Jpn. J. Cancer Res. 87, 831–836, August 1996

Selective Enhancement by Tumor Necrosis Factor-α of Vascular Permeability of New Blood Vessels Induced with Agarose Hydrogel-entrapped Meth-A Fibrosarcoma Cells

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We have previously developed a simple and quantitative method for assessment of *in vivo* tumor cell-induced angiogenesis by means of microencapsulation of tumor cells in agarose hydrogel and mouse hemoglobin ELISA (mHb-ELISA). In this article, we report that the new blood vessels induced with agarose-encapsulated tumor cells have the same sensitivity to tumor necrosis factor-α (TNF-α) as the original solid-tumor vessels. Agarose beads (average diameter=200 μm), in which Meth-A fibrosarcoma cells were microencapsulated, were subcutaneously implanted in non-syngeneic ddY mice. Ten days later, extensive angiogenesis was observed on the implanted sites of Meth-A agarose beads, whereas no new blood vessels were induced with cell-free agarose beads. The vascular permeability of the new blood vessels induced with agarose-microencapsulated Meth-A cells was selectively and significantly enhanced by the i.v. injection of TNF-α, and it reached the maximum level at 2 h after the injection of TNF-α. At 4 h after the injection of TNF-α, the vascular permeability was reduced to the basal level. This permeability profile in Meth-A agarose beads in ddY mice is very similar to that in Meth-A solid tumor in syngeneic BALB/c mice. On the other hand, TNF-α-treatment did not affect the vascular permeability of other normal tissues or inflammatory tissue in ddY mice. These results strongly suggest that the new blood vessels induced with agarose-microencapsulated tumor cells have the specific characteristics of tumor vessels. Our *in vivo* angiogenesis assay system should be useful not only to screen anti-angiogenetic agents, but also to elucidate the mechanism of tumor angiogenesis.

Key words: Tumor angiogenesis — Agarose-microencapsulation — Meth-A fibrosarcoma cells — TNF-α — Vascular permeability

Angiogenesis, the formation of new blood vessels, plays important roles in normal physiological processes such as embryonic development, as well as in wound healing and tissue regeneration.[1,2] Further, the formation of new capillaries is closely related to tumor growth and metastasis.[3] Since Folkman proposed that angiogenesis inhibitors could be used to suppress tumor growth,[4] the *in vitro* anti-angiogenic activities of numerous compounds have been reported.[5–7] However, the clinical application of such anti-angiogenic compounds for cancer chemotherapy is still limited. This is because no quantitative and reproducible *in vivo* angiogenesis assay system has yet been established, because only embryogenic or inflammatory new blood vessels have been used for screening of anti-tumor angiogenic compounds, and because the anti-angiogenic compounds detected by *in vitro* assay have not always inhibited tumor angiogenesis *in vivo*. It is hoped that an *in vivo* screening system for anti-tumor angiogenic drugs will allow these problems to be surmounted.

Recently, we developed a novel *in vivo* tumor cell-induced angiogenesis assay system by means of the tumor cell-microencapsulation technique using agarose hydrogel and mouse hemoglobin (mHb) enzyme-linked immunosorbent assay (mHb-ELISA).[8] This assay system is simple, rapid, quantitative and reproducible, and thus superior to conventional assay systems, such as the chorioallantoic membrane of the chick embryo and rabbit cornea methods.[9,10] Furthermore, our assay system enabled us to evaluate *in vivo* the angiogenic activity of human tumor cells in normal mice, because agarose hydrogel-microencapsulated cells are isolated from the host immune system. However, it has not been studied in detail whether the new blood vessels induced with tumor cells in our system have tumor vessel-specific characteristics.

In this study, to clarify whether the newly formed capillaries in our system have the same properties as those of Meth-A tumor vessels, we examined whether the vascular permeability of the new blood vessels induced with agarose-microencapsulated Meth-A tumor cells was increased after the i.v. administration of tumor necrosis factor-α (TNF-α). Systemic administration of TNF-α selectively opens the interstitial space at the tumor vascular wall, leading to efficient penetration of blood components into the tumor tissue.[11,12] The TNF-α sensitivity of

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the vascular endothelial cells, thus, can be considered as a marker of tumor vascular endothelial cells.

MATERIALS AND METHODS

Animals Female BALB/c mice (5 weeks old) and female ddY mice (5 weeks old) were purchased from Shimizu Experimental Animal Co., Ltd., Kyoto.

