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# A Quantitative *in vivo* Method of Analyzing Human Tumor-induced Angiogenesis in Mice Using Agarose Microencapsulation and Hemoglobin Enzyme-linked Immunosorbent Assay

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**This study was conducted to develop a quantitative assay system for use in the *in vivo* evaluation in mice of angiogenesis induced by human tumor cells. The human epidermoid carcinoma cells, A431 cells, were cultured on microcarriers. Microcarrier-attached A431 cells (A431-MC) were microencapsulated with agarose hydrogel to isolate them from the immune system of the C57BL/6 mice after subcutaneous dorsal midline implantation. The agarose hydrogel-microencapsulated A431 cells (Aga-A431 cells; diameter = 300 $\mu$m) survived for at least 10 days *in vitro*, and the proliferation profile of the Aga-A431 cells was indistinguishable from that of non-microencapsulated A431 cells. The Aga-A431 cells were subcutaneously injected into mice with an 18-gauge needle. Ten days later, few vessels had formed at the site implanted with cell-free agarose beads, whereas notable angiogenesis was observed at the site implanted with Aga-A431 cells. The degree of angiogenesis was evaluated by measurement of the hemoglobin content in the implanted site using a mouse hemoglobin (mHb) enzyme-linked immunosorbent assay (ELISA) system. This mHb-ELISA system has the advantages of great simplicity and reproducibility. The measured mHb content of new blood vessels at the site implanted with agarose beads was in good agreement with the amount of angiogenesis observed under a stereoscopic microscope. This assay system enabled us to evaluate the angiogenesis induced by xenogeneic cells, such as human tumor cells. Thus, our novel method may be useful for the study of the angiogenic potential of various human tumor cells and in research on the anti-angiogenic properties of various agents.**

Key words: Tumor angiogenesis — A431 cells — Agarose microencapsulation — Mouse hemoglobin ELISA

Angiogenesis is an important physiological property of vascular endothelial cells (EC), typically seen during embryonic development,[1–3] the adult female reproductive cycle,[4, 5] and wound healing.[6, 7] It is well-known that the growth of new blood vessels also occurs in some pathological conditions, including tumor growth and metastasis.[8–10] Continuous tumor angiogenesis is indispensable to the growth of solid tumors, because it provides the tumor's nutrient and oxygen supply. Thus, the inhibition of tumor angiogenesis may provide a new approach to cancer therapy.[11, 12]

Recently, *in vivo* tumor angiogenesis was postulated to consist of the following sequential events: (i) tumor cells produce and secrete various angiogenic factors, such as fibroblast growth factors (FGF), vascular endothelial growth factor (VEGF), and transforming growth factor $\beta$ (TGF-$\beta$); (ii) these factors stimulate the EC of the parent vessels, and the activated EC induce enzymatic degradation of the basement membrane; (iii) EC begin to migrate toward the angiogenic stimulus and a solid sprout is created; (iv) EC invade and proliferate, increasing the length of the sprout; (v) EC differentiate to tube formation and link up with each other; and (vi) new basement membranes and new capillary networks are formed.[13–15] Each of these postulated tumor angiogenetic steps (i)–(vi) has been studied in detail by using *in vitro* angiogenesis assay as part of the on-going research effort to elucidate the mechanisms of tumor angiogenesis and to design an angiogenic inhibitor. However, *in vivo* tumor angiogenesis is by contrast a very complicated multifactorial phenomenon, and as a result the anti-angiogenetic drugs identified by the *in vitro* assays have not always inhibited tumor angiogenesis *in vivo*. In fact, TGF-$\beta$ induced marked angiogenesis *in vivo*, whereas it significantly suppressed EC growth *in vitro*. Therefore, a quantitative *in vivo* angiogenesis assay system is necessary for the comprehensive evaluation of angiogenesis.

