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Comparison of the Growth Curves of Cancer Cells and Cancer Stem Cells

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Abstract: A fundamental problem in cancer research is identification of the cells responsible for tumor formation. The latest field of cancer research has revealed the existence and role of cancer stem cells (CSCs). These findings support the idea that malignancies originate from a small fraction of cancer cells that show self-renewal and multi- or pluripotency. Identification of this CSC population has important implications for the management of cancer patients, including diagnostic and predictive laboratory assays as well as novel therapeutic strategies that specifically target CSCs. In this study, we investigated the growth rates of CSC populations for comparison with cancer cell lines. To construct the growth curves, blood-derived CSCs were isolated from patients with breast, colon, or lung cancer and cultured *in vitro*. Quantitative real-time PCR was then performed to identify CSCs in the samples. We found that CSCs did not follow the common pattern of a typical growth curve of mammalian cells in contrast to the cancer cell lines. This observation of rapidly growing CSCs indicates their involvement in tumor formation.

Keywords: Cancer stem cells, growth curves, Nanog, Oct3/4, Sox2.

INTRODUCTION

Tumor metastasis is a major cause of cancer morbidity and mortality. However, the precise mechanisms underlying metastasis development remain poorly understood. Metastasis involves migration of malignant cells from a primary tumor to distant sites where these cells establish secondary tumors. Circulating tumor cells (CTCs), which were first detected in the blood of a cancer patient in 1869, are thought to represent tumor cells in transit, and some of which will result in metastases [1]. These cells are capable of intravasation from a primary tumor, undergoing phenotypic alterations that enable intravascular survival, extravasation from the blood vessel, implantation into a target tissue, and proliferation to form a tumor metastasis [2]. CTCs can be detected and enriched by various methodologies. Therefore, CTC analyses may be considered as a real-time "liquid biopsy" in cancer patients. This biopsy allows characterization of specific subpopulations of CTCs and may revolutionize cancer detection and management [3]. According to Cohen *et al.*, 2008, CTCs can serve as a prognostic factor for patients with metastatic colorectal cancer [4]. The ability to identify patients with poor prognoses and those who will progress quickly may have broad clinical implications.

There is increasing evidence that a subset of CTCs has stem-like characteristics. A large body of research has described stem cells in normal tissues, which are capable of self-renewal through asymmetric cell division while generating committed progenitor cells whose descendants may eventually differentiate and carry out tissue-specific functions [5]. More recently, studies of neoplastic tissues have provided evidence that a population of cells within a tumor has the ability to self-renew. This population has been characterized as cancer stem cells (CSCs). CSCs constitute a small minority of neoplastic cells and are defined by their ability to generate new tumors. For this reason, they have also been termed "tumor-initiating cells" [6].

Identification of the stem cell population in a blood sample appears to be crucial. The CSC model has strong translational and clinical relevance, because it can explain many of the features of resistances against cancer therapies [7]. Furthermore, characterization of this stem cell population may identify potential targets for prospective therapies. In this study, the growth rates were compared between cancer cell lines and CSCs.

MATERIALS AND METHODS

Cell lines and culture conditions. The following carcinoma cell lines were obtained from the European Collection of Cell Cultures (UK): T47D and MDA- MB 231 representing breast cancer, HCT-116 and HT55 representing colon cancer, and CALU-1 and COR-L 105 representing lung cancer. All cell lines were cultured in the appropriate cell culture medium with the appropriate amount of heat inactivated fetal bovine serum (Biochrom, S0615, UK) and 2 mM L-glutamine (Sigma, G7513, Germany) in a humidified incubator at 37°C with 5% CO_2. Media were changed at 90% confluence. Cells were harvested by trypsinization (0.25% trypsin/EDTA, Invitrogen, 25200-072, CA) during their exponential growth phase.

Blood sample collection. Cancer cells were obtained from female patients (40–63 years old) with breast, colon, or

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non-small cell lung carcinoma. All patients were at stage 4 of the disease and provided informed consent. Peripheral blood (20 ml) was collected from each patient and placed in a tube containing EDTA (Vacutainer, BD, 368860, USA) as an anticoagulant and then rotated for 30 min to prevent coagulation.