Microencapsulation of Meth-A cells in agarose hydrogel Meth-A fibrosarcoma cells were maintained by serial intraperitoneal passage in BALB/c mice. Meth-A cells were microencapsulated in agarose microbeads following the method originally developed by Nilsson *et al.*[13, 14] with a minor modification. Briefly, 0.1 g of Agarose-LGT (Nacalai Tesque Inc., Kyoto) and 3 ml of phosphate-buffered saline were mixed and autoclaved in a 50 ml glass centrifuge tube. The tube was cooled and kept at 37°C in a water bath. One ml of Hanks' balanced salt solution (HBSS) containing 5×10^7 Meth-A cells was pre-incubated at 37°C, then mixed with the above agarose solution. Liquid paraffin (10 ml), which had been autoclaved and cooled to 37°C, was poured into the mixture and the whole was emulsified by manual shaking to obtain the desired bead size. The centrifuge tube was immersed into an ice bath for 10 min to gel the agarose solution. The agarose beads were washed 3 times with HBSS to remove the liquid paraffin.

Implantation of agarose microbeads and cotton in mice In order to induce inflammatory blood vessels, cotton (3–4 mg/site) was implanted subcutaneously in the dorsal aspect of 5-week-old female BALB/c mice on day 0, and 3×10^5 Meth-A cells (in 0.1 ml) were implanted intradermally in the abdomen of the same mice on day 6. Meth-A cells microencapsulated with agarose hydrogel (Aga-Meth-A cells; 300 μl/site) were implanted subcutaneously in the right dorsal aspect of 5-week-old female ddY mice, and cell-free agarose beads that contained the same amount of agarose were implanted subcutaneously in the left dorsal aspect of the same mice on day 6. The amount of mHb and the vascular permeability of blood vessels in the sites implanted with Aga-Meth-A cells were determined on day 17.

mHb-ELISA The mHb-ELISA was performed as described previously.[8] Briefly, the mHb-ELISA system employs rabbit anti-mHb IgG (solid-phase antibody), rabbit biotinylated anti-mHb IgG (sandwich antibody), and horseradish-peroxidase-avidin D (Vector Laboratories Inc., Burlingame, CA). The color was developed with (3,3′,5,5′-tetramethylbenzidine, Dojindo, Kumamoto) substrate, and the absorbance at 450 nm, referred to that at 655 nm, was measured with a microplate reader.

Determination of change of vascular permeability after administration of TNF-α MPEG-TNF-α [1000 Japan Reference Unit (JRU)/mouse],[15–17] in which 56% of the lysine amino groups of natural human TNF-α (Hayashibara Biological Laboratories Inc., Okayama) are coupled with polyethylene glycol (PEG), was injected i.v. into the mice anesthetized with sodium pentobarbital. At 0.5, 1.5, 2.5 and 3.5 h later, Evans blue (Wako Pure Chemical Ind. Co., Ltd., Osaka), which binds with albumin in blood, suspended in saline [0.2% (w/v)] was administered i.v. at 0.2 ml/mouse. At 30 min after the injection of Evans blue, the mouse was killed by abdominal arterial exsanguination. Then the brain, lung, liver, spleen, kidney, skin, blood, Meth-A tumor, cotton, and agarose beads were recovered and weighed separately. The Evans blue of the collected tissue was extracted by immersion for 48 h in 1 or 2 ml of dimethyl sulfoxide (Wako Pure Chemical Ind. Co., Ltd.).[18] The absorbance (A) at 640 nm of the Evans blue extracted from each tissue was measured to estimate the vascular permeability. The vascular permeability (VP) ratio was calculated according to the following equation.

$$\mathrm{VP}=\frac{\text{A/gram of tissue after the administration of MPEG-TNF-}\alpha}{\text{A/gram of tissue without the administration of MPEG-TNF-}\alpha}$$

Statistical methods The significance of the changes in VP ratio was evaluated by using Student's t test.

