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Letter to the Editor

USE OF HISTOCULTURE AND GREEN FLUORESCENT PROTEIN TO VISUALIZE TUMOR CELL HOST INTERACTION

Dear Editor:

Three-dimensional collagen sponge gel matrix-supported histocultures involves culturing blocks of tissues on top of the sponge gels (Leighton et al., 1960; Freeman and Hoffman, 1986). Histoculture provides the supporting matrix to allow various tissues to be maintained in the living state with their native tissue architecture for very long periods (for discussion, *see* Hoffman, 1991).

To study tumor cell invasion of normal tissue *in vitro*, Margolis et al. (1995) implanted aggregates of tumor cells externally tagged with a fluorescent dye onto living tissue blocks in histoculture; the tumor cells spread inside the tissue in the first hours after implantation and then began to leave the aggregate and invade the surrounding tissue. However, the external fluorescent tag can function for only a very few cell generations before it becomes too dilute.

To fully understand how tumor cells colonize host target organs and progress in host tissue (Hart and Saini, 1992), it is necessary to have an experimental system where the process can be replicated, visualized, and followed as it occurs over long time periods and many cellular generations.

To achieve these goals, we have accomplished the following two enabling steps: (a) utilized three-dimensional collagen sponge gel matrix-supported histoculture developed by Leighton (1957), Leighton et al. (1960), and (Freeman and Hoffman, 1986) and (Hoffman, 1991) to histoculture human tumor cells colonizing target host tissue; (b) enabled the visualization of tumor cells in the background of live host tissue by transfection of the jellyfish (*Aequorea victoria*) green fluorescent protein (GFP) gene [Chalfie et al. (1994); Heim et al. (1995); Zolotukhen et al. (1996) (Chishima et al, 1997a, b, c)]. We show here that histoculture of host tissue involved with GFP-expressing tumor cells (Chishima et al, 1997d) offers critical advantages enabling tumor cells to colonize and progress in host tissue under *in vivo*-like conditions with concomitant fluorescence visualization during the entire process.

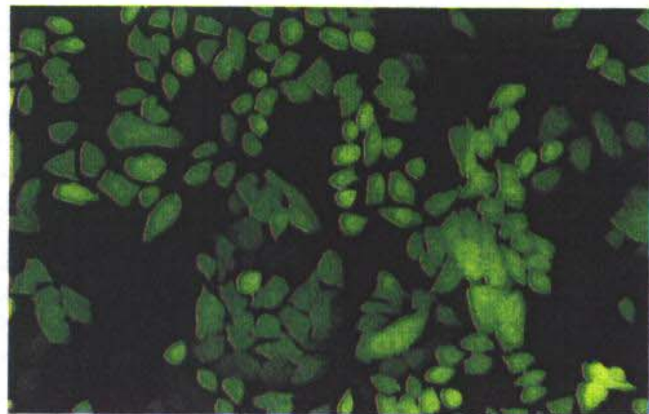

FIG. 1. The human lung cancer cell line Anip 973 was transfected with the pED-mtx^r dicistronic expression vector in which the humanized green fluorescent protein (GFP)-S65T and DHFR genes were transcribed. The stable high-expression Clone-26 was selected in 50 nM methotrexate (MTX) *in vitro*. (Original magnification = ×200).