A few *in vivo* angiogenesis assay systems, typified by the chick embryo chorioallantoic membrane (CAM) method[16, 17] and the rabbit or rat cornea method,[18, 19] have been reported, but the evaluation of angiogenesis induced in all conventional systems is qualitative and subjective, relying on visualization of tumor-induced blood vessel growth, with measurement of the length or

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area of vessels formed on the surface of the tumor tissue. We report herein the development of a rapid, and quantitative assay system for the *in vivo* evaluation of tumor angiogenesis.

## MATERIALS AND METHODS

**Animals and cells** C57BL/6 mice (male, 6 weeks old) and Japanese white rabbits (male, 10 weeks old) were purchased from Shimizu Experimental Animal Co., Ltd., Kyoto. A431 cells, a human epidermoid carcinoma cell line that secretes FGF and VEGF,[20-22] were obtained from RIKEN Cell Bank, Tsukuba. The A431 cells were serially subcultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS). Smooth muscle cells (SMC) were isolated from calf aorta by mechanical scraping and cultured with DMEM containing 10% FCS.

**Culture of A431 cells on microcarriers** Porous microcarriers were kindly donated by Asahi Kasei (Tokyo). Microcarriers were hydrated with $Ca^{2+}$-$Mg^{2+}$-free Dulbecco's phosphate-buffered saline [PBS(−)] and autoclaved. After being washed with PBS(−), the microcarriers were suspended in DMEM containing 10% FCS and incubated at 37°C in 5% $CO_2$ on 100-mm petri dishes. After 12 h incubation, A431 cells ($1\times10^6$ cells/dish) were plated on the petri dishes to attach the microcarriers. Microcarrier-attached A431 cells (A431-MC) were cultured with DMEM supplemented with 10% FCS.

**Microencapsulation of A431-MC with agarose hydrogel** The A431-MC were microencapsulated in agarose microbeads following the method originally developed by Nilsson *et al.*[23,24] with a slight modification. Briefly, a 4% (wt%) AGAROSE-LGT (Nacalai Tesque Inc., Kyoto) solution (1 ml) in Eagle's minimum essential medium (MEM, Nissui Seiyaku Co., Ltd.) was autoclaved in a 50-ml glass centrifuge tube, then cooled and kept at 37°C in a water bath. The A431-MC in DMEM (1 ml, containing 0.5 ml of A431-MC) were mixed with this agarose solution at 37°C. Then liquid paraffin (20 ml), which had been autoclaved and cooled to 37°C, was poured into the mixture. The liquids were emulsified with manual shaking to the desired bead size (diameter; 300 μm), and the mixture was immersed into an ice bath for 10 min to gel the agarose solution. The A431-MC-microencapsulated agarose beads were washed 3 times with PBS(−) to completely remove the liquid paraffin. The viability and the growth profile of A431 cells microencapsulated in agarose beads were measured by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide, Dojindo, Kumamoto) assay according to the method described by Mosmann.[25]

**Implantation of agarose microbeads in mice** A 300 μl portion of A431-MC-microencapsulated agarose beads, or of cell-free agarose beads as a control, in PBS(−) was subcutaneously injected near the dorsal midline of the C57BL/6 mice with an 18-gauge hypodermic needle without any surgical operation. Ten days later, the mice were killed under general anesthesia and the area implanted with the beads was exposed. The agarose gel pellet was cut from the skin, and homogenized with 1 ml of Tris-HCl-buffered solution (TBS). Centrifugation was applied at 30,000*g* for 10 min, and the supernatant was subjected to mouse hemoglobin (mHb)-enzyme-linked immunosorbent assay (ELISA) (Fig. 5).

**mHb-ELISA** The mHb was prepared according to Huntsman's method.[26] Rabbits were immunized with 50 μg of mHb in Freund's complete adjuvant followed by three injections of 10 μg of mHb in Freund's incomplete adjuvant at 1-week intervals. The IgG fraction of antiserum was prepared by using a protein A-agarose (Bio-Rad, California) column. The binding specificity of rabbit anti-mHb IgG was examined by western blotting.