Lymphocyte isolation. Whole blood cells were layered over Biocoll separating solution 1077 (Biochrom, L6115, UK). Peripheral blood mononuclear cells (PBMCs) were collected after density gradient centrifugation, washed with phosphate buffered saline (Sigma, P3813), and then cultured in the appropriate medium until reaching a sufficient number to isolate CSCs.

Microsphere formation assay. This assay is based on the ability of CSCs to form microspheres and was used demonstrate CSC-like cells among CTCs. Light microscopy was used for assessment and evaluation.

Growth curves. To construct growth curves, the cells were cultured under the appropriate conditions for 10 days. CSCs were cultured in DMEM/F12 (Invitrogen, 31331-028, CA) supplemented with epidermal growth factor, (Sigma, E9644) [8], fibroblast growth factor-2 (Sigma, F0291), leukemia inhibitory factor, (Chemicon, Millipore, ESG1106, Germany), and 2-mercaptoethanol (Gibco, 21985-023, CA). Leukemia inhibitory factor was used to prevent differentiation and maintain self-renewal [9]. Fibroblast growth factor-2 is as a potent inhibitor of tumor cell proliferation [10]. The cells were detached by trypsinization (0.25% trypsin/EDTA) every day and the total cell number was counted using a NC-100+ NucleoCounter system (Chemometec, Denmark) for the cell lines and a NEUBAUER slide for CSCs.

Quantitative real-time RT-PCR analysis. Quantitative real-time RT-PCR (qRT-PCR) was performed to evaluate the expression of "stemness" markers. Total RNA from human embryonic stem cells (ESCs) (Celprogen, 36101RNA, CA) was used as a reference for the gene expression levels of stemness markers. Total RNA was extracted from CSCs using the TRIZOL reagent (Invitrogen, 15596-026). Genomic DNA was removed from the RNA preparations by DNase treatment (RNase-Free DNase Set, Qiagen, 79254, Germany). cDNA was prepared from 1 μg total RNA by reverse transcription using a first strand cDNA synthesis kit (Fermentas, K1612, Canada) and Oligo (dT) primer. The cDNA was used as a template for qRT-PCR using Maxima SYBR Green qPCR Master Mix (Fermentas, K0222) and specific primers designed with Gene Expression 1.1 software (Table **1**). The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, and extension at 72°C for 45 sec. A final extension step was performed at 72°C for 10 min followed by melting curve analysis.

Statistical analysis. The present study used random sampling between patients with different types of cancer. qRT-PCR results were evaluated according to the Kolmogorov-Smirnov test for distribution. All samples had a normal distribution.

RESULTS

The growth curves of the cancer cell lines and CSCs from the three types of cancer are shown in (Fig. **1**).

Table 1. Primer sequences.

Gene	Primer Sequence (5'–3')
18S	Forward:TGCCCTATCAACTTTCGATGGTAGTC
	Reverse: TTGGATGTGGTAGCCGTTTCTCA
Nanog	Forward:CGTGTGAAGATGAGTGAAACTG
	Reverse: GGATGGGCATCATGGAAA
Oct3/4	Forward: AGGAAGCTGACAACAATG
	Reverse: ACTCGGTTCTCGATACTG
Sox2	Forward: CTCGCCCACCTACAGCAT
	Reverse: GCTGGCCTCGGACTTGAC
CD34	Forward: GCATCACTGGCTATTTCCT
	Reverse: TCCGTGTAATAAGGGTCTTC
Nestin	Forward: GAGACACCTGTGCCAGCCTTTCTTA
	Reverse:CTGGGCTCTGATCTCTGCATCTACAG

The presence of CSCs among the cultured CTCs isolated from patient whole blood was demonstrated using cellular- and molecular-based methods. Microsphere formation in semi-suspension, which is the result of the ability of CSCs to form spherical colonies, is shown in Fig. **2** [11]. The gene expression of representative stemness markers was evaluated by qRT-PCR to demonstrate the stem-like character of the cultured cells. Fig. **3** shows the relative expression of all molecular markers compared with that in ESCs according to the Livak method [12].

