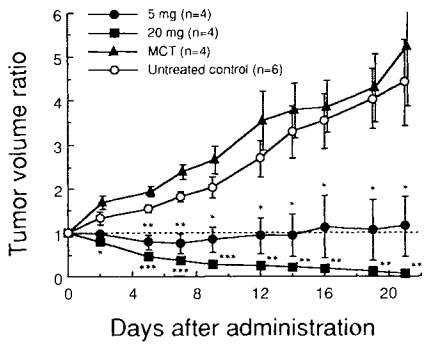

Fig. 4. Antitumor activity of TNP-470 MCT solutions and plain MCT against week-4 VX-2 carcinoma in rabbits after i.a. injection. Each point represents the mean ± S.E. Significantly different from the corresponding point for the untreated control by Student's *t*-test (*;P<0.05, **;P<0.01, ***;P<0.001).

and release of the angiogenesis inhibitor around the tumor should be responsible for the distinct antitumor activity.

Figure 4 shows the effect of i.a. administration of TNP-470 MCT solutions on week-4 tumors. Untreated tumors again grew extensively, and volume increased 4.4 times during the following 3-week period. Plain MCT showed little initial suppression in this experiment, which was inconsistent with the results observed in the case of week-3 tumors. The MCT solution containing 5 mg of TNP-470 provided persistent suppression of tumor growth with transient regression. When the MCT solution containing 20 mg of TNP-470 was administered intra-arterially, the tumor volume was gradually reduced to the volume ratio of 0.06 three weeks after administration, and three out of the four tumors had disappeared. In addition, these noticeable antitumor effects were not accompanied by severe effects on body weight, suggesting the therapeutic usefulness of the TNP-470 MCT solution in the treatment of unresectable enlarged tumors. The reason why initial suppression by plain MCT as was observed with the week-3 tumors was not clearly observed with the week-4 tumors, and why the MCT solution containing 5 mg of TNP-470 caused little regression of the week-2 and the week-4 tumors while it caused noticeable regression of the week-3 tumors may be attributed to differences in vascular development from the tumor to the femoral artery and/or in the tumor itself, the extent of necrosis in the tumors, and an insufficient dose of the oil solution in light of the vascular volume of the tumor in case of the week-4 tumors.

As we expected, a synergetic antitumor effect was observed with coadministration of the TNP-470 oil solution and conventional chemotherapeutic drugs such as MMC and ADM which were suspended in the TNP-470 sesame oil solution and administered intra-arterially. Figure 5 shows the antitumor activity of the TNP-470 sesame oil solutions with and without ADM or MMC. Coadministration of TNP-470 and 1 mg of ADM or MMC in oil solution resulted in enhanced antitumor activity compared with TNP-470 alone, and the combination of ADM and TNP-470 tended to cause the strongest growth suppression. We also examined the antitumor effect of coadministration of TNP-470 MCT solution and these anticancer drugs, but the antitumor activity was not obviously enhanced. The reason for this phenomena re-

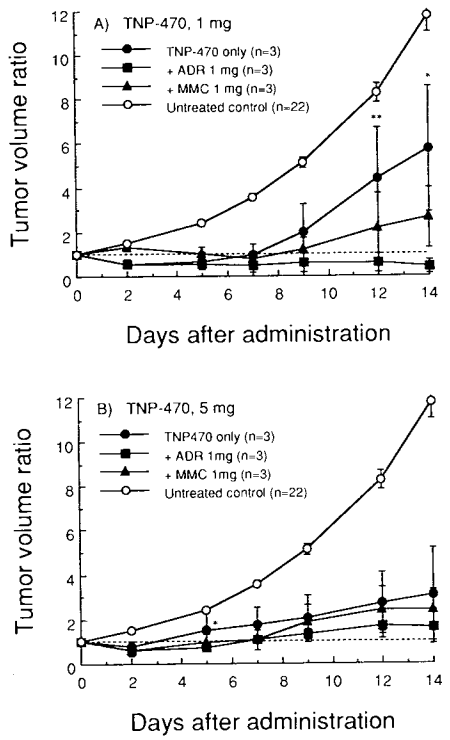

Fig. 5. Antitumor activity of a single i.a. injection of sesame oil solutions containing TNP-470 (1 mg (A) and 5 mg (B)) against week-2 VX-2 carcinoma in rabbits upon coadministration with ADM (1 mg) or MMC (1 mg). Each point represents the mean ± S.E. Each point for the treated groups, except for day 2 for + MMC in (A), was significantly different from the corresponding point for the untreated control by Student's *t*-test (*;$P<0.05$, **;$P<0.01$, no asterisk;$P<0.001$).

mains to be determined, but it is assumed to be due to the already strong suppression achieved by the TNP-470 MCT solution alone and the solubility of these anticancer drugs in the oil solution.