RESULTS

Previously we reported that, with the use of tumor cell-microencapsulation in agarose hydrogel, new blood

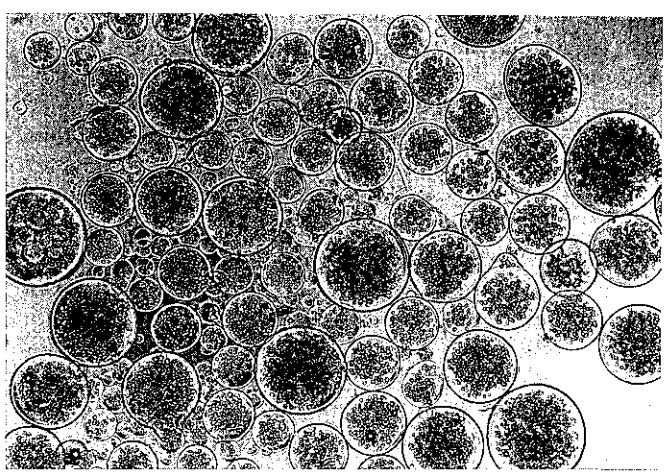

Fig. 1. A phase contrast micrograph of agarose microbeads containing Meth-A cells. Meth-A cells were microencapsulated in 2.5% (wt%) agarose hydrogel (encapsulation density: 1.25×10^7 cells/ml). The average bead diameter is about 200 μm, and the number of the entrapped cells is approximately 50 cells/bead.

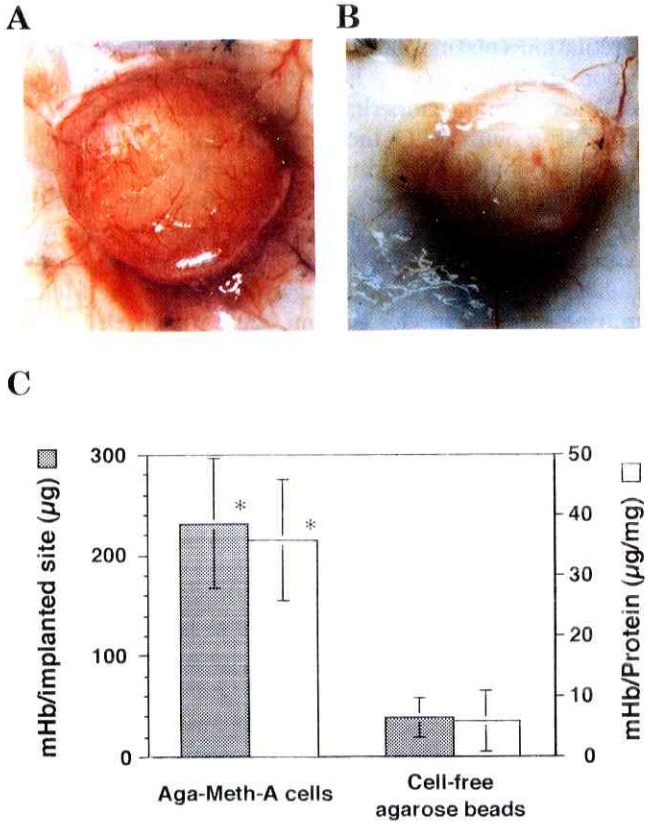

Fig. 2. Angiogenesis induced by Meth-A cells in agarose microbeads in ddY mice. Agarose microbeads with or without Meth-A cells were subcutaneously implanted near the dorsal midline of ddY mice. Eleven days later, agarose gel pellets were observed under a stereoscopic microscope (A, agarose beads with Meth-A cells; B, cell-free agarose beads), and the mHb in the vessels was quantified with mHb-ELISA (C). Each value is the mean±SEM, n=6. *Significantly different from cell-free agarose beads ($P<0.01$).

