Selection for Experimental Metastatic Ability of Heterologous Tumor Cells in the Chick Embryo after DNA-mediated Transfer

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ABSTRACT

The chick embryo is an immune-deficient host able to support growth of a wide variety of transformed cells. Since growth of normal cells is not observed, this system appears to be generally useful for investigating malignant properties of different cells. Recently, we developed a sensitive assay to quantitate and select for rodent cells able to survive and grow in embryonic chick organs following i.v. injection (Cancer Res., 42: 4018-4025, 1982). We envisage this assay as a model system for studying aspects of the metastatic process. We have used DNAs from murine and human melanoma cell lines (which grow well in chick embryos after i.v. injection) to transfect murine LTA cells (which do not grow in chicks after i.v. injection). From the transfected LTA cells, we were able to isolate clones which grow well in the chick after i.v. injection. Such clones were not observed in untransfected LTA cells or with LTA cells transfected with LTA DNA. These experiments clearly demonstrate the feasibility of using the chick embryo as a host system to study genes involved in growth control alteration of the sort seen in malignant transformation.

INTRODUCTION

The growth and metastatic spread of tumors are complex processes involving interactions between tumor cells and normal tissues. To investigate these interactions, it is desirable to exploit different host systems in which aspects of these processes may be investigated. The chick embryo is a relatively immune-deficient host that has been used to study tumorigenic properties of a wide variety of transformed cells (8, 15, 16, 18). As an alternative to using tumor formation as an end point, we developed recently a ouabain selection assay to recover viable rodent tumor cells for further study (5). This assay extends the sensitivity of the chick embryo in testing experimental metastatic properties of tumor cells by permitting early detection and quantitation of viable cells in embryonic organs following i.v. injection. This allows a kinetic description of cell number over time and permits discrimination among cell types the numbers of which (a) decline, (b) remain relatively constant (mimicking quiescence), and (c) increase to varying degrees in, e.g., embryonic liver following i.v. injection. Data using this assay (5) and from histological studies (8, 15, 16, 18) suggest that many tumor cells fall into Categories b and c, while normal cells fall into Category a.

The genetic basis for differences between tumorigenic and normal cells has been the subject of much recent work (3, 19, 22) and remains an important question in our understanding of steps involved in malignant transformation and tumor progression. Because (a) the ouabain-plating assay in the chick embryo can detect small differences in vivo growth ability following i.v. injection, (b) such differences appear to reflect properties relevant to malignant transformation, and (c) the embryonic immune system permits the testing of cells expressing foreign antigens, we sought to determine the feasibility of using this assay to select for changes in tumorigenic properties in rodent cells altered by DNA-mediated gene transfer.

MATERIALS AND METHODS

Cells and Cell Culture. LTA cells, murine Tk\textsuperscript{-}-negative and adenine phosphoribosyltransferase-negative L-cells (7, 17), were a gift from C. P. Stanners, McGill University, Montreal, Quebec, Canada. B16F1 murine melanoma cells (9, 10) were a gift from I. J. Fidler, M. D. Anderson Hospital and Tumor Institute, Houston, TX. IGR37, a human melanoma cell line established from a metastatic tumor (11), was a gift from S. K. Liao, McMaster University, Hamilton, Ontario, Canada. Cells were maintained in tissue culture in \( \alpha \)-minimal essential medium with (B16F1, IGR37) or without (LTA) added nucleosides (Grand Island Biological Co., Grand Island, NY), supplemented with 10% fetal calf serum (Flow Laboratories, McLean, VA). Cells were routinely subcultured by trypsinization (0.1% Bacto-trypsin; Difco Laboratories, Detroit, MI). Cells for injection into chicks were reseeded in appropriate medium plus fetal calf serum as listed above. Plating efficiency (colony-forming ability) was determined by plating known numbers of cells in 60-mm dishes in medium plus varying concentrations of ouabain as described previously (5).

Assay for Growth in Chicks following i.v. Injection. "Experimental metastasis" in the chick was assayed by i.v. injection and cell quantitation as described previously (5). Briefly, windows were cut in the shells of 11-day embryos (White Leghorn, obtained from a local hatchery), and cells were injected in a volume of 0.1 ml, using a 30-gauge needle, into CAM veins. Windows were sealed with melted paraffin, and the embryos were returned to the incubator (Roll-X; Marsh Manufacturing, Inc., Garden Grove, CA), maintained at 37° and ~60% relative humidity, for varying lengths of time. Livers were then dissected and were either fixed in formalin for histological sectioning or were enzymatically dissociated into a single cell suspension and plated as described previously (5) in 2 \( \times \) 10\textsuperscript{-4} ml ouabain in medium appropriate for the cell type. At this concentration of ouabain, chick cells rapidly die, while rodent cells grow normally, permitting quantitation of rodent cells in embryonic liver by counting ouabain-resistant colonies in the plates. Livers were generally dissected 7 days after cells were injected, but for kinetic studies, livers were dissected at varying times, from immediately after injection to 7 to 10 days later; embryos left for 10 days were just beginning to hatch.