DISCUSSION

Generation of a growth curve can be useful to evaluate the growth characteristics of a cell line. The lag phase indicates the population doubling time and saturation density. A growth curve of mammalian cells in suspension culture is a semi-logarithmic sigmoidal plot of cell density as a function of time (Fig. **4**). Segment 1 of the curve is called the "lag phase" of growth. Cells in this phase undergo little or no division (i.e., the slope of the growth curve for the lag phase is approximately 0). In segment 2, the slope of the growth curve changes from 0 to a positive value as the cells enter the terminal lag phase. In this phase, cell division has begun and its rate is increasing constantly. When the slope of the curve reaches a maximum value (segment 3), the cells are in the exponential growth phase and are undergoing division at their maximum rate. The cells continue growing at this rate until they enter the "early stationary phase" (segment 4). In this phase, the cell proliferation rate decreases, probably because of nutrient depletion or accumulation of deleterious waste products. The cells will eventually enter the stationary phase of growth (segment 5), where the slope of the curve once again approaches 0. If the cells remain in the stationary phase for too long, cell division ceases, resulting in a net decrease of cell number [13].

In our study, the growth curves of cancer cell lines followed the above-mentioned sigmoidal plot, in contrast to

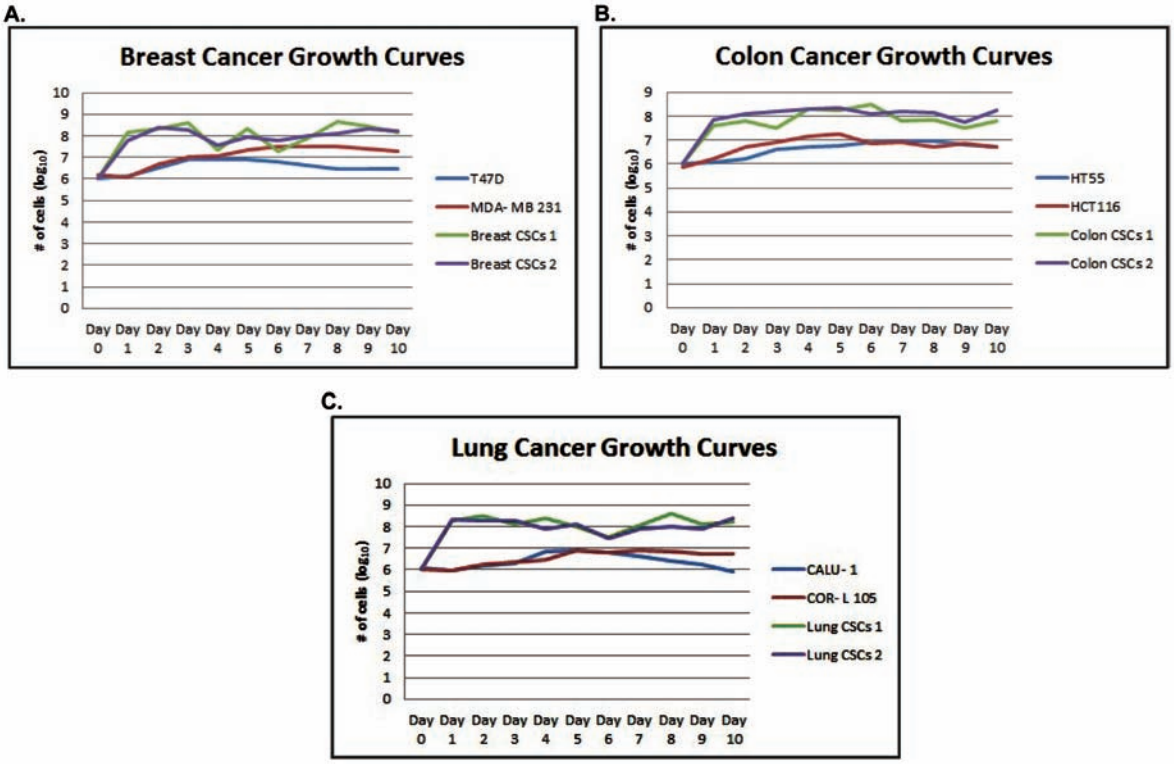

Fig. (1). Growth curves of each cancer type.

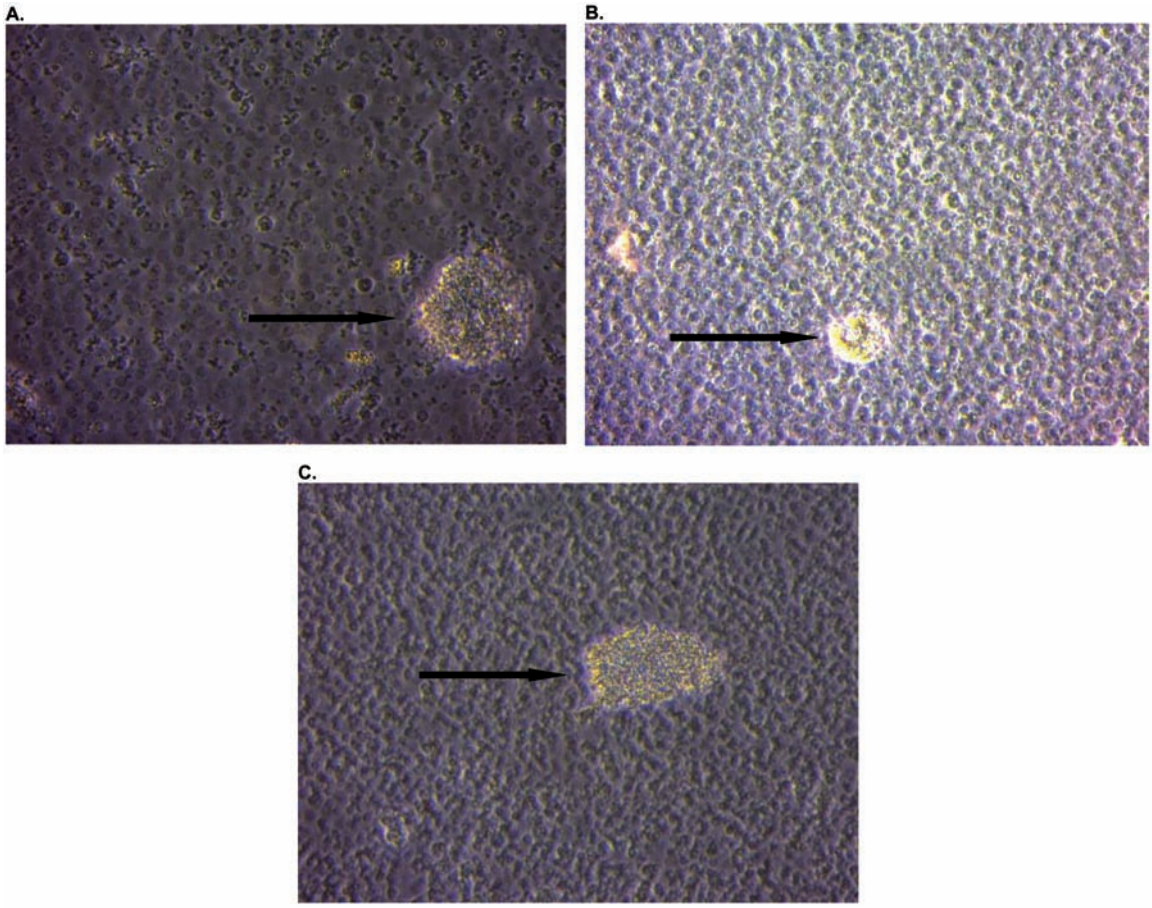

Fig. (2). Representative microspheres (indicated by arrows) in semi-suspension under an inverted light microscope. Breast, lung, and colon CSCs are shown (**A**, **B**, and **C** respectively).

CSC growth curves that generally did not follow a particular growth pattern. CSCs reached their maximum cell number earlier than that of the cancer cell lines. This characteristic resembles the rapid growth of a tumor and can explain the role of CSCs as tumor-initiating cells, and indicates their involvement in metastasis. Although the CSCs did not grow at the same rate as the other cancer cells, at least for lung and breast CSCs, the growth curves appeared to follow a common pattern. This pattern showed fluctuations in total cell number. To confirm this growth pattern, more growth curves must be obtained from cells harvested from male and female patients at different stages of each of the three types of cancer. It should be shown whether the growth pattern obtained in this study applies to all disease stages or just stage IV.

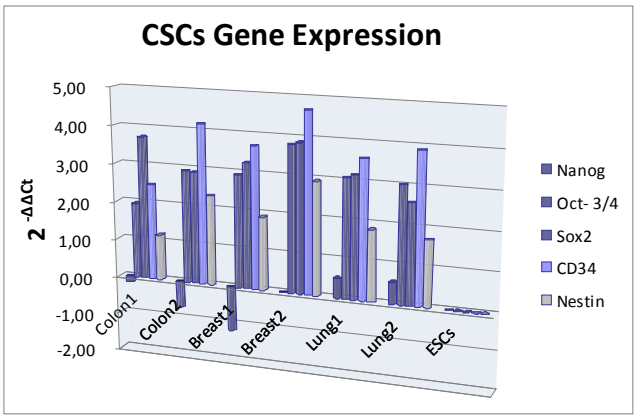

Fig. (3). Quantitative real-time PCR results.

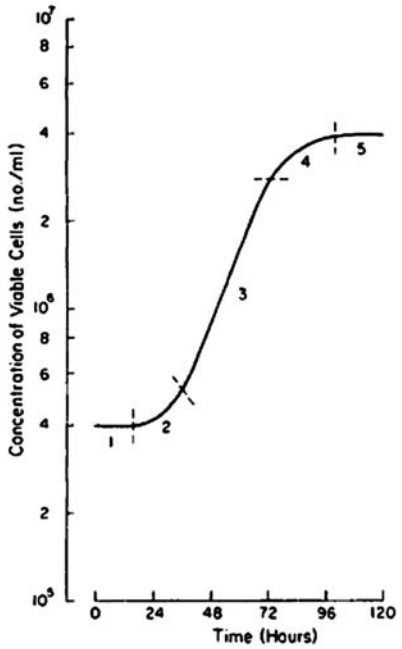

Fig. (4). Typical growth curve of mammalian cells.

Researchers have tried to use growth curve analysis in clinical studies. The effects of various drugs on tumor cell proliferation can be studied in growth curve analyses. By treating cancer cells with candidate drugs, it is possible to examine the percentages of live and dead cells at various times points. It has been shown that most drugs are often present at cytotoxic concentrations within a few hours following administration. However, lower concentrations that inhibit cell proliferation may be present for longer, but this effect is likely to be short compared with the interval between treatments.

Although growth curve analysis has proved to be a useful tool in research and clinical development, it is still based on an imperfect model of tumor growth. The fact that growth curves do not take into account the changes in proliferative rates, which might occur after individual cycles of treatment, often without changes in tumor volume, makes it a technique with some limitations. Furthermore, it is not always easy to measure the proliferation of cells from tissue samples or very small cells. Other methods need to be combined with growth curve analysis to overcome its limitations.

We examined the gene expression of stemness markers Nanog, Oct3/4, Sox2, Nestin, and CD34 [14-16]. Nanog is a transcription factor that plays an important role in the self-renewal of undifferentiated ESCs. Its function is correlated with other factors such as Oct3/4 [also known as POU5F1 (POU class 5 homeobox 1)]. The regulation and maintenance of the pluripotent state are areas of intense research, and it is clear that Oct3/4 and Nanog are key regulators in these processes [17, 18]. Oct3/4 is initially active in the oocyte, and it remains active even during the preimplantation period. It forms a dimmer with Sox2 and binds to DNA. At least part of their function in pluripotent cells is mediated via a synergistic interaction between them to drive transcription of target genes. Currently known targets of Sox2-Oct3/4 synergy are Fgf4, Utf1, and Fbx15, as well as Sox2 and Oct3/4 themselves [19]. Studies have claimed that this dimmer activates the Nanog protein, and this molecular cascade is responsible for the capability of tumor cells to differentiate into any cell type of the three germ layers (pluripotency) [20-22]. Another early expressed protein during development is Nestin. It is not expressed in adults because Nestin is replaced by tissue-specific intermediate filament proteins. We also examined expression of the gene encoding the hematopoietic progenitor cell antigen CD34. Proteins of the CD34 family are expressed by a variety of pluripotent cells and tissue stem cells. All CD34 family members are expressed by hematopoietic stem cells and/or more differentiated multipotent hematopoietic progenitors. It is possible that this family has a specific role in stem cell behavior, and several stem cell-related functions may be performed by anti-adhesion proteins that have a potent ability to establish apical domains [23-25].

CONCLUSIONS

Identification of the CSC subset among CTCs collected from blood samples of cancer patients is crucial. Investigation of CSCs may reveal novel targets that could overcome issues of drug resistance, improve therapeutic efficacy, and make cancer treatment more successful and perhaps even curative while obviating systemic toxicity [26]. Construction of a growth curve for comparison with existing curves is a simple and quick method to predict the existence of CSCs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Declared none.

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