The mHb-ELISA was developed by the sandwich method using rabbit anti-mHb IgG (solid phase), rabbit biotinylated anti-mHb IgG (primary antibody) and horseradish peroxidase (HRP)-avidin (enzymatic marking, Vector Laboratories Inc., California). A 96-well ELISA plate was coated with rabbit anti-mHb IgG (50 μl/well, 10 μg/ml in 0.05 *M* bicarbonate-buffered solution, pH 9.6) at 4°C overnight. The plate was washed 3 times with TBS containing 0.05% Tween-20 (TTBS), then 250 μl of 1% bovine serum albumin (BSA) in TBS was added for blocking at room temperature for at least 2 h. The plate was washed with TTBS, and the sample solution or mHb, each of which was diluted in TTBS containing 0.1% BSA, was added (50 μl/well) and allowed to react for 2 h at room temperature. The plate was washed 3 times with TTBS followed by the addition of rabbit biotinylated anti-mHb IgG (50 μl/well) and further incubation for 2 h. The plate was washed with TTBS 3 times, then HRP-avidin (50 μl/well, 1:10000-diluted) was added and allowed to react at room temperature for 1 h. Again, the plate was washed with TTBS 3 times, then the color was developed with 3,3′,5,5′-tetramethylbenzidine (TMBZ, Dojindo) substrate. The absorption of the plates was read with a microplate reader at 450 nm with the reference wavelength at 655 nm.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting** SDS-PAGE was done by the method established by Laemmli.[27] The electrophoresed gel was overlaid with a 0.2-μm nitrocellulose membrane and proteins were transferred electrophoretically for Western blotting. Then the nitrocellulose membrane was blocked with 1.5% gelatin/TBS at room

temperature for 2 h. A rabbit anti-mHb IgG and a goat anti-rabbit IgG coupled to HRP (Zymed, California) were used in the immunoblot assay as the primary antibody and the secondary antibody, respectively.

## RESULTS

**Viability of A431 cells in agarose microbeads** The proliferation profile of A431 cells, cultured on porous cellulose microcarriers (diameter=150 $\mu$m), was nearly identical with that of A431 cells cultured in the usual way (Fig. 1). A431 cells cultured on microcarriers for 3 days were microencapsulated with 2% (wt%) agarose hydrogel, and the agarose-microencapsulated A431 cells not only survived but also proliferated for at least 10 days (Fig. 2). Additionally, the proliferation of A431 cells in agarose microbeads was equivalent to that of A431 cells cultured on plates or microcarriers. These findings indicate that the procedure of agarose-microencapsulation does not impair the proliferative ability of the A431 cells.

**Angiogenesis induced by agarose microbeads entrapping A431 cells** The agarose microbeads encapsulating the A431 cells were on average 300 $\mu$m in diameter (Fig. 1). These agarose beads were subcutaneously injected into mice with an 18-gauge needle. Marked new capillary vessel formation at the agarose-pellet site was observed on day 10 (Fig. 3). The agarose-microencapsulated bovine aorta-derived SMC did not induce any new blood vessel formation (data not shown). There were in general few vessels on the implanted site of the cell-free agarose beads that were used as negative controls.

**Establishment of mHb-ELISA and evaluation of *in vivo* angiogenesis** To measure *in vivo* angiogenesis accurately, we established an mHb-ELISA system. First, rabbit anti-mHb IgG was prepared and its binding specificity was examined by western blotting. The subunits of mHb are composed of four peptide chains ($\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$) each with a molecular weight of 16100 Da. Many bands other than that of mHb were recognized from the supernatant prepared by homogenizing the A431-MC-agarose beads pellet. Thus, polyclonal anti-mHb IgG prepared by immunization of rabbits was confirmed to recognize only mHb among the proteins found by SDS-PAGE at the implanted site of A431-MC-agarose beads (Fig. 4).

