INTRODUCTION

Despite recent advances in treatment of primary tumors, metastasis remains the major cause for failure in cancer treatment (Weinstat-Saslow and Steeg, 1994; Fidler and Ellis, 1994; Liotta and Stetler-Stevenson, 1993). By better understanding the mechanisms of the metastatic process, it will be possible to identify the steps that are most susceptible to therapeutic intervention (Woodhouse et al., 1997; Zetter, 1998; Chambers et al., 1999). To address questions regarding the in vivo formation of metastases, a way is needed to directly observe metastasis as it occurs over time. High resolution intravital videomicroscopy (IVVM) permits the study of events in the metastatic process that were previously inaccessible by commonly used in vivo and in vitro assays (Chambers et al., 1992, 1995; Morris et al., 1993; I. C. MacDonald et al., 1998). Although ‘spontaneous’ and ‘experimental’ end-point metastasis assays allow for quantification of the number and sizes of metastases in various organs, they do not provide information on how these metastases develop. IVVM provides an observation window which allows access to steps in hematogenous metastasis that previously were difficult to observe and impossible to quantify.

Visualization of tumor cells by IVVM requires that the cells carry a marker to distinguish them from the surrounding normal tissue. The naturally expressed marker melanin is extremely valuable for the detection of melanoma cells (Chambers et al., 1992; Luzzi et al., 1998); however, some cells may escape detection because of the heterogeneous expression of melanin within cell populations. Exogenous fluorescent markers have therefore been employed. Calcein-AM is a cytoplasmic marker that gives a clear outline of the cell profile (MacDonald et al., 1992) but is lost from the cells with time, limiting its use to a period of approximately 24 hours from the time of uptake into the cells. Fluorescent plastic nanospheres (Morris et al., 1994) are useful cell markers which do not fade with time but, since they are not distributed uniformly within cells, visualization of the cell boundary is difficult. Moreover, consecutive dilution of the label occurs with each cell division, rendering cells virtually undetectable after two to three division cycles.

SUMMARY

High resolution intravital videomicroscopy has provided a powerful tool for directly observing steps in the metastatic process, and for clarifying molecular mechanisms of metastasis and modes of action of anti-metastasis therapeutics. Cells previously have been identified in vivo using exogenously added fluorescent labels, limiting observations to a few cell divisions, or by natural markers (e.g. melanin) expressed only by specific cell types. Here we tested the utility of stable green fluorescent protein (GFP)-transfected cells for monitoring and quantifying sequential steps in the metastatic process. Using CHO-K1 cells that stably express GFP, we document the visualization and quantification by intravital videomicroscopy of sequential steps in metastasis within mouse liver, from initial arrest of cells in the microvasculature to the growth and angiogenesis of metastases. Individual, non-dividing cells, as well as micro- and macrometastases could clearly be detected and quantified, as could fine cellular details such as pseudopodial projections, even after extended periods of in vivo growth. We quantified the size distribution of micrometastases and their locations relative to the liver surface using 50 μm thick formalin-fixed tissue sections. The data suggest preferential growth and survival of micrometastases near the liver surface. Furthermore, we observed a small population of single cells that persisted over the 11 day observation period, which may represent dormant cells with potential for subsequent proliferation. This study demonstrates the advantages of GFP-expressing cells, coupled with real-time high resolution videomicroscopy, for long-term in vivo studies to visualize and quantify sequential steps of the metastatic process.

Key words: Green fluorescent protein (GFP), Metastasis, Pseudopodia, Angiogenesis, Mouse liver, Intravital videomicroscopy

Cellular expression of green fluorescent protein, coupled with high-resolution in vivo videomicroscopy, to monitor steps in tumor metastasis

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divisions. Clearly, what is needed is an endogenous tumor cell marker that is stably expressed over long periods of time in vivo by all cells of the population injected and by their progeny, permitting their detection by IVVM.

Transfection of tumor cells with green fluorescent protein (GFP) (Misteli and Spector, 1997) cDNA produces a heritable, stable cytoplasmic marker that allows cells to be detected for long-term observations in vivo. To date, the use of GFP-expressing tumor cells has permitted visualization of individual metastatic cells and end-point spontaneous metastases in freshly excised organ specimens (Chishima et al., 1997a-c; T. J. MacDonald et al., 1998; Yang et al., 1998, 1999). It has also permitted quantification of the motility of tumor cells within primary tumors in the mammary fat pad, by means of in vivo time-lapse confocal microscopy (Farina et al., 1998). However, real-time monitoring by IVVM of sequential steps in the metastatic process over the long term, using GFP-expressing cells, has not (to our knowledge) been carried out previously.