During chemoembolization therapy, compensative development of collateral circulation bypassing the occluded arteries often becomes a problem (11, 12). We, therefore, examined the possibility of enhancing the antitumor activity of PLGA msp containing MMC (MMC msp) by administering the TNP-470 MCT solution concomitantly. Ethylcellulose msp containing MMC were reported to be an effective embolizing agent in early clinical experiments (13, 14). In Fig. 6, growth profiles of tumors after i.a. administration of MMC msp (1 mg as MMC) suspended in MCT with and without TNP-470 are shown. Tumor growth in all groups treated was significantly suppressed ($P<0.001$) compared with untreated control, but no statistical difference was detected in the three groups treated. However, in contrast to the tendency of tumor regrowth in the group receiving MMC msp only, tumor growth was completely suppressed for the 2 weeks following administration of MMC msp and 5 mg of TNP-470. This observation could indicate the inhibition of the development of collateral circulation around the tumor by TNP-470, as the origin of collateral circulation is reported to be the functioning tiny vessels around occlusions in the case of the liver (11, 12).

With our present selective delivery system, we have confirmed long-lasting retention of TNP-470 at the tumor site after i. a. administration of the TNP-470 MCT solution *in vivo* (10) and sustained release of TNP-470 from the MCT solution *in vitro* (8). We next tried to confirm that TNP-470 released from the MCT solution could actually inhibit angiogenesis using the CAM assay for our preliminary evaluation. Figure 7 shows the features of the CAM on which a droplet (5 μl) of plain MCT or MCT solution containing TNP-470 (2.5 or 25 μg) was placed and left for 2 days. Plain MCT and a low dose of the TNP-470 MCT solution (2.5 μg) had no obvious effect on the membrane vasculature, while the MCT solution containing 25 μg of TNP-470 induced an avascular zone on the membrane after 2 days of contact, indicating that TNP-470 was released from the MCT solution in an active form. TNP-470 produces half-maximal cytostatic inhibition of various endothelial cells at approximately 10~80 pg/ml, and cytotoxicity against these cells was observed at concentrations higher than 3 μg/ml and *in vitro* (15, 16). In this experiment, as the release of TNP-470 from the MCT solution follows first-order kinetics with a half-life of 2.0 day^{-1}, approximately half the TNP-470 was released during the 2-day contact period. Therefore, since the regional TNP-470 concentration on the CAM possibly exceeded the cytotoxic level, partial destruction of constructed vessels was observed in the case of the high dose of TNP-470. Half-maximal cytostatic inhibition for various tumor cells ranged from 500 pg/ml to 2 μg/ml TNP-470 *in vitro* (16). Therefore, the initial release of TNP-470 from the MCT solution, which was selectively retained around the tumor after i. a. administration may have led to a high drug concentration at the tumor site and thereby caused regression of the proliferated tumor as could scarcely be attained by systemic administration. This striking regression should be maintained for a long time period by the inhibition of angiogenesis at lower drug concentrations as the drug is slowly released from the oil base. In addition, remarkable antitumor effects were obtained without any influence on normal tissues because of selective delivery of the drug to the tumor. Indeed, in rats bearing Walker 256 carcinosarcoma in the liver, initial TNP-470 concentration in the tumor after administration of the MCT solution *via* the hepatic artery was 22 times higher than that in normal liver tissue and a relatively high level was

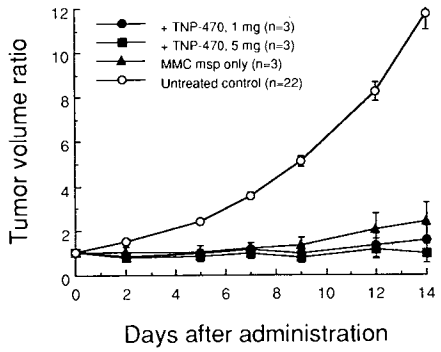

Fig. 6. Antitumor activity of a single i.a. injection of MMC msp suspended in MCT with or without TNP-470 (1 mg and 5 mg) against week-2 VX-2 carcinoma in rabbits. Each point represents the mean ± S.E. Each point for the treated groups was significantly different ($P<0.001$) from the corresponding point for the untreated control by Student's *t*-test.