DNA Transfection and Selection for Growth in Chicks. Total genomic DNA was prepared from logarithmically growing cells by phenol extraction (14). Genomic DNA, either alone or with cloned TK DNA (12) (a gift from C. P. Stanners, McGill University), was introduced into LTA cells using the calcium phosphate precipitation procedure of Graham and van der Eb (12, 13). Cotransfection experiments with TK DNA served as a check on the efficiency of the transfection procedures and permitted determination of the extent of transfection in the chick embryos.

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selection, using HAT medium (21), of cells competent to take up DNA. HAT-resistant colonies were pooled from individual transfection plates and were injected into chicks (5 × 10⁵ cells/embryo). Cells persisting in liver 7 days later were again pooled, grown briefly in tissue culture (in medium containing HAT and ouabain), and injected into chicks. Such enrichment cycling was repeated several times for each transfectant line, with cells always kept in HAT medium when in tissue culture. When genomic DNA was used alone, without TK DNA, individual plates of potential transfectants were trypsinized, and cells were injected into chicks at 5 × 10⁶ cells/embryo. Cells from this series were also cycled several times through chicks, as described above but without HAT in the medium, to enrich for cells able to grow in chicks after i.v. injection.

RESULTS

Responses of Human, Rodent, and Chick Cells to Ouabain.
The ouabain-plating assay for recovery of rodent cells from embryonic chick liver depends on the sensitivity of chick cells to the cytotoxic drug ouabain, relative to rodent cells (5). We sought to determine whether this assay could also be used to detect and recover human cells injected into CAM veins. The dose response of human melanoma IGR37 cells to ouabain, relative to results published previously for chick and rodent cells (5), is shown in Chart 1. Several other human tumor cell lines showed a dose response nearly identical to that shown for IGR37 cells (data not shown). Characteristic species differences in ouabain sensitivity have been reported previously, and the data presented here are in agreement with published data for both normal and transformed human cells (see Ref. 2 for review). Because human cells are thus more sensitive to ouabain than are chick cells, the ouabain-plating assay cannot be used to detect growth of human cells in chick organs. The assay can, however, readily detect rodent cells present in chick organs (5). Furthermore, resistance to ouabain has been shown to be a dominant genetic trait in rodent cells (1, 2), permitting transfer of human DNA into rodent cells with little likelihood of affecting their ouabain resistance and suggesting that experimental metastatic ability of such cells could be determined using the ouabain-plating assay. As expected, resistance of LTA cells to ouabain remained unchanged after the DNA transfections and passages through the chick described below.

Characterization of Recipient Cells for DNA Transfection.
As a recipient cell line for DNA transfection, we required a rodent cell type the numbers of which declined steadily in chick liver following i.v. injection. We have shown previously that murine LTA cells are of this phenotype (5). Because LTA cells grow poorly in chicks after i.v. injection and because they have been shown to be good recipient cells for DNA transfection (12), this cell line was chosen as a DNA recipient cell line.

Our aim was to select transfectants with increased ability to grow in chick liver after i.v. injection, relative to untransfected LTA cells. Thus, it was necessary to determine the reproducibility of background numbers of viable LTA cells persisting in liver. Chart 2a shows the results of replicate experiments in which LTA cells (5 × 10⁵/embryo) were injected, and numbers of viable LTA cells in liver were determined 7 days later. The background level of LTA cells surviving in liver after 7 days was reproducible in replicate experiments. Most embryos contained fewer than 20 cells/liver, but occasional embryos contained nearly 10⁵ cells/liver. In no case have we detected more than 10⁴ LTA cells/liver under these conditions.

In order for transfectant growth to be detected, numbers of transfectants recovered from liver had to be greater than the LTA background of up to 10⁵ cells. A single transfectant arrested initially in liver would be unlikely to grow in 7 days to exceed this background (e.g., one cell arrested in liver and dividing every 24 hr would produce only 128 cells in liver 7 days later and would not be detected above the LTA background). Our strategy, therefore, was to enrich for transfectants able to grow by pooling cells recovered from liver (consisting presumably of both control LTA cells and subthreshold numbers of growing transfectants) and cycling these cells through additional rounds of injection and plating of livers. As a control for this procedure, we cycled control LTA cells repeatedly through the liver (Chart 2b). After 3 such cycles, numbers of LTA cells persisting in liver were no greater than the 10⁴ threshold seen in Chart 2a. We conclude that cycling of LTA cells through liver does not select for LTA cells with enhanced growth ability.

