Mouse sarcoma L1 cell line holoclones have a stemness signature

J. Miloszewski*, M. Gos*, M. Przybyszewska*, H. Trembacz*, M. Koronkiewicz† and P. Janik*

*Cell Biology Department, Maria Skłodowska-Curie Cancer Centre and Institute of Oncology, Warsaw, Poland and †Flow Cytometry Laboratory, National Medicine Institute, Warsaw, Poland

Received 2 March 2009; revision accepted 11 August 2009

Abstract

Accumulating data suggest that cancers contain a fraction of cells called cancer stem cells (CSCs), that may be responsible for upkeep and relapses of disease. In experimental settings, CSCs are regarded as most effective at tumour initiation in *in vivo* assays. Since the first isolation of cancer stem cells from acute myeloid leukaemia in 1994, cancer stem cells have been identified in human solid tumours and they have also been found in the established cell lines, based on ability of CSCs to form *in vitro* colonies of a specific morphology, called holoclones. Our study examined the ability of a mouse sarcoma cell line, derived from a lung metastasis of a BALB/c mouse and established as a stably growing line (L1), to produce holoclones *in vitro*. We aimed to verify a stemness signature of the holoclone cells. The L1 cell line was found to form holoclone colonies *in vitro*, which were shown to contain a percentage of CSC-like cells. A fraction of the L1 cells was able to repopulate the original cell line, and presented an increased clonogenic and metastatic potential (18th passage). In addition, MTT assay and flow cytometry of the side population fraction revealed that these cells were more resistant to chemotherapeutic drugs than the original cell line, and over-expressed the anti-apoptotic genes, *GRP78* and *GADD153*. We conclude that mouse L1 sarcoma cell line contains CSC-like cells.

Introduction

Emerging evidence suggests that tumour expansion is driven by cancer stem cells, which make up a small

fraction of cells capable of self-renewal through asymmetric as well as symmetric division, and characterised by increased resistance to chemotherapeutic agents (1). Cancer stem cells share many properties with normal, tissue-residing stem cells, from which according to one of the hypotheses, they may arise following accumulation of somatic mutations and epigenetic changes under microenvironmental pressure. The cancer stem cell (CSC) hypothesis of carcinogenesis is an attractive explanation of functional heterogeneity that is commonly observed in solid tumours. This hypothesis assumes hierarchical organization of cells within a tumour, with a subpopulation of stem-like cells responsible for sustaining tumour growth (2,3).

The first evidence of cancer stem cell existence was demonstrated in 1994 in Dick's laboratory, where this rare population of cells was isolated from AML patients. These cells, when injected into NOD/SCID mice, formed leukaemic blasts (4). The first report on stem cells in solid tumours was published in 2003 by Clarke and colleagues, who isolated and characterised CSCs from breast tumours (5). Since then, cancer stem cells have been found in many other solid tumours, including brain, colon, pancreas, prostate and lung cancers (6–10). CSCs have also been shown to be present in established, immortalised cell lines and can be separated from the general population by flow cytometry, either with the use of specific antibodies, or as a small side population (SP) with high Hoechst 33342 dye efflux, related to their multidrug resistance properties. Another successful method of CSC separation is based on their ability to form dense colonies of a specific morphology, called holoclones (11, 12). Holoclones obtained from different cell lines contained cells with cancer stem cell characteristics (11–13).

In our study, a mouse L1 sarcoma cell line derived from BALB/c lung metastasis, and established as a stably growing line (14), was examined. Based on morphological criteria, holoclone and paraclone cell populations were separated. Further analysis of biological properties

Correspondence: J. Miloszewski, Cell Biology Department, Maria Skłodowska-Curie Cancer Centre and Institute of Oncology, WK Roentgena 5, 02-781 Warsaw, Poland. Tel.: 48225462472; Fax: 48226449184; E-mail: joannam@coi.waw.pl

of the holoclone-forming cells revealed their stemness signature.