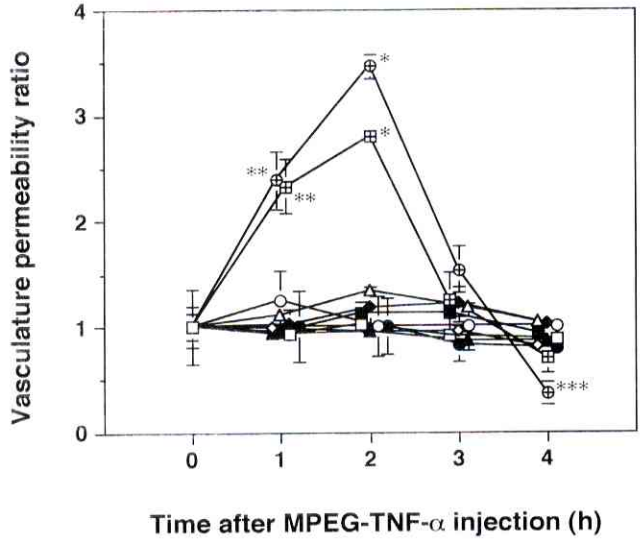

Fig. 3. Effect of systemic pretreatment with MPEG-TNF-α on the permeability ratio of Evans blue-albumin from blood vessels. Mice were given i.v. injection of MPEG-TNF-α (1000 JRU/mouse) and i.v. injection of 0.2 ml of 0.2% Evans blue suspended in saline at 30 min before the time indicated. The mice were then killed at the time indicated, and the Evans blue in the collected tissues was quantified as described in "Materials and Methods." Control-basal levels without pre-injection of MPEG-TNF-α are shown for time 0. □, brain; ■, lung; ▲, liver; △, spleen; ◇, kidney; ◆, skin; ⊕, tumor (Meth-A); ●, cotton; ⊞, Aga-Meth-A cells; ○, cell-free agarose beads. Each point represents the mean± SEM, n=4–16. Statistical significance compared to the 0-time value: * $P<0.001$; ** $P<0.005$; *** $P<0.01$.

vessels induced with human tumor cells could be observed in C57BL/6 mice.[8] In this study, we examined whether the new blood vessels induced with tumor cells encapsulated in agarose hydrogel had the same properties as those induced with the original tumor cells. The agarose beads had an average diameter of 200 μm, and encapsulated about 50 Meth-A cells/bead (Fig. 1). Aga-Meth-A cells were subcutaneously implanted in non-syngeneic ddY mice on day 1. On day 11, many new blood vessels were apparent at the sites implanted with the Aga-Meth-A cells, as in the case of Meth-A solid tumors inoculated in syngeneic BALB/c mice (Fig. 2A). The cell-free agarose microbeads induced almost no new blood vessel formation (Fig. 2B). To quantitate the newly formed capillaries, we examined the mHb levels of the vessels with mHb-ELISA. The mHb level of new blood vessels at the site implanted with Aga-Meth-A cells was markedly higher than that of the cell-free agarose beads (Fig. 2C). These results suggest that the Meth-A cells entrapped in agarose microbeads induced angiogenesis.

Fig. 3 shows the TNF-α sensitivity of new blood vessels induced with Aga-Meth-A cells. TNF-α selectively induces hemorrhagic necrosis of the tumor vascular system, but does not affect the blood vessels in normal tissues.[19, 20] Therefore, the TNF-α sensitivity of new capillaries induced with Aga-Meth-A cells was used as an index to examine whether the capillaries have the properties of tumor-specific endothelial cells. The anti-tumor potency of MPEG-TNF-α is 100-fold greater than that of native TNF-α because of the improvement of bioavailability,[17] and the dosage of MPEG-TNF-α of 1000 JRU/mouse used in this study has been confirmed in a previous study to be sufficient to induce hemorrhagic necrosis of Meth-A tumors at 24 h after i.v. injection

without side effects.[15] The VP of tumor blood vessels formed in Meth-A solid tumors was increased at 1 h after i.v. injection of MPEG-TNF-α, and its maximum level was observed at 2 h after TNF-α-treatment (3.5 times higher than the control-basal level). At 3 h after the injection of TNF-α, the VP returned to the control-basal level, and it was lower than the control-basal level at 4 h. The VP of new capillaries at the site implanted with Aga-Meth-A cells showed a time-dependent increase, and its level at each time was similar to that in the Meth-A solid tumors (2.8 times higher than the control-basal level at 2 h after the injection of TNF-α). At 3 h after TNF-α-treatment, the VP declined, and it was slightly lower than the control-basal level at 4 h. Therefore, on treatment with TNF-α, the new blood vessels induced with Aga-Meth-A cells showed the same VP profile as that in the Meth-A solid tumors. On the other hand, the permeability of the vessels in normal tissues, such as the brain, lung, liver, spleen, kidney and skin, or inflammatory sites in which cotton had been implanted, was little changed by i.v. injection of MPEG-TNF-α.

DISCUSSION

Angiogenesis is essential to tumor growth and metastasis, providing nutrition and oxygen. Tumors have been extensively studied in efforts to elucidate their mechanism of angiogenesis and to develop novel anti-tumor agents, and various *in vivo* tumor angiogenesis assay systems have been developed by many laboratories.[9, 10, 21–23] Recently, we developed a simple, rapid and quantitative assay system for selective evaluation of *in vivo* tumor angiogenesis.[8] This method, using microencapsulation of tumor cells in agarose beads and an mHb-ELISA system, can also be used to assay human tumor angiogenesis in normal mice because the cells entrapped in the agarose microbeads are isolated from the host immune system. This feature is expected to be particularly useful.