Cotransfection with a selectable gene such as TK has been shown to select for a subset of recipient cells competent to take up transfected DNA (12). Because this procedure would effectively reduce the numbers of untransfected LTA cells, cotransfection with TK was used in a number of experiments. In a control experiment, we cotransfected LTA cells with LTA genomic DNA plus TK DNA. HAT-resistant cells were cycled 3 times through liver (Chart 2c; Table 1, Experiment 5). These cells did not show enhanced growth properties relative to untransfected LTA cells (Chart 2, a and b). Transfection with TK DNA alone, without genomic DNA carrier, gave similar results (data not shown). We conclude that the transfection procedure alone,
using genomic DNA from LTA cells and cloned TK DNA, is not sufficient to alter the growth properties of LTA cells in the chick.

Characterization of Donor Cell Lines for DNA Transfection. For donor DNA, we chose tumor cells which grew readily in chick liver following i.v. injection. We have shown previously that murine B16F1 melanoma cells are of this phenotype and, in fact, grow rapidly enough to form macroscopic tumors in many chick organs within 7 days of injection (5). As described above, human cancer cells cannot be detected by the ouabain-plating assay; thus, we chose a human tumor line that grew in chick sufficiently rapidly to form detectable tumors. Injection of IGR37 human melanoma cells produced small tumors, visible by eye, in liver 7 days after injection. Fig. 1 shows histological sections of embryonic chick livers after injection of (a) IGR37 human melanoma cells, (b) B16F1 murine melanoma cells, and (c) LTA cells. Significant tumor cell growth is observed with both the melanoma cell lines, and these were used as sources of donor DNA in transfection studies.

DNA Transfection and Selection for Increased Ability to Grow in Chicks. Cells were obtained from 5 independent DNA transfection experiments and were tested for altered ability to grow in chicks after i.v. injection. These experiments are summarized in Table 1, and details of the sequential cycling through chicks are presented in Chart 2c (as mentioned above) and in

Chart 3. In Experiments 1 to 4, cells were obtained that were clearly above the LTA background threshold determined above (10^6 cells recovered per liver 7 days after i.v. injection of 5 x 10^6 cells) after one or 2 enrichment cycles through chicks. This enhanced growth ability was observed with both B16F1 (Experiment 1) and IGR37 (Experiments 2 to 4) DNA. In addition, both
cotransfection with genomic DNA plus cloned TK DNA and preselection in HAT medium (Experiments 1 to 3) and transfection with genomic DNA alone (Experiment 4) produced cells with enhanced growth ability, suggesting that the TK gene is not responsible for the altered growth phenotype. This conclusion is consistent with the observation (above) that transfection with TK DNA alone (without genomic carrier) or cotransfection with LTA genomic DNA and TK DNA failed to alter the LTA growth phenotype.

Increased numbers of cells recovered from liver 7 days after injection could be due to an increase in growth ability or to increased trapping of cells in liver. We have shown previously that the percentage of cells trapped initially in liver for a variety of cell types is between 0.1 and 2.0% of the original inoculum (5). In order to determine if the phenotypic alteration observed following DNA transfection is an alteration in growth ability or in initial trapping efficiency, we examined the kinetics of cell number recovered from liver over time following injection (Chart 4). Cells transfected with B16F1 DNA and cycled 4 times through the chick clearly have enhanced growth ability in chicks relative to control LTA cells, although they grow at similar rates in vitro (data not shown). Both cell types are found in equal numbers in liver immediately following injection (~1.0 to 2.0% of the original inoculum). LTA cells subsequently decline in number in liver, while numbers of B16F1-transfected cells increase, with a doubling time in chicks of ~50 hr [B16F1 cells proliferate with a ~16-hr doubling time in the chick (5)]. Cells transfected with IGR37 DNA were also trapped in chick liver at ~1.0 to 2.0% of the original inoculum (data not shown). Kinetic assays on these cells, however, were not reproducible because of the instability of their altered phenotype. Within 1 to 2 weeks of in vitro growth (in HAT medium for cells cotransfected with TK) following selection in the chick, cells transfected with human IGR37 DNA reverted to a nearly normal LTA growth phenotype with only an occasional embryo containing more than background numbers of rodent cells. In contrast, the enhanced growth phenotype was fairly stable in cells transfected with murine B16F1 DNA. The requirement for enrichment cycling in chicks for detection above background levels, coupled with the phenotypic instability of the human transfectants, does not allow for precise determination of transfection frequency. However, since all transfection experiments with B16F1 and IGR37 DNA yielded positive results, transfection frequency must be at minimum one positive colony per transfection plate (of 20 to 30 µg genomic DNA and 5 x 10⁶ LTA cells).