The standard curve of mHb-ELISA was sigmoid, and the minimum amount detectable with this system was 10 ng/ml mHb. Each sample solution was prepared from A431-MC-microencapsulated agarose beads which had

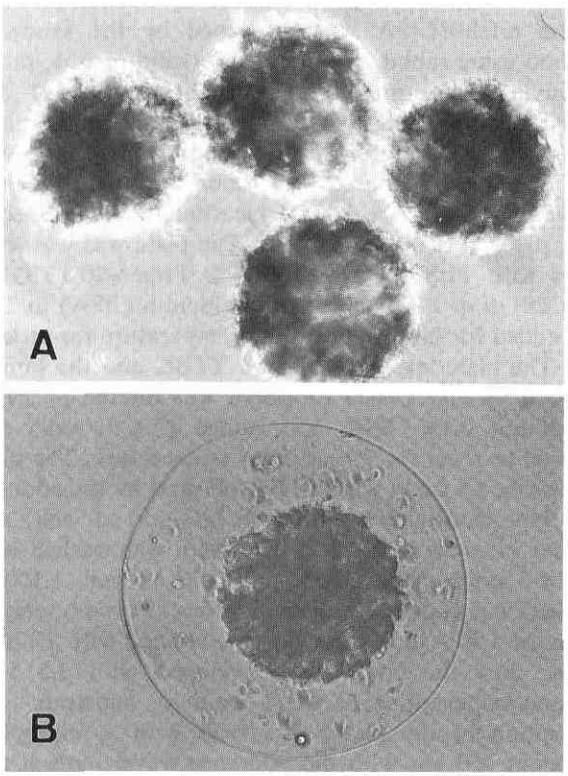


Fig. 1. Phase contrast micrographs of A431 cells cultured on microcarriers and entrapped in agarose beads. The cellulose microcarrier is 150 $\mu$m in diameter and has 30 $\mu$m pores on the surface. A431 cells cultured on microcarriers were treated with an MTT reagent on day 3. A431 cells are recognized as black figures upon MTT formazan production (A). A431 cells cultured on microcarriers for 10 days were microencapsulated in 2% (wt%) agarose hydrogel. The average bead size was about 300 $\mu$m (B).

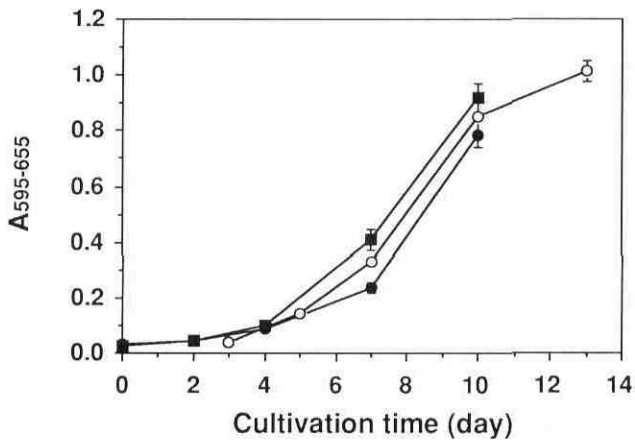


Fig. 2. Proliferation of A431 cells *in vitro*. A431 cells were cultured on a 24-well culture plate (■) or on microcarriers (●). After culture on the microcarriers for 3 days, A431 cells were microencapsulated in 2% agarose beads and cultured on a 24-well culture plate (○). The viability of the cells was evaluated with an MTT assay. Each point represents the mean±SD, n=3.