In this investigation, we studied whether the newly formed capillaries induced with the tumor cells encapsulated in agarose beads have the properties of tumor-specific capillaries. The TNF-α sensitivity of new blood vessels induced with Aga-Meth-A cells was examined. TNF-α effectively inhibits tumor growth and selectively causes tumor necrosis in tumor-bearing mice.[19, 20] Tumor hemorrhagic necrosis selectively occurs in the tumor vasculature, but does not occur in the blood vessels in normal tissues. Furthermore, TNF-α does not induce any necrotic changes at the inflammation site.[20] By utilizing the difference in sensitivity to TNF-α between tumor vessels and normal vessels, we examined whether the newly formed capillaries induced in our *in vivo* angiogenesis assay system had the specific characteristics of tumor vessels. Because TNF-α rapidly disappears from the circulation (plasma half-life; 3.5 min) after entering the blood stream,[15] a very high dose is required to obtain sufficient tumor hemorrhagic necrosis. The modification of TNF-α with PEG increased its anti-tumor potency and also markedly reduced the toxic side-effects.[15–17] Instead of native TNF-α, we therefore used MPEG-TNF-α, in which 56% of the lysine amino groups were coupled with PEG, at 1000 JRU/mouse. This dose was sufficient to induce hemorrhagic necrosis in the Meth-A murine sarcoma model without acute toxicity.

The new blood vessels induced with Aga-Meth-A cells were very sensitive to TNF-α. The VP of the newly formed capillaries induced with Aga-Meth-A cells in non-syngeneic ddY mice was increased by the i.v. administration of MPEG-TNF-α, and its course of change was similar to that observed in Meth-A tumors of syngeneic BALB/c mice. Many investigators have reported essentially the same mechanism of the hemorrhagic necrosis induced with TNF-α.[18–20] Briefly, blood vessels in the tumor show hyperpermeability at 1–2 h after i.v. injection of TNF-α, and adsorption of fibrin-like substances on the luminal surface is seen at 3–6 h, followed by complete blood circulation blockage owing to thrombus formation at 24 h. The increased permeability of the newly formed vessels in our system at 2 h after the administration of TNF-α would reflect the cytotoxicity of TNF-α on tumor vascular endothelial cells, while normalized permeability at 3 h would reflect the cessation of the circulation of the blood because of the adsorption of fibrin-like substances on the luminal surface of the tumor vascular endothelial cells.

The blood vessels in animals with cell-free agarose pellets or agarose pellets encapsulating normal primary cells (bovine smooth muscle cells) did not show any change of permeability (data not shown). The blood vessels in normal tissue and in the inflammation site also did not show any change of permeability. These results suggest that the newly formed capillaries induced with Aga-Meth-A cells have the specific characteristics of tumor vessels, but not of inflammatory vessels, and that the angiogenesis induced with agarose pellets is dependent on the characteristics of the microencapsulated cells.

Previously, we reported that a factor(s) secreted from tumor cells converted normal endothelial cells into tumor-like endothelial cells.[24] The newly formed blood vessels induced with Aga-Meth-A cells might acquire the properties of tumor vessels via soluble angiogenic factor(s) secreted from Meth-A cells in agarose microbeads. In our previous report,[8] we noted that human tumor angiogenesis was observed in the mouse after introduction of human tumor cells encapsulated in agarose beads. The new blood vessels induced with human tumor cells microencapsulated in agarose microbeads in

normal mice have the same characteristics as the vessels formed in human tumor tissues. These observations indicate that our *in vivo* angiogenesis assay system can be used to evaluate the angiogenesis induced with human tumor cells.

The results described herein suggest that our angiogenesis assay system will be helpful in the screening of novel tumor-angiogenesis inhibitors and in the clarification of the mechanism of tumor angiogenesis. Moreover, we think that our *in vivo* angiogenesis assay system can be used to evaluate the sensitivity to TNF-α of the vascular endothelial cells in various tumor types and to clarify the specific characteristics of the tumor vessels induced by various tumors.

ACKNOWLEDGMENTS

This work was supported by a Grants-in-Aid for Scientific Research (06672180) from the Ministry of Education, Science, Sports and Culture of Japan.

(Received February 13, 1996/Accepted May 8, 1996)

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