A Novel HSV-1 Virus, JS1/34.5−/47−, Purges Contaminating Breast Cancer Cells From Bone Marrow

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Abstract

Purpose: Oncolytic herpes simplex virus type 1 (HSV-1) vectors show considerable promise as agents for cancer therapy. We have developed a novel recombinant HSV-1 virus (JS1/34.5−/47−) for purging of occult breast cancer cells from bone marrow of patients. Here, we evaluate the therapeutic efficacy of this oncolytic virus.

Experimental Design: Electron microscopy was used to determine whether human breast cancer and bone marrow cells are permissive for JS1/34.5−/47− infection. Subsequently, the biological effects of JS1/34.5−/47− infection on human breast cancer cells and bone marrow were established using cell proliferation and colony formation assays, and the efficiency of cell kill was evaluated. Finally, the efficiency of JS1/34.5−/47− purging of breast cancer cells was examined in cocultures of breast cancer cells with bone marrow as well as bone marrow samples from high-risk breast cancer patients.

Results: We show effective killing of human breast cancer cell lines with the JS1/34.5−/47− virus. Furthermore, we show that treatment with JS1/34.5−/47− can significantly inhibit the growth of breast cancer cell lines without affecting cocultured mononuclear hematopoietic cells. Finally, we have found that the virus is effective in destroying disseminated tumors cells in bone marrow taken from breast cancer patients, without affecting the hematopoietic contents in these samples.

Conclusion: Collectively, our data show that the JS1/34.5−/47− virus can selectively target breast cancer cells while sparing hematopoietic cells, suggesting that JS1/34.5−/47− can be used to purge contaminating breast cancer cells from human bone marrow in the setting of autologous hematopoietic cell transplantation.

High-dose chemotherapy is an aggressive treatment intended to completely eradicate neoplastic cells and is frequently used to offer improved relapse-free survival to high-risk patients with a spectrum of cancers, including breast cancer, germ cell tumors, neuroblastoma, and hematologic malignancies. However, high-dose chemotherapy is severely myelosuppressive and hence needs to be followed with autologous hematopoietic cell transplantation.

Advanced cancer (9–12), there is a risk that the patients harvested stem cells may be contaminated with disseminated tumor cells, which can directly contribute to relapse (13). In this context, a number of ex vivo purging strategies have been developed, to more effectively reduce the tumor burden in stem cell grafts and minimize treatment-associated causes of relapse. These include ex vivo chemotherapy (14), CD34+ (stem) cell enrichment (15), immunotoxins (16), immunomagnetic removal of tumor cells (17), and the use of cytotoxic herpes simplex virus (HSV; ref. 18) and adenoviruses (19). To date, the available ex vivo purging methods seem to be limiting and can compromise the functional integrity of the hematopoietic graft (20–23), resulting in reduced efficacy of this approach. Hence, there is a significant need for the development of new approaches to stem cell purging.

Oncolytic viruses genetically programmed to replicate within cancer cells and to directly induce toxic effects via cell lysis or apoptosis are currently being explored in the clinic. To date, only a small group of oncolytic viruses have been used clinically. These include engineered adenovirus, herpes, and vaccinia DNA viruses with tumor selectivity (and two wild-type simplex virus (HSV; ref. 18) and adenoviruses (19). To date, the available ex vivo purging methods seem to be limiting and can compromise the functional integrity of the hematopoietic graft (20–23), resulting in reduced efficacy of this approach. Hence, there is a significant need for the development of new approaches to stem cell purging.

The ability to exploit the unique features of HSV type 1 renders this virus an attractive tool for cancer therapy and offers
a number of significant advantages over other viral vectors. Specifically, HSV-1 infects a broad range of cell types with high efficiency and is cytolytic by nature (i.e., the replicative life cycle of the virus results in host cell destruction). Further, the well-characterized large genome of HSV-1 contains many non-essential genes that can be replaced (up to 30 kb) with multiple therapeutic transgenes. Furthermore, the virus remains episomal within the infected cells, precluding the risk of insertional mutagenesis. Additionally, many antiviral drugs are available as a safeguard against unfavorable replication of the virus (24).

Attenuated, replication-competent HSV vectors that replicate in dividing cells and result in cell death with in situ viral spread are available but are generally incapable of replicating in normal tissue (25). To improve the use of such viruses, we have constructed a novel recombinant HSV-1 vector (JS1/ICP47/C0), featuring deletions of the ICP34.5 and ICP47 genes and, consequently, shows tumor-selective replication and enhanced antigen presentation in HSV-infected cells (26). Furthermore, JS1/ICP47/C0 incorporates a mutation to increase the expression of the HSV US11 gene, to enhance replication of HSV ICP34.5 mutants in tumors (26). Additionally, JS1/ICP47/C0 exhibits enhanced oncolytic activity, as it is based on a more potent clinical isolate of HSV (JS1) rather than the attenuated laboratory strains previously used by others (26). In the present study, we have examined the cytolytic effects of JS1/ICP47/C0 on a spectrum of human breast tumor cell lines and have evaluated the efficacy of JS1/ICP47/C0 as an ex vivo hematopoietic cell-purging agent.