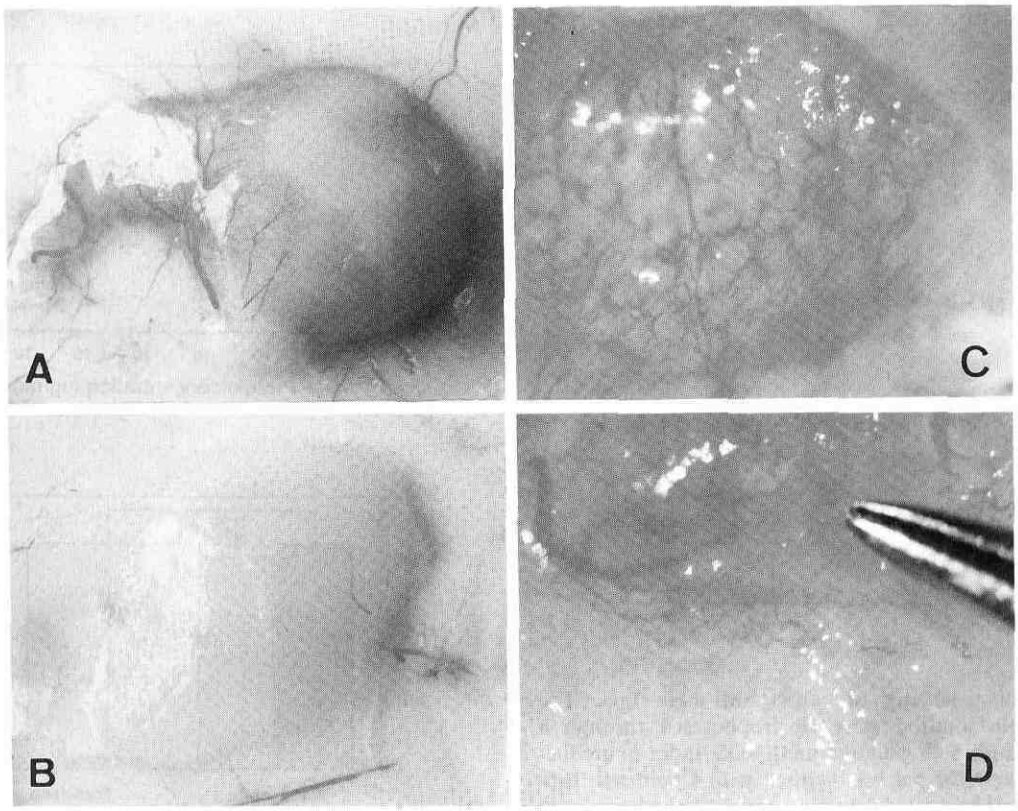


*Fig. 3. Angiogenesis induced by A431 cells in agarose beads. A431 cells were microencapsulated in 2% agarose hydrogel on day 10 after cultivation on microcarriers. Agarose beads were subcutaneously implanted near the dorsal midline of the C57BL/6 mice. Treated mice were killed, and the agarose gel pellet was exposed on day 10 for observation under a stereoscopic microscope. A, The surface of implanted agarose beads with A431 cells; B, Cell-free agarose beads (negative control); C, D, The inside of an agarose gel pellet containing A431 cells. These beads induced notable angiogenesis at the implanted site. Further, the agarose beads were surrounded by a thick granuloma. In contrast, there was little induction of new vessels or invasion of fibroblasts in the case of cell-free agarose beads.*

been implanted into the mice. When the serially diluted sample solution was determined by mHb-ELISA, the absorbance values formed a curve parallel to the standard curve (Fig. 5).

Using our novel *in vivo* quantitative angiogenesis assay system, we measured the mHb in the A431-agarose pellet site on day 10 after implantation by mHb-ELISA. The mHb level of the site implanted with A431-agarose microbeads was markedly higher than that of the site implanted with the cell-free agarose beads (Fig. 5). These results were in good accordance with the results of stereoscopic observation of the agarose pellet.

## DISCUSSION

Solid tumors invariably induce tumor angiogenesis, that is, tumor neovascularization, with extensive and rapid growth. Thus, the inhibition of tumor angiogenesis may offer an approach to the eradication of solid tumors. Fotsis *et al.*[28] reported that 2-methoxyoestradiol not only strongly inhibited tumor angiogenesis, but also markedly suppressed solid tumor growth. However, more detailed knowledge of the mechanisms of tumor angiogenesis and the development of a quantitative *in vivo* tumor angiogenesis assay method are necessary for the design of new anti-angiogenic agents.