Materials and methods

Cell culture and isolation of holoclones and paraclones

The L1 sarcoma cells derived from BALB/c lung metastasis (14) were cultured in DMEM supplemented with 10% foetal bovine serum and standard antibiotics. To study colony morphology, subconfluent cells after trypsin/EDTA treatment, were seeded at low density in Petri dishes and colony formation was observed. Colonies classified as holoclones or paraclones were observed.

For the subsequent *in vivo* and *in vitro* experiments, cells were harvested in trypsin/EDTA and resuspended in a fresh medium to obtain low-density single-cell suspension. Then, 100 µl of this suspension was dispensed into each well of a 96-well culture plate and each well was observed under a microscope. The single-cell wells were observed daily. When a colony grew to confluence, cells were transferred to a flask, maintained nearly to confluence and frozen for further studies.

Evaluation of biological properties of the L1 holoclones

To assess the cells' ability to form colonies, 100 L1 cells or their holoclones (L1H) and paraclones (L1P) were suspended in culture medium and plated in Petri dishes. Cells were allowed to grow for 6 days and the number of colonies was scored after fixing in methanol and staining with Giemsa dye. To evaluate metastatic potential, 2×10^5 of L1, L1H or L1P cells were resuspended in 0.2 ml of 0.85% saline and injected intravenously into BALB/c mice. Cells of the 4th and 18th passages were injected into lateral tail vein. The mice were sacrificed 21 days after tumour cell inoculation, lungs were excised, fixed and stained. Lung tumour colonies were counted using a dissecting microscope.

Migration assay was carried out according to the method originally described by Repesh (15). Samples containing 2×10^4 of L1 cells or L1H and L1P in 200 µl of a serum-free medium were placed in the upper compartments of Costar Transwells and incubated for 16 h. Then cells from the lower parts of filters were fixed in methanol and stained with Giemsa dye. Cells from each filter were counted using phase-contrast microscopy and the results were expressed as a number of migrating cells/eight fields.

For determination of multidrug resistance, the MTT test was performed. Cells and their holoclones were cultured in RPMI-1640 medium without phenol red to 80% confluency and exposed to doxorubicin (1.5 µg/ml) for 24 h at 37 °C in humidified, 5% CO₂ atmosphere.

Then, MTT (3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide, Sigma, St Louis, MO, USA) was added and cells were incubated for 4 h; purple formazan crystals (which indicate mitochondrial dehydrogenase activity in metabolically active cells) were solubilized with acidified isopropanol. Absorption was measured at 570 nm using a microplate reader.

Cell proliferative activity

The L1 cells and their holoclones or paraclones were harvested by trypsinisation, washed once in phosphate-buffered saline (PBS) and re-suspended in an ice-cold PBS at a final concentration of 2×10^6 cells/ml. Cell suspensions were processed for Ki-67 expression according to the procedure described by Larsen (16). Briefly, 500 µl of permeabilizing DNA staining solution [PBS containing Nonidet P-40 (NP40) 0.5% v/v, propidium iodide (PI) 20 µg/ml, RNase 0.25 mg/ml, EDTA 0.5 mM, pH 7.2] was added to 1 ml of cell suspension for 15 min. Next, 40 µl of FITC-conjugated monoclonal Ki-67 antibody or FITC-conjugated control antibody (BD Biosciences-Pharmingen, San Diego, CA, USA) was added to cell suspensions and incubated for 30 min at 0–4 °C with gentle agitation. Labelled cells were analysed by multiparameter flow cytometry using FACS Calibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Hoechst 33342 SP cell analysis

L1 cells and their holoclones were resuspended in DMEM at a concentration of 1×10^6 cells/ml. After 90 min incubation at 37 °C with 5 µg/ml Hoechst 33342 (Sigma) alone or with verapamil (Isoptin; Abbott, Abbott Park, IL, USA) as a control, cells were centrifuged and resuspended in ice-cold PBS at a concentration of 10^6 cells/ml. For the flow cytometric analysis, FACS was configured for the dual emission wavelength analysis. To exclude debris, cells were gated based on forward and side light scatter. The SP cell gate was defined on the L1 cells. Analyses were carried out using FACS Vantage (Becton Dickinson).

Molecular studies

Total RNA was extracted from the L1 cells and from their holoclones using TRIzol reagent, according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed to cDNA using the Advantage RT-PCR Kit. Real-Time PCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on 7000 Real-Time PCR system (Applied Biosystems). Reaction mixtures

(25 µl) were subjected to a two-step programme: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analysed using ABI PRISM 7000 SDS Software. Relative fold difference in gene expression was calculated by $\Delta\Delta C_t$ method using *rRNA* gene as normalization control.

Poly A+ RNA was purified using Atlas Pure Total RNA Labelling System (Clontech, BD, Otsu, Shiga, Japan) according to the manufacturer's instructions. Global gene expression analysis was performed using Atlas mouse 1.2 cDNA expression array. To quantify intensity of hybridization, results were analysed using ImageQuant software and to identify differentially expressed genes, the AtlasImage program (Clontech, BD) was used.

L1H and L1P cells at the 4th passage were used throughout all experiments.

Results

To select the cancer stem cell-like population from the L1 cell line, a method based on colony morphology described by McKenzie (11) and Locke (12) was applied. After 5 days, the original L1 cells plated at a low density formed three types of colonies, holo-, para- or meroclones. The holoclones take the form of compact round colonies, paraclones form loose irregular colonies (Fig. 1a,b), while meroclones present intermediate features. The holoclones observed consisted of tightly packed, round, small cells, while paraclones contained larger, diffused cells (Fig. 1). Holoclones were cultured for several days before the L1H cells were collected. After several passages, these cells lost their initial morphological and phenotypical properties and were indistinguishable from the original cell line. Similarly, paraclones were propagated for several days to collect paraclone cells, L1P.

Biological properties of holoclone cells were compared to those of the original cell line and, when necessary, also with those of paraclones. Colony formation assay showed that the L1 holoclone cells formed more colonies than the original cell line (Fig. 2, $P = 0.0012$) and more colonies than paraclone cells. Experimental plates contained paraclones as well as holoclones (Fig. 2).

Proliferative activity of holoclone-forming cells was evaluated using the Ki-67 index. There was a trend of lower proliferative activity in holoclones than of original cells ($35.4 \pm 2.70\%$ versus $47.03 \pm 4.43\%$ Ki-67 positive cells from the total population), but this difference was not statistically significant ($P = 0.0944$). Paraclone cells had the same proliferative activity as native L1 cells ($42.1 \pm 4\%$ versus $47.03 \pm 4.43\%$ Ki-67 positive cells from the total population).

The L1H cells of the early, 4th passage, injected into the mouse lateral tail vein, produced significantly less

Figure 1. Holoclone (a) and paraclone (b) of L1 cell line. Magnification $\times 100$.

lung metastases (1.2 ± 0.4) than the control L1 line (18.6 ± 2.3). However, after 18 passages, holoclone cells changed their properties and formed many more lung metastases (32.0 ± 12.0) than either the original cell line or holoclones of the early passages (Table 1). Paraclones of the 4th passage produced nearly the same number of lung metastases as the L1 cells (12.9 ± 7.2 versus 18.6 ± 2.3), while cells of the 18th passage formed only 7.3 ± 1 metastases.

Figure 2. Plating efficiency of L1 cells and their subpopulations. Results expressed as mean number and type of colonies per dish $\pm$ SD.

Table 1. Number of lung metastases in the BALB/c mice after intravenous administration of the L1 and L1H cells

L1	L1H	L1P
18.6 $\pm$ 2.3 <i>n</i> = 14	4th passage 1.2 $\pm$ 0.4 <i>n</i> = 16	4th passage 12.9 $\pm$ 7.2 <i>n</i> = 14
	18th passage 32.0 $\pm$ 12.0 <i>n</i> = 7	18th passage 7.3 $\pm$ 1.0 <i>n</i> = 7

Results expressed as mean number of colonies per lung per mouse. *n*, number of repetitions.

In addition, holoclone cells tended to have lower migration properties (44 ± 5.3 cells/8 fields) compared to the original L1 cells (51 ± 9.2 cells/8 fields), but no statistical significance was found ($P > 0.05$).

The MTT assay showed that holoclone cells were less sensitive to doxorubicin treatment ($28.1 \pm 3.6\%$ of dead cells) than the original L1 cell line ($49.8 \pm 5.1\%$ of dead cells), while its paraclones had almost the same sensitivity to doxorubicin ($43.7 \pm 5\%$ dead cells) as that of L1 cells. We also analysed the size of the side population of the original L1 cells and its holoclones by flow cytometry, based on the ability of cells to efflux Hoechst 33342 dye. The efflux was blocked by incubation of cells with verapamil to confirm SP phenotype. The side population was estimated at 0.23% of the original L1 cell line and

increased to 0.34% in holoclones, while in paraclones it was only 0.02% (Fig. 3). The observed proportion of SP was not related to *MDR1* gene expression, as evaluated by the Real-Time PCR technique.

Molecular studies with the use of Atlas Mouse 1.2 cDNA expression array revealed several differences between the L1 holoclones and the original cell line. In particular, genes which encoded growth arrest and DNA damage-inducible protein 153 (not observed in original cell line), 78-kDa glucose regulated protein (Grp78; 39.7-fold difference), ezrin (7.21-fold), Cek5 receptor protein tyrosine kinase ligand (5.14-fold), cathepsin A (5.02-fold) and cathepsin D (4.63-fold) were found to have significantly elevated expression in the L1H cells.

Discussion

We aimed to investigate whether the L1 cell line contains a population of cancer stem cell-like cells. It is generally accepted that cancer stem cells, besides their renewal potential and an ability to differentiate, have higher resistance to unfavourable conditions, including influence of chemical agents (17). Therefore, SP in the L1 cell line was assessed. The SP is a small fraction of cells containing stem cells, distinguished by their ability to efflux Hoechst dye (18). Our L1 cell line was shown to comprise about 0.23% SP cells. This was the first indication that cancer stem cells may be present in this line.

In 2005, Locke and co-workers showed that cancer stem cells were also present in well-characterised human epithelium-derived cell lines (for example, DU-145, MCF-7, PC3). These cells were found to form specific types of colonies called holoclones, consisting of tightly packed, small cells. Therefore, we selected holoclones from our mouse sarcoma L1 cell line and characterised their biological and molecular features.

The holoclone cells were found to have higher clonogenic potential than cells of the original line and to form colonies of different size and shape, while the original cell line produced mainly meroclones and paraclones. This observation suggested that the holoclone cells had self-renewal potential, and that selected cells could repopulate the original L1 cell line. In addition, holoclones were found to contain a larger fraction of SP (0.34% versus 0.23% in the original cell line and 0.02% in paraclones). Therefore, selection of cells based on their colony morphology seemed to be a useful method to enrich the stem cell fraction. Moreover, the results of *in vivo* experiments showed that cells of the early passage holoclones formed no metastases or produced only single metastatic colonies in the lungs of BALB/c mice. After several passages, as L1H cells almost re-created the original cell line, their metastatic potential was significantly higher as compared to

Figure 3. L1, L1H and L1P cell lines' side populations. Cells were analysed for the Hoechst 33342 dye efflux. Cytograms show (a) L1 cells, (b) L1 cells with verapamil, (c) L1H cells, (d) L1H cells with verapamil, (e) L1P cells, (f) L1P cells with verapamil.

L1 control cells. A fraction of cancer stem cells present in holoclones seemed to be able to differentiate into progenitor cells, which might be responsible for higher metastatic potential of the cells after several passages.

Cancer stem cells, like adult stem cells, are slow-cycling cells. As indicated by Ki-67 antigen expression, holoclone cells showed lower proliferative activity than whole cell line or paraclone cells.

A further CSC-specific property of the selected holoclones was an increased resistance to chemical agents. The L1 holoclones were less sensitive to doxorubicin than the original cell line and the cells of paraclones.

Holoclone cells also had higher expression of genes coding for the anti-apoptotic proteins, GADD153 and GRP78 (19,20). Moreover, the level of the anti-apoptotic Bcl-2 protein was higher in holoclones than in control cells and in paraclone cells (data not shown), which suggests that holoclone cells presented more efficient anti-apoptotic mechanisms.

In conclusion, holoclones obtained from the mouse sarcoma L1 cell line contained cells which displayed several features of cancer stem cells. A selected fraction of these CSC-like cells was able to repopulate the original cell line, had higher clonogenic potential and was more resis-

tant to chemotherapeutic drugs. These results show that holoclone cells of the L1 cell line present a stemness signature. Thus, the L1 cell line provides a new model to study pharmacosensitivity of CSCs, both *in vitro* and *in vivo*.

References

- 1 Zhou J, Zhang Y (2008) Cancer stem cells. Models, mechanism and implication for improved treatment. *Cell Cycle* **7**, 1360–1370.
- 2 Adams JM, Strasser A (2008) Is tumor growth sustained by rare cancer stem cells or dominant clones? *Cancer Res.* **68**, 4018–4021.
- 3 Alison MR, Islam S, Lim SM (2009) Number crunching in cancer stem cells market. *Breast Cancer Res.* **11**, 302.
- 4 Lapidot T, Sirard C, Vormoor J, Mudroch B, Hoang T, Caceres-Cortes J *et al.* (1994) A cell initiating human myeloid leukemia after transplantation into SCID mice. *Nature* **367**, 645–648.
- 5 Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. USA* **100**, 3983–3988.
- 6 Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J *et al.* (2003) Identification of a cancer stem cell in human brain tumors. *Cancer Res.* **63**, 5821–5828.
- 7 Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ (2005) Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res.* **65**, 10946–10951.
- 8 O'Brien CA, Pollett A, Gallinger S, Dick JE (2007) A human colon cancer cell capable of initiating tumor growth in immunodeficient mice. *Nature* **445**, 106–110.
- 9 Li CH, Heidt DG, Dalerba P, Burant ChF, Zhang L, Adsay V *et al.* (2007) Identification of pancreatic cancer stem cells. *Cancer Res.* **67**, 1030–1037.
- 10 Eramo A, Lotti F, Sette G, Pilozi E, Biffoni M, Di Virgilio A *et al.* (2007) Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ.* **15**, 504–514.
- 11 MacKenzie IC (2005) Retention of stem cell patterns in malignant cell lines. *Cell Prolif.* **38**, 347–355.
- 12 Locke M, Heywood M, Fawell S, Mackenzie I (2005) Retention of intrinsic stem cell hierarchies in carcinoma derived cell lines. *Cancer Res.* **65**, 8944–8950.
- 13 Li H, Chen X, Calhoun-Davies T, Claypool K, Tang D (2008) PC3 human prostate carcinoma cell holoclones contain self-renewing tumor initiating cells. *Cancer Res.* **68**, 1820–1925.
- 14 Janik P, Bertram JS, Szaniawska B (1981) Modulation of lung tumor colony formation by a subcutaneously growing tumor. *J. Natl. Cancer Inst.* **66**, 1155–1158.
- 15 Repesh LA (1989) A new in vitro assay for quantitating tumor cell invasion. *Invasion Metastasis* **9**, 192–208.
- 16 Larsen JK, Christensen IJ, Christiansen J, Mortensen BT (1991) Washless double staining of unfixed nuclei for flow cytometric analysis of DNA and a nuclear antigen (Ki-67 or bromodeoxyuridine). *Cytometry* **12**, 429–437.
- 17 Dean M (2006) Cancer stem cells. Redefining the paradigm of cancer treatment strategies. *Mol. Interv.* **6**, 140–148.
- 18 Ho MM, Ng AV, Lam S, Hung J (2007) Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res.* **67**, 4827–4833.
- 19 Oyadomari S, Mori M (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.* **11**, 381–389.
- 20 Reddy RK, Mao C, Baumeister P, Austin RC, Kaufman RJ, Lee AS (2003) Endoplasmic reticulum chaperone protein GRP78 protects cells from apoptosis induced by topoisomerase inhibitors: role of ATP binding site in suppression of caspase-7 activation. *J. Biol. Chem.* **278**, 20915–20924.