In the present report we document the visualization and quantification by IVVM of sequential steps in the metastatic process of GFP-expressing CHO-K1 cells within mouse liver, from initial arrest of cells in the microvasculature to their eventual growth into macroscopic tumors in which angiogenesis is occurring. We were able to clearly observe details such as cytoplasmic projections, even many days after injection of the cells, and to follow the growth of micrometastases over extended periods of time; furthermore, individual non-dividing cells were readily detectable long after injection. The use of GFP-expressing tumor cells constitutes a powerful new tool to further expand the capabilities of high resolution intravitral videomicroscopy, in order to study cellular and molecular details of the metastatic process.

MATERIALS AND METHODS

Cell culture

CHO-K1-GFP is a Chinese hamster ovary cell line previously transfected (Chishima et al., 1997b) with the pED mtx2 expression vector containing the highly-fluorescent S65T variant (GFP-S56T) of the wild-type GFP gene and the DHFR gene as a selectable vector containing the highly-fluorescent S56T variant (GFP-S56T) of the DHFR gene. The cells were routinely maintained (Weiss et al., 1992; Morris et al., 1993). For each sample 10,000 cell counts were done. The same was done to quantify the relationship between two specific factors, regression functions were obtained by linear regression analyses. Data on distribution of tumor foci with respect to the liver surface were analyzed using three parameters for comparison: mean distance, standard deviation, and maximum values. A level of $P<0.05$ was regarded as statistically significant.

Stability of GFP fluorescence intensity in vitro

To characterize the stability of GFP fluorescence over time, cells were grown with or without MTX-selective medium and were passaged every fourth day over a 24-day period. After each cell passage fluorescence intensity was assessed by flow cytometry (Epics XL-MCL, Coulter, Miami, FL). For each sample 10,000 cell counts were performed in duplicate.

Intravital videomicroscopy

Female SCID mice, 6-7 weeks of age (Charles River, St Constant, Quebec), were cared for in accordance with standards of the Canadian Council on Animal Care, under an approved protocol of the University of Western Ontario Council on Animal Care. Mice were anesthetized using a ketamine/xylazine mixture (1.6 mg of ketamine and 0.08 mg of xylazine per 15 g body mass) administered by intraperitoneal injection. A suspension of $3 \times 10^6$ CHO-K1-GFP cells in 0.15 ml of DMEM supplemented with 10% FCS was injected via a mesenteric vein to target the liver, as previously described (Morris et al., 1993). Tempesic analgesic (0.1 mg/kg body weight) was administered subcutaneously as mice awoke and 18 hours after surgery.

Mice were examined by IVVM (Chambers et al., 1995; I. C. MacDonald et al., 1998) immediately (up to 90 minutes) after injection of cells or 1, 2, 3, 4, 7, 9, and 11 days later. The animals were anesthetized with sodium pentobarbitol (60 mg/kg i.p.), after which a portion of a liver lobe was exposed and the mouse placed on a viewing platform on the stage of an epi-fluorescence inverted microscope (Nikon Diaphot TMD). Oblique transillumination was provided by a fiber optic light source to enhance cell contrast and epi-illumination (450-490 nm) was used to excite GFP. Real-time images obtained using a video camera with extended red sensitivity (Panasonic, WV 1550), were viewed on a video monitor and recorded on SVHS videotape. The animal’s temperature was monitored and maintained at $37^\circ$C using a heat lamp. Anesthesia was maintained with supplemental administration of sodium pentobarbitol as required.

Histology

Mice were sacrificed by anesthetic overdose after IVVM. The livers were fixed in 10% neutral buffered formalin (pH 7.6) and sectioned (~50 μm thick) using a Vibratome Series 1000 sectioning system (Technical Products International, St Louis, MO) as described previously (Luzzi et al., 1998). Tissue was not paraffin-embedded because GFP fluorescence is lost during treatment with organic solvents (Chalfie et al., 1994). Sections were examined microscopically using the green fluorescence of tumor cells to identify them and to quantify tumor number, tumor size, and shortest distance between the tumor center and the periphery of the liver. To eliminate double- and triple-counting of the same micrometastasis in serial tissue sections, unbiased stereological correction was used (Weibel, 1979).

Statistical analysis

Statistical analyses were performed using a Statistical Analysis Software, Version 6 for Windows (SAS Institute Inc., Cary, NC). To assess the relationship between two specific factors, regression functions were obtained by linear regression analyses. Data on distribution of tumor foci with respect to the liver surface were analyzed using three parameters for comparison: mean distance, standard deviation, and maximum values. A level of $P<0.05$ was regarded as statistically significant.

RESULTS

Stability of GFP fluorescence intensity in vitro

The fluorescence intensity of CHO-K1-GFP cells maintained in the presence vs absence of MTX over a 24 day period in vitro was quantified (Fig. 1). For cells grown in the presence of MTX the median fluorescence intensity at day 3 was 105 (arbitrary units) compared to 81 units at day 24. In the absence of MTX, cells showed a median fluorescence intensity of 95 units, similar to that of cells maintained under selective pressure at day 24. Thus the in vitro fluorescence intensity of the cells was sufficiently stable to permit detection in vivo, even in the
absence of selective pressure. Non-fluorescent cells were not detected among cells maintained with or without MTX.

**Cell arrest in liver sinusoids, extravasation, and early growth of micrometastases**

Cytoplasmic expression of GFP permitted IVVM identification of individual tumor cells (10-15 μm in diameter) and their morphology against the faint yellow autofluorescence of the liver tissue and the dark red appearance of the blood vessels. GFP permits resolution of a high level of detail of cellular morphology at all stages of the metastatic process. At 90 minutes after injection, all observed cancer cells remained within sinusoidal vessels near terminal portal venules in acinar zone 1 (Fig. 2A). Occasionally cells near branch points were forced into several sinusoids at once, resulting in odd shape deformations (Fig. 2A). Twenty-four hours after initial arrest, most cells had completed extravasation and others were in the process of extravisating. Pseudopodial projections extending through the vascular wall from tumor cells were observed during this process, and some extravasated cells were found wrapped around the abluminal surfaces of vessels. Other extravasated cells were seen to extend long slender projections to the avascular region immediately below the liver capsule. These projections could be as long as 60 μm and GFP permitted resolution of their detailed morphology, such as the bifurcation seen in Fig. 3A. Extravasated tumor cells, located within the liver tissue between sinusoids (Fig. 2B), remained viable upon completion of the extravasation process, as indicated by the persistence of their green fluorescence.

Multicellular micrometastases present by day 4 (Fig. 2C), ranged in diameter from 20 to 380 μm and at higher magnification individual cells within them could be clearly seen by IVVM, especially at the tumor periphery. Views of one of these micrometastases taken at two different focal depths (‘optical slicing’) are shown in Fig. 3B,C. At a deeper plane of focus (Fig. 3B), the center of the metastasis is clear but the peripheral cells appear out of focus. In contrast, at a plane nearer the surface of the liver (Fig. 3C) the same peripheral cells appear in sharper focus than the interior cells, indicating that these peripheral cells had invaded the avascular layer located just below the liver capsule. Details of individual pseudopodia from micrometastases were clearly visible (Fig. 3B,C). Larger micrometastases separated by normal tissue are present by day 9, many of them showing pseudopodial projections extending from their periphery into the surrounding tissue (e.g. Fig. 2D). Solitary individual tumor cells were also present in the tissue at this and later times, visible under IVVM solely because of their GFP fluorescence. Three such cells detected in close proximity (≥250 μm) to two metastases at day 11 are shown in Fig. 3D. Based on the observed blood flow directions within vessels of this area, it is unlikely that these cells had spread via the vasculature from the adjacent tumors. Such cells could represent undivided cells remaining from the original injection or might have migrated within the liver parenchyma from the adjacent metastases.

**Development of tumor microvasculature**

Microvessels were first seen within the metastases at day 11. By fluorescence illumination the vessels appeared dark against the green fluorescence of the tumor tissue and had a highly distorted configuration, as illustrated in Fig. 3E. By transillumination at higher magnifications, the movement of individual blood cells was clearly seen using IVVM. Red blood cell (RBC) motion was measured in vessels that passed from normal to tumor tissue. Typically, relatively steady flow (e.g. 53.6 μm/second ±2.2 (s.d.) in a 10.5 μm vessel) became far more sluggish within the tumor tissue, with complete stasis in some branches. Fig. 4A,B illustrates the highly irregular

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**Fig. 1.** Stability of GFP fluorescence intensity in vitro. Fluorescence intensity was analyzed by flow cytometry of CHO-K1-GFP cells suspended in medium. (A) Fluorescence intensity distribution of cells maintained in media containing MTX (1.5 μM) as a selection pressure against non-GFP expressing cells. The analysis was performed 3 days after initial cell plating. (B) Fluorescence intensity profile of the same cell population 24 days after the initial reading. The cells were continuously maintained in MTX (1.5 μM). No significant changes were observed in the general shape and position of the distribution. (C) Fluorescence intensity profile of CHO-K1-GFP cells originating from the initially observed (A) cell population, but continuously grown after that point in the absence of MTX. This mimics the selection-free in vivo environment of the mouse liver. Readings were taken every 4 days and did not differ from the beginning and end-point data presented.
morphology often seen, and Table 1 gives the corresponding diameters and RBC velocities at selected locations.

**Size distribution and locations of micrometastases**

Quantification of the size distribution of micrometastases (Fig. 5) and their locations relative to the liver surface (Fig. 6) was performed following the real-time IVVM observations, using 50 μm thick formalin-fixed tissue sections. Such quantification was made possible through the identification of single cells and micrometastases in these sections via their expression of GFP. Only single cells were present up to day 1 but by day 2, small micrometastases of 2-4 cells accounted for 11.8% of the total tumor foci observed (Fig. 5). By day 3, the proportion of single cells had decreased to 38% of the tumor foci observed, and by day 11 this proportion had decreased to only 2%. The numbers of micrometastases increased dramatically by day 3: approximately 40% of the total foci consisted of small micrometastases 20-80 μm in diameter, and a further 20% had reached diameters up to 140 μm. However, by day 4 these numbers had changed to 15% and 65%, respectively, indicating that significant growth of the micrometastases had occurred in the meantime. By day 7, micrometastases up to 380 μm diameter were found, although the great majority were ≤260 μm in size. By day 9 the range of diameters was even greater, with a few micrometastases up to 560 μm being found. By day 11 the median size of micrometastases had obviously increased further (Fig. 5), but the range of diameters was smaller, with micrometastases measuring 140-380 μm.

The initial distribution of tumor cells immediately after injection (Fig. 6, top panel) showed cells located throughout the organ, and these data are consistent with a random distribution. The volume of tissue available for trapping cells decreases with distance from the surface (see Fig. 6 inset) and, therefore, for a random distribution of cells the heights of the bars should decrease with distance from the surface, as was found. This pattern was typical for tumor foci (single cells and
micrometastases) up to day 4, but by day 7 a different distribution had emerged: tumor foci were found only within ~400 μm of the surface, whereas at greater depths (down to ~900 μm, the center of the organ) they had disappeared. This change in distribution was statistically significant, based on linear regression analysis using three parameters from the data shown in Fig. 6 for comparison: mean distance ($P=0.037$), standard deviation ($P=0.013$), and maximum values ($P=0.018$). Thus, the data suggest a preferential growth and survival of micrometastases near the liver surface and failure of cells located more centrally to survive.

**DISCUSSION**

In the past, GFP has been used to detect metastatic cells in freshly excised tissue samples (Chishima et al., 1997a-c; T. J. MacDonald et al., 1998; Yang et al., 1998, 1999), and for studies of cell motility within primary tumors in the mammary fat pad by in vivo time-lapse confocal microscopy (Farina et al., 1998). The success of these studies suggested that the use
of GFP-expressing cells would facilitate in vivo investigations of the metastatic process by IVVM, which until now have been limited in certain respects by the cell labeling techniques available. Utilization of GFP as a cell label for in vivo experiments requires that cells be stably transfected, expressing GFP over the long term. The CHO-K1-GFP cells used in the present study exhibited stable fluorescence (as demonstrated by flow cytometry) even after 24 days maintenance in vitro in medium deprived of selective pressure; moreover, the entire cell population remained fluorescent under these conditions. This indicates suitability of the cells for long-term use in vivo, and we demonstrate here the advantages of GFP-expressing cells for visualization and quantification, by real-time IVVM, of sequential steps in the metastatic process.

GFP-expressing tumor cells injected into the portal circulation could be seen clearly at subsequent stages of metastasis: initial arrest of cells in the liver microvasculature, extravasation, growth into micrometastases, and continued growth into macroscopic tumors in which angiogenesis took place. Previously, such in vivo visualization of tumor cells had been possible only by using exogenous markers such as Calcein-AM or fluorescent nanospheres, and/or the natural marker melanin in melanoma cells (I. C. MacDonald et al., 1992, 1998; Luzzi et al., 1998). Calcein-AM showed the detailed morphology of individual cells but disappeared within ~24 hours. Fluorescent nanosphere labeling lasted longer, until diluted out by 2-3 cell divisions; however, visualization of cell boundaries was difficult because of non-uniform distribution of nanospheres throughout the cytoplasm. Limitations of the endogenous marker melanin include its expression by only specific cell types (e.g. melanoma); furthermore, some cells may escape detection because of the heterogeneous expression of melanin.

Fig. 5. Survival and growth of single cells and micrometastases. GFP made possible the visualization and quantification of single cells and small micrometastases which would have been difficult or impossible to detect by other methods. The percentage of the total observed foci which consisted of single undivided cells (white bars) decreased with time, from 100% at day 1 (not shown) to only 2% at day 11. Micrometastases (black bars) increased in size progressively over the 11 day period, as shown by the changes in size distribution.
of melanin within cell populations. In contrast, use of GFP as a cytoplasmic marker (which can be transfected into any cell type of interest) enabled us to visualize in vivo both the general outlines of cells and fine morphological details such as long slender pseudopodial projections, for extended periods of time.

Pseudopodial projections from solitary individual cells were more clearly visible with GFP than any we have seen previously using other markers, and never before have we seen bifurcations of pseudopodia in vivo (Fig. 3A). Furthermore, to our knowledge pseudopodial projections from cells around the periphery of micrometastases have not previously been detected in vivo (except for melanoma in chick embryo chorioallantoic membrane: Chambers et al., 1992), and the present study using GFP revealed such projections in micrometastases of all sizes. In larger micrometastases these projections were often directed particularly toward other micrometastases close by, implying some form of interaction between them. The fact that GFP-expressing single cells and micrometastases could be seen so clearly by IVVM and also in formalin fixed thick tissue sections opened the way, for the first time, for detailed quantification of the growth of micrometastases and their locations relative to the liver surface, and how these parameters changed with time. Of particular interest was the finding that micrometastases seem to show preferential growth and survival near the liver surface (confirming the results of Dingemans et al., 1985, 1994) and that those that are located more centrally fail to survive. This raises the question of whether the tissue close to the liver surface contains preferentially the factors necessary for metastatic growth. A further advantage of GFP-expressing cells is the increased contrast between brightly fluorescent tumor tissue and blood vessels within it. The ability to visualize and quantify blood vessel development in metastases in vivo will greatly facilitate studies of angiogenesis and the testing of effects of anti-angiogenic agents on metastatic development.

We recently showed that, contrary to what is generally believed, a significant population of melanoma cells remain within the liver tissue by 13 days after injection, as solitary, dormant cells, and that most small micrometastases that formed by day 3 failed to continue to grow (Luzzi et al., 1998). Detection of these solitary cells and micrometastases was only possible due to their melanin content (and nanosphere labeling for undivided cells), and we could not have done the study on other cancer cell types. However, transfection and expression of GFP makes possible such studies on any cancer cell type of interest. Here we have used CHO-K1 cells, and the present findings together with our recent data (Luzzi et al., 1998) point to the existence of a previously unappreciated phenomenon. We have identified a population of single undivided cells surviving in the tissue up to two weeks after injection. These apparently dormant cells could have the potential to be activated at a later time and commence growth. If this situation also occurs clinically, these dormant cells will represent a critically important population to understand and control. The present study shows that GFP is superior to previously used cancer cell markers and, used in combination with intravital videomicroscopy of intact organs, GFP allows for reliable in vivo detection and quantification of sequential steps in metastasis. This method should provide a powerful tool to further our understanding of mechanisms involved in metastasis and angiogenesis, and allow for real-time monitoring and evaluation of the effects of therapeutic agents targeted at specific key steps of metastasis.

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