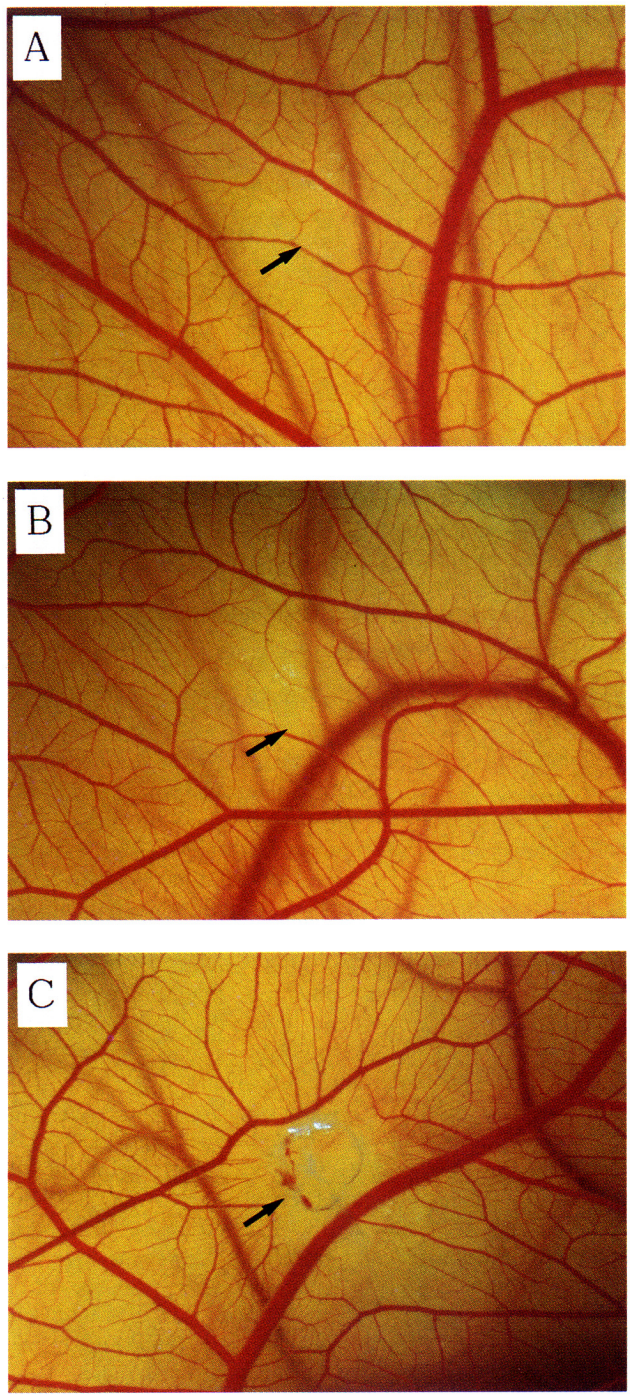

Fig. 7. Effects of MCT droplets (5 μl) containing or not containing TNP-470 on neovascularization in the CAM assay. Samples were placed on the CAM and left for 2 days. Arrows indicate the position of the oil droplets. (A); plain MCT, (B); MCT solution containing 2.5 μg of TNP-470, (C); MCT solution containing 25 μg of TNP-470.

maintained in the tumor for over 2 weeks; further the MCT solution containing 0.5 ~ 5 mg of TNP-470 caused remarkable tumor regression by single intrahepatic arterial administration without any severe side effects (10).

In conclusion, in the rabbit tumor model, a single injection of the TNP-470 MCT solution into the femoral artery supplies blood to the tumor provided striking regression of even well-developed tumors without severe effects on body weight, indicating the potential of the TNP-470 MCT solution for use in clinical cancer therapy.

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