Materials and Methods

Cells. Human breast cancer cell lines MDA-MB-231, MDA-MB-453, MCF-7, T47D, ZR-75-1, and Cal51 were obtained from the American Type Culture Collection (Manassas, VA). HBbSV161, normal human mammary epithelial cells immortalized with SV40 T-antigen, were a generous gift from Prof. M O’Hare (Ludwig Institute, London). All cell lines were grown in DMEM supplemented with 10% FCS, 10 mmol/L L-glutamine, and 100 units/mL penicillin and 200 µg/mL streptomycin. The cells were maintained in a humidified atmosphere at 37°C and 5% CO₂.

Virus. All viruses used in this study (JS1 WT, JS1/ICP34.5/C0/ICP47/C0, and JS1/ICP34.5/C0/ICP47/C0/GFP) are based on the clinical JS1 strain of HSV1 and are schematically presented in Fig. 1. All viruses were provided by BioVex Ltd. (Oxford, United Kingdom) as purified, high-titer viral stocks.

Bone marrow cell preparation. Bone marrow samples were obtained from high-risk breast cancer patients. Bone marrow samples were diluted in HBSS, layered on Ficoll-Paque Plus, and centrifuged for 30 minutes at 2,100 × g. The mononuclear enriched cell interface was extracted, washed twice in PBS, and resuspended in red cell lysis buffer [0.155 mol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L EDTA (pH 7.4)] for 2 minutes to remove RBC. The remaining bone marrow cells were then pelleted at 1,000 × g for 10 minutes at 4°C, washed in PBS, and counted. Bone marrow cells were cultured in Myelocult long-term culture medium (Stem Cell Technologies, London, United Kingdom) and supplemented with 20 µg/mL human granulocyte macrophage colony-stimulating factor, 10 ng/mL stem cell factor, and interleukin-3.

Purification of primary human breast tumor cells. Primary breast tumor cells were purified according to the method of Kothari et al. (27). Briefly, fresh breast tumor tissue was cut up and digested at 37°C with type IA collagenase (1 mg/mL) in RPMI 1640 supplemented with 5% FCS and 2 mmol/L L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 50 units/mL polymyxin B, and 2.5 mg/mL amphotericin B for 2 to 5 hours. Undigested tissue was removed by filtration using a 50-µm pore followed by a 28-µm pore nylon mesh. The tumor epithelial cells were purified using superparamagnetic, polystyrene beads (Dynal Biotech, Bromborough, United Kingdom) coated with a mouse IgG1 monoclonal antibody (Ber-EP4) specific for two (34 and 39 kDa) isoforms of the EP4 glycopolypeptide membrane antigen expressed on the cell surface of mammary epithelial cells and then cultured in breast cancer culture media consisting of DMEM/F-12 (1:1) supplemented with 5% FCS, 5 mg/mL insulin, 10 mg/mL apotransferrin, 100 µmol/L ethanolamine, 1 µg/mL hydrocortisone, 10 ng/mL epidermal growth factor, 15 mmol/L HEPES, 2 mmol/L L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 50 units/mL polymyxin B, and 2.5 mg/mL amphotericin B. Purified primary breast cancer cells were cultured for at least 48 hours before use.

Viral infection of breast cancer cell lines. Breast cancer cell lines were seeded at 1 × 10⁵ well in six-well dishes and infected after 24 hours with JS1/ICP47/C0 at multiplicity of infections (MOI) of 0.01, 0.1, and 1. The virus was removed after 2 hours, and the cells washed twice with PBS. At 24, 48, and 72 hours after infection, the medium was removed, and the viable adherent cells were counted using a hemocytometer and trypan blue used to identify viable cells. Control cells were mock infected with serum-free DMEM. The assay was done in duplicate.

Viral infection of human bone marrow cells. Freshly prepared bone marrow cells were infected in suspension using virus at MOIs of 0.1 or 1. The cells were then incubated for 2 hours with intermittent rocking. The virus was removed by centrifuging, and the cells washed twice with PBS twice at 1,500 × g. The cells were then used in granulocyte macrophage colony-forming unit assays or long-term culture. Control cells were mock infected with serum-free DMEM.