The most recently developed *in vivo* angiogenesis assays, include sponges impregnated with, or matrigel supplemented with, materials inducing angiogenesis.[29, 30] However, these methods can be used to assess only the already known factors in angiogenesis. Plunkett *et al.*[31, 32] reported that calcium alginate beads entrapping Lewis lung carcinoma cells induced angiogenesis in BALB/c mice. It is well-known that calcium alginate beads are extremely unstable and fragile *in vivo*, and inflammatory angiogenesis is therefore often induced by entrapped

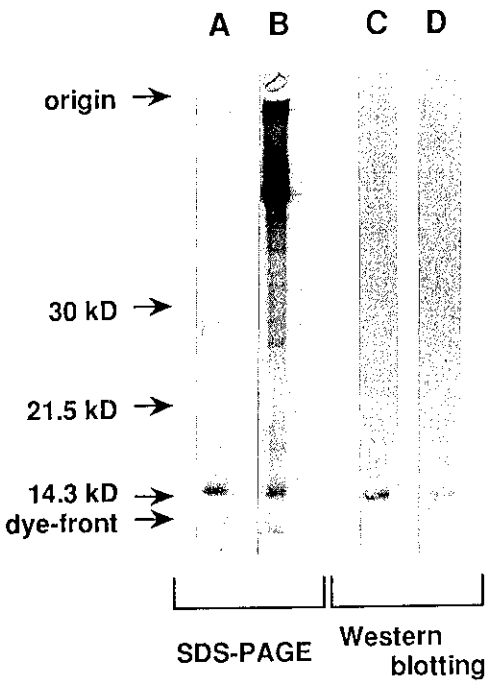


Fig. 4. Binding specificity of rabbit anti-mHb IgG. The mHb and sample solution were electrophoresed through a 17% polyacrylamide slab gel containing SDS under nonreducing conditions, and the gel was stained with Coomassie brilliant blue R250. The molecular mass marker standards used were Rainbow protein molecular weight markers (Amersham). Lane A, mHb (5 $\mu$g/lane); B, sample solution (10 $\mu$g/lane). Western blotting developed with rabbit anti-mHb IgG and HRP-labeled goat anti-rabbit IgG. Lane C, mHb (5 $\mu$g/lane); D, sample solution (10 $\mu$g/lane).