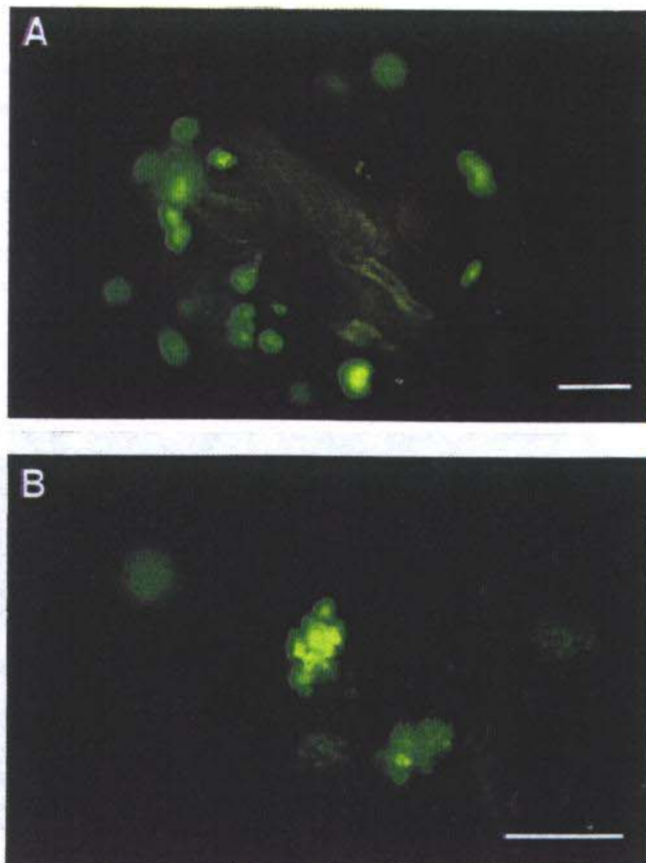

FIG. 2. Micrometastases in fresh lung tissue. *Bars* = 300 µm (*A*); 200 µm (*B*). (*A*) Clone-26 cells made numerous small metastases that ranged from 15 µm to 180 µm in the lung tissue seen in severe combined immune deficient (SCID) mice 5 wk after 7.5 × 10^6 cells were injected in the tail vein. (*B*) High magnification of the human lung tumor colonies in the SCID-mouse lung. These colonies are approximately 160 µm in diameter.

Human cell line. The human lung adenocarcinoma cell line (Anip 973) was obtained from Harbin Medical University (Harbin, China).

DNA manipulations and expression vector constructions. The dicistronic expression vector (pED-mtx^r) was obtained from Genetics Institute (Cambridge, MA) (Kaufman et al, 1991). The expression vec-

tor containing the human coden-optimized hGFP-S65T gene was purchased from CLONTECH Laboratories (Palo Alto, CA). To construct the hGFP-S65T-containing expression vector, phGFP-S65T was digested with *Hind* III in order to blunt the end. The hGFP entire coding region was then excised with X*ba* I. The pED-mtx^r vector was digested with P*st* I, blunted at the end, and further digested with X*ba* I. The hGFP-S65T cDNA fragment was then unidirectionally subcloned into pED-mtx^r.

Cell culture, transfection, subcloning. Anip 973 cells were cultured in RPMI 1640 (Life Technologies, Baltimore, MD) containing 10% fetal calf serum (FCS) (Gemini Bio-products, Calabasas, CA) and 2 m*M* L-glutamine. For transfection, near-confluent Anip 973 cells were incubated with a precipitated mixture of LipofectAMINE™ Reagent (Life Technologies, Baltimore, MD), and saturated amounts of plasmid for 6 h before being replenished with fresh medium. Anip 973 cells were harvested by trypsin/EDTA 48 h posttransfection. The cells were subcultured at a ratio of 1:15 into selective medium that contained 50 nM methotrexate (MTX). Cells with stably integrated plasmids were selected by growing transiently transfected cells in the MTX-containing medium. Clones were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin/EDTA. The clones were amplified and transferred by conventional culture methods. Clone-26 was selected due to its high-intensity GFP fluorescence and stability in the absence of MTX (Chishima et al, 1997b, c).

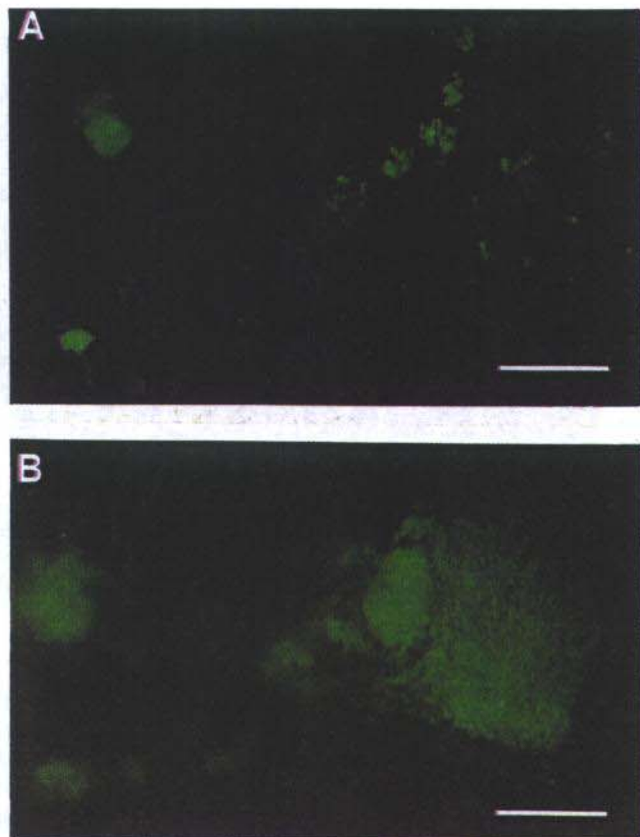

FIG. 3. *In vitro* histoculture of the fresh severe combined immune deficient (SCID)-mouse lung tissue involved with green fluorescent protein (GFP)-transfected Anip cells. *Bars* = 300 μm. (*A*) Fresh lung tissues that contained GFP-Anip micrometastases were histocultured on sponge gel matrixes for 3 d. Metastatic colonies grew *in situ*. (*B*) The same field as Fig. 3 *A*. The colonies grew on the sponge gel for 24 d.

Animals and experimental metastases. Six-wk-old SCID/SCID severe combined immune deficient (SCID) mice were injected intravenously with a single dose of 7.5×10^7 Clone-26 cells. Cells were first harvested by trypsinization and washed three times with cold serum-containing medium, then kept on ice. Cells were then injected in a total volume of 0.5 ml within 40 min of harvesting. The nude mice were sacrificed 5 wk after tumor cell injection.

In vitro *tumor progression in histoculture.* Whole lung tissues were aseptically removed from the scid mice. The lung tissues were divided into pieces of approximately 2–3 mm in diameter, which were then placed on prehydrated collagen sponge gels (Upjohn Co., Kalamazoo, MI). The gels were floated in 24-well plates at the air-water interface in RPMI 1640 medium containing 20% fetal calf serum (FCS) (Gemini Bio-products), 2 m*M* L-glutamine, and penicillin. The histocultures were incubated at 37° C in a humidified atmosphere containing 95% air and 5% CO_2. The lung tumor colony growth in the host lung tissue was observed for more than 1 mo. in histoculture.

Microscopy. Light and fluorescence microscopy were carried out using a Nikon microscope equipped with a Xenon lamp power supply and Leica stereo fluorescence dissecting microscope equipped with a mercury lamp power supply. GFP filter sets (Chromatechnology Corp., Brattleboro, VT) were used for fluorescent analysis.

Transfection of GFP expression vector to human lung cancer cells. The GFP expression vector transfected cells were able to grow in levels of MTX up to 50 nM. The MTX-resistant Anip 973 cells developed a striking increase in GFP fluorescence after subcloning compared to transiently transfected cells. A subclone that expressed GFP the strongest was isolated and termed Anip 973 Clone-26 (Clone-26) (Fig. 1) (Chishima et al, 1997b, c).

Lung colonization by the GFP-transfected lung tumor cells in nude mice. 7.5×10^6 Clone-26 cells were injected in the tail vein of SCID mice. Three wk after injection, numerous micrometastatic colonies were detected in the whole lung tissue (Fig. 2). Colonies had developed up to approximately 550 μm. The Clone-26 cells in the mouse lung were strongly fluorescent and expressed stable high-level GFP *in vivo* even in the absence of MTX.

Progression of lung colonization by the GFP-transfected lung tumor cells in histoculture. Clone-26-seeded scid mouse lungs were removed from the mice and then histocultured on sponge gels. Tumor colonies further developed and spread rapidly in the lung tissue over time in histoculture (Fig. 2 *A,B*). After only 1 wk of histoculture, tumor cells started to invade and colonize the supporting collagen sponge gel. After 2 wk histoculture, tumor cells formed satellite colonies in the sponge gel distant from the primary colonies in the lung tissue (Fig. 2). The colonies were growing faster in histoculture than the metastatic colonies in the SCID mice, perhaps due to the absence of inhibitory substances found only *in vivo* that keep tumor cells in a degree of dormancy. Tumor colonies confirmed growth in histoculture for more than three weeks (Figure 3).

We have also demonstrated visualization of host organ colonization and tumor progression *in vitro* using histoculture of nude mouse organs involved with GFP-transfected human lung adenocarcinoma cells (Chishima et al, 1997d). The present study indicates that the scid mouse lung is possibly even a better substrate for invitro colonization by a human lung tumor.

Khokha and Groom (1995) and Koop et al. (1995) visualized extravasation and initial seeding steps in tumor metastasis using external fluorescently labeled tumor cells *in vivo*. They demonstrated that the growth regulation of cells that have already extravasated and seeded potential target organs is rate-limiting, and seemingly the most important step in the metastatic process. However, their system did not permit long-term visualization of colonization.

Margolis et al. (1995) visualized the migration of external fluorescently labeled lung tumor cells in host mouse lung in histoculture. This study was not able to visualize the long-term growth progression of metastatic colonies in host organs because of the limitation of exogenously labeled fluorescent tumor cells whose intensity decreased as the tumor cells divided. Tumor cells with GFP, on the other hand, pass on the gene to daughter cells and can be followed continuously. GFP fluorescence visualizable in fresh tissue also has a great advantage over reporter genes such as lac-Z, which can only be visualized by removing the tissue from culture or the animal and processing it (Lin et al., 1990), which distorts the tumor and host tissue structure.

To overcome these limitations, we have utilized the green fluorescent protein of the jellyfish *Aequorea victoria* as a stable genetic marker to visualize tumor cells colonizing host tissue in histoculture.

Using the tumor scid mouse lung chimeric histoculture methods developed in the present study, GFP fluorescence facilitates the understanding of tumor colonization. This model also provides many opportunities for understanding of metastasis and to develop therapies of the lethal aspect of cancer.

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