Viral infection of purified primary breast tumor cells. Purified primary breast cancer tumor cells were cultured for at least 48 hours before infection with JS1/ICP47/C0/GFP at MOI 1. Control cells were
mock infected with serum-free DMEM. At 24, 48, and 72 hours, the cells were examined by fluorescence microscopy for green fluorescent protein (GFP) expression. Viable cells were counted on a hemocytometer using the trypan blue exclusion assay.

**Clonogenic assay.** Granulocyte macrophage colony-forming unit assays were carried out in triplicate using Methocult, a methylcellulose medium. After infection, 1 x 10^5 bone marrow cells were suspended in 1 ml of Methocult H4230 supplemented with 100 μL of cytokine mix [stem cell factor, granulocyte colony-stimulating factor, interleukin-3, and granulocyte macrophage colony-stimulating factor and 1.5 μL (3 units) of erythropoietin], plated onto 35-mm dishes, and incubated in a humidified atmosphere at 37°C with 5% CO2. The plates were scored at days 7 and 14 for myeloid colonies (granulocyte macrophage colony-forming unit) and day 14 for erythroid colonies (burst-forming unit-erythroid).

5-Chloromethylfluorescein diacetate labeling of MDA-MB-231 cells. CellTracker 5-chloromethylfluorescein diacetate (CMFDA), chloromethyl derivative of fluorescein diacetate (Molecular Probes, Leiden, the Netherlands) was used for long-term tracing of MDA-MB-231 cells. CMFDA passes freely through the cell membrane into the cytoplasm where it undergoes a glutathione S-transferase-mediated reaction producing a cell-impermeable reaction product. The cells were stained in suspension according to manufacturer’s instructions. Briefly, MDA-MB-231 cells were resuspended 200 μL serum-free DMEM containing 5 μmol/L CMFDA and incubated under normal culture conditions for 30 minutes, with intermittent rocking. The cells were then resuspended in serum-free medium for 30 minutes and washed twice in PBS.

Purging cancer cells from cocultures of bone marrow and breast cancer cells. CMFDA-labeled MDA-MB-231 cells were added to freshly prepared bone marrow cells from high-risk breast cancer patient at ratios of 10%. Cocultures were then infected with JS1/34.5 /47 at MOI 0.1 as described above. At 0, 3, and 6 days after infection, samples were harvested for data acquisition by fluorescence-activated cell sorting (FACS) analysis. Control cells were mock infected with serum-free DMEM.

**FACS analysis.** MDA-MB-231 cells were labeled with CellTracker green CMFDA as above. Dead cells were identified by the addition of 50 nmol/L TO-PRO-3 (Molecular Probes) immediately before analysis. Samples were analyzed on a dual laser FACS Calibur (Becton Dickinson, San Jose, CA) with a blue laser (488 nm) used to excite CMFDA and a red diode laser (635 nm) used to excite TO-PRO-3. CMFDA fluorescence was detected using a 530/30 band-pass filter in front of the detector, and TO-PRO-3 fluorescence was detected with a 660/16 band-pass filter. Debris and cell aggregates were excluded based on their forward and side scatter characteristics. At least 50,000 events per sample were collected, and data were analyzed using CellQuest software (Becton Dickinson). The percentage of CMFDA and unlabeled cells that were live and dead was calculated for each sample.

Electron microscopy of MCF-7 cells. Adherent cells were grown on sterilized fibronectin-coated Thermaxx coverslips (Agar Scientific Ltd., Stanstead, United Kingdom) for 24 hours. The cells were infected at ~70% confluence and processed for microscopy at the indicated time points. Specifically, the cells were fixed in 3% glutaraldehyde in 0.1 mol/L cacodylate buffer for 30 minutes and post-fixed in 1% osmium tetroxide. The cells were then dehydrated through ascending concentrations (50-100%) of ethanol and embedded in Spurr’s resin. The Thermaxx coverslip was then removed before cutting, and ultrathin sections (60-80 nm) were stained in uranyl acetate and Reynold’s.
lead citrate. Electron microscopy and interpretation of data were done by Dr. J. Moss (Division of Investigative Science, Imperial College).

**Reverse transcription-PCR analysis for CK19 gene expression.** Freshly prepared bone marrow cells (1 × 10⁶) from high-risk breast cancer patients were infected with JS1/34.5 /47 as describe above and maintained in culture for 6 days. Control cells were mock infected with serum-free DMEM. Total RNA was harvested from each sample, and cytokeratin 19 (CK19) mRNA levels were analyzed by real-time reverse transcription-PCR (RT-PCR). Control RT-PCR analysis for mRNA dosage was carried out for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR was carried out according to the manufacturer’s protocols on an Applied Biosystems (Warrington, United Kingdom) 7700 Real-time PCR system, using Assay-On-Demand primers for CK19 and GAPDH.