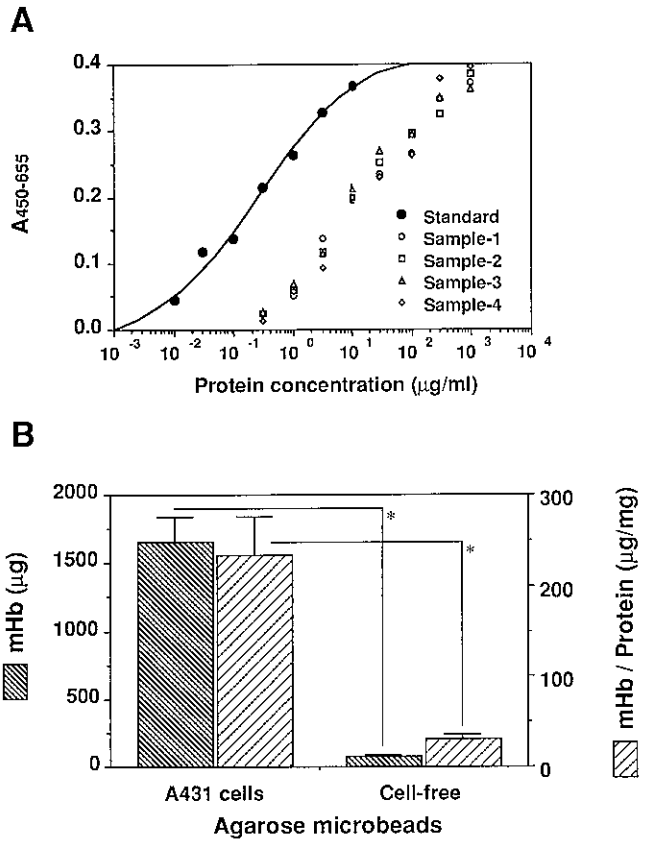


Fig. 5. Evaluation of angiogenesis induced by A431 cells entrapped in agarose beads with mHb-ELISA. A431 cells were microencapsulated in 2% agarose hydrogel. Cell-free 2% agarose beads were used for the negative control. The agarose beads were subcutaneously implanted near the dorsal midline of C57BL/6 mice. On day 10, the implanted tissues were collected, and the mHb levels were determined by mHb-ELISA. Protein levels in the implanted tissues were measured by the Bio-Rad protein assay. A: The standard curve of the mHb-ELISA showed high sensitivity and reproducibility. Each sample solution was prepared from A431-MC-microcapsulated agarose beads which had been implanted in the mice. B: The sites implanted with the agarose beads containing A431 cells showed significantly higher mHb content than that of the sites implanted with the cell-free agarose beads, in agreement with the stereoscopic microscopy observations (Fig. 3). Each value is the mean$\pm$SE, n=10. $*$ $P<0.001$ significantly higher than the value with cell-free agarose beads.

heterogenous cells. Further, these methods are disadvantageous because of the use of radioisotopes or because of low sensitivity. Therefore, we have established a new quantitative method for assay of *in vivo* tumor angiogenesis in normal mice.

The agarose hydrogel-microencapsulation of A431 cells was sufficiently non-disruptive that all of the encapsulated A431 cells were viable and their growth profile was indistinguishable from that of normally cultured A431 cells for at least 10 days (Fig. 2). Comparable results were also obtained with other cells, such as SMC (data not shown). The time-dependent increase in cell number strongly suggests that the agarose microbeads provided a microenvironment that is nutritionally adequate to support cell proliferation.

The A431 cell-microencapsulating agarose beads induced marked angiogenesis on day 10 after subcutaneous implantation, whereas no new capillary blood vessels were observed in the case of cell-free agarose beads (Fig. 3). In addition, agarose-microencapsulated SMC, that is, heterogeneous cells in relation to the host mice, did not induce angiogenesis. These results clearly demonstrated that the agarose beads were very stable, and their contents were isolated from the host's immune system, indicating that our novel assay system can be used to evaluate the tumor angiogenesis alone. A431 cells are reported to secrete potent angiogenic factors including

FGF and VEGF.[20-22] Presumably new capillary blood vessels were induced by the release from the microbeads of angiogenic factors produced and secreted by immobilized A431. Further studies are needed on the mechanisms of A431 cell-induced angiogenesis.

To quantitatively evaluate *in vivo* angiogenesis, we attempted to measure the mHb level in the agarose pellets using mHb-ELISA. The amount of mHb in A431-agarose pellets was significantly higher than that in cell-free agarose pellets. There was good correspondence between the stereoscopic microscopy observations and the mHb-ELISA value for the agarose pellet. Thus, this ELISA system was confirmed to be useful for quantitative analysis of *in vivo* angiogenesis. This ELISA system could also be applied in other *in vivo* angiogenesis assays, including the CAM, cornea and dorsal air sac methods, as a quantitative method for angiogenesis measurement.

In summary, the assay system reported herein presents the advantages that it is easy to perform and yields quantitative results that can be used to evaluate angiogenesis induced by human tumors in a normal mouse. We believe that our assay system will contribute to studies of the angiogenesis induced by human tumors *in vivo*. We have also used this system to assess the inhibitory effect of several compounds reported to have anti-angiogenic activity. Our assay system may be useful in the identification of anti-human tumor angiogenic agents and in the examination of the mechanisms of *in vivo* tumor-induced angiogenesis.

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