**DISCUSSION**

We have described here an increase in the ability of LTA cells to grow in chick embryos following i.v. injection, mediated by transfection of genomic DNA from 2 sources, a human and a murine melanoma cell line. These experiments demonstrate the feasibility of using the chick embryo in an in vivo assay for quantitatively detecting small changes in growth ability resulting from DNA transfection. The assay described here thus offers the potential for detecting and identifying genes that are involved in malignant progression.

This assay has advantages over other "experimental metastasis" assays in testing cells modified by DNA transfection. The immature immune system of the chick embryo is relatively tolerant of foreign cell surface antigens which may be expressed on cells following heterologous DNA transfection. Furthermore, altered cells can be readily recovered from the embryo as viable clones for further study. Finally, the ouabain-plating assay is sufficiently sensitive to quantitatively detect subtle differences in growth ability after i.v. injection, even if such differences are quite small. We demonstrate here that this assay is sufficiently sensitive to discriminate between cells that decline in number in liver after injection and cells that grow with a relatively slow apparent doubling time of ~50 hr, a division rate insufficient to produce visible tumors (macro- or microscopic) during the 7- to 10-day assay time.

The increase in growth ability of the transfectants is likely to be the result of specific gene(s) from the donor DNA, since the transfection procedure alone (using genomic LTA DNA) did not produce an enhanced growth phenotype. Preliminary Southern blots on cells transfected with human IGR37 DNA have detected some human DNA in these cells. However, the lack of stability of the enhanced growth phenotype in the cells transfected with human DNA will make characterization of the transfected sequences difficult. Secondary transfections of DNA from cells with increased growth ability into LTA cells have thus far failed to yield cells with increased growth ability (data not shown), perhaps indicative of (a) a large genetic target size in the primary transfections, or (b) loss of the transfected human DNA sequences from murine cells. Transfection of cloned bacterial genes into mammalian cells has been reported to result in unstable phenotypes (4). It is intriguing that the altered growth phenotype reported here is stable in the interspecies transfectants (murine B16F1 DNA into murine LTA cells), while the interspecies transfectants (human IGR37 DNA into murine LTA cells) are relatively unstable. Recently, Schaefer et al. (20) have reported similar instability of a different phenotype (growth in soft agar) in transfection with human lymphocyte DNA into mouse NIH/3T3 cells. These observations with DNA transfection studies parallel those...
obtained for chromosome retention in inraspecies and interspecies hybrids (6). It is interesting to speculate that these examples of instability may reflect a basic phenomenon of interspecies DNA instability.

Tumor progression from normal to highly malignant phenotypes is a complex process thought to involve interplay between a series of genetic changes. Attempts to identify some of these changes have been the subject of much recent work (e.g., the identification of oncogenes; see Refs. 3, 19, and 22 for reviews), and the role of such changes remains subject to much speculation. The assay used here, permitting in vivo assessment of tumor cell properties coupled with the capability for detailed quantitation of cellular growth ability, should provide a useful tool in the understanding of in vivo properties of cells at different stages of tumor progression and should permit identification of genetic changes (in a variety of species) responsible for altered phenotypes. We have begun recently an examination of the role of oncogenic viral sequences in growth in the chick embryo following i.v. injection. These studies make use of a further advantage of this assay, namely, the ability of the embryos to be maintained at different temperatures, permitting testing of cells bearing temperature-sensitive mutations.

The specific nature of the transfected gene(s) responsible for the phenotypic alteration observed here remains to be described, although such genes appear to be conserved across species, since DNA from both mouse and human cells can alter the growth properties of mouse cells when tested in chicken embryos. In this system, the percentage of an inoculum that is initially arrested in embryonic liver is relatively constant for both tumorigenic and normal cell types. Differences between cell types thus result from differential ability to increase in number in liver following initial arrest. Factors important in this process might include ability to escape from the circulation, ability to grow in the new 3-dimensional tissue structure, and responses (or non-responses) to nutritional or hormonal factors in the circulation. Any gene that is responsible for controlling either the survival of a tumor cell or its subsequent growth in animals is of potential interest in our understanding of tumorigenicity and metastasis. Regardless of the nature of the gene(s) responsible for the phenotypic change reported here, these experiments suggest that the assay used here has potential value in the study of genes, from a variety of species, contributing to the malignant phenotype.

REFERENCES

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Fig. 1. Histological sections of 18-day embryonic chick livers 7 days after CAM vein injection of IGR37 human melanoma cells ($10^6$ cells/embryo) (a), B16F1 murine melanoma cells ($10^6$ cells/embryo) (b), and LTA cells ($10^6$ cells/embryo) (c). Examples of tumor growth in a and b are marked with arrows, while livers injected with LTA (c) showed no signs of tumor growth and could not be distinguished from uninjected control livers. H & E.
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