**Results**

**Biological effect of JS1/34.5 /47 on breast cancer cell lines in vitro.** To evaluate the biological effect of JS1/34.5 /47 on human breast cancer cells, a series of breast cancer cell lines were infected with JS1/34.5 /47 at MOIs of 0.01, 0.1 and 1. The cell lines used represent estrogen receptor α–positive (T47D, MCF-7, and ZR-75-1) and estrogen receptor α–negative (MDA-MB-453, MDA-MB-231, and Cal51) tumor cell types. Additionally, normal human mammary epithelial cells immortalized with SV40 virus (HBrSV161) were also examined. The results presented in Fig. 2 show that irrespective of estrogen receptor status, all cells were infected with JS1/34.5 /47 and were killed efficiently. Moreover, at MOIs of 0.1 and 1, the virus seemed to cause immediate and complete inhibition of proliferation followed by cell death, as early as 48 hours after infection. Treatment of the cells with virus at MOI of 0.01 seemed to delay the onset of cell death to 72 hours and is accompanied with a limited initial proliferation. Nonetheless, nearly all the mammary cell lines examined exhibit 100% kill by 144 hours.

**Biological effect of JS1/34.5 /47 on bone marrow cells in vitro.** The biological effects of JS1/34.5 /47 on bone marrow cells were examined by assessing the effect of HSV infection on the clonogenic capacity of these cells. To this end, samples from six patients were either mock infected (vehicle only) or infected with JS1/34.5 /47−, JS1/34.5 /47−GFP, or JS1 WT at MOIs of 0.1 and 1. The clonogenic capacity of the cells was determined by evaluating granulocyte macrophage colony-forming unit and burst-forming unit-erythroid after infection. Figure 3 shows the results presented as mean colony-forming unit/burst-forming unit from the six patients expressed as a ratio relative to the mock-infected control value of 1. The data show that over a period of 14 days, the relative effects of JS1 WT, JS1/34.5 /47−, and JS1/34.5 /47−GFP were similar. On average, an ~20% decrease in the clonogenic capacity of the bone marrow cells was observed at MOI 0.1. However, statistical analysis (the paired t tests) of these results showed that these differences were not statistically significant (P > 0.05). In comparison, the clonogenic potential of the cells was more severely affected at MOI of 1. Based on these results, it was decided to use JS1/34.5 /47− at an MOI of 0.1 for further experiments.

**Infectivity of human breast cancer cells.** Electron microscopy was used to ascertain whether human breast cancer cells are permissive to JS1/34.5 /47− infection and replication. To this end, MCF 7 cells were infected with JS1/34.5 /47 at MOI 0.1 and examined by electron microscopy over a period of 72 hours. Comparison of control uninfected cells to infected MCF7− cells shows no detectable change in cell morphology at 2 hours after infection (Fig. 4). However, at 24 hours, progeny virions were seen in the nucleus, cytoplasm, and intercellular space. Additionally, the cells have increased in size, exhibit bigger nuclei and marginalized nucleosomes, and have lost cytokeratin filaments. There was an absence of apoptotic death up to 72 hours, but lytic, necrotic death was observed from 48 hours. Similar results were obtained when several other breast cancer cell lines were infected with JS1/34.5 /47− as well as JS1 WT (data not shown).

**Infectivity of primary human mammary tumor cells.** To establish whether primary human mammary tumor cells are permissive to JS1/34.5 /47− infection, primary mammary tumor cells were purified from fresh breast tumors using a purification protocol developed in our laboratories (27). Because the number of purified primary tumor cells obtained from fresh breast tumor specimens is limiting, primary tumor cells purified from five specimens were pooled and infected with JS1/34.5 /47−/GFP at MOIs of 0.1 and 1 and evaluating granulocyte macrophage colony-forming unit (CFU-GM) and burst-forming unit-erythroid (BFU-e) at the indicated times. The mean colony-forming unit/burst-forming unit from the six patients was expressed as a ratio relative to the mock-infected control value of 1. Points, mean; bars, SE.
Infectivity of bone marrow cells. To examine the ability of JS1/34.5/47 virus to infect human bone marrow cells, electron microscopy was done on bone marrow cells treated with JS1 WT and JS1/34.5/47 at MOI 0.1 to assess viral replication, using MCF-7 cells as a positive control. Bone marrow cells were found not to be permissive to JS1/34.5/47 infection and replication because progeny virions were not found within these cells at any time during the assay, and because the cells seemed to be indistinguishable from control mock infected cells (Fig. 6). Background apoptosis and necrotic lysis was similar in cells cultured with or without virus suggesting that bone marrow cells were either not permissive to HSV infection, or that they were infected but did not support HSV replication. Similar results were obtained when these cells were infected with JS1 WT (data not shown).

To further assess infectivity of bone marrow cells, the bone marrow cells infected with JS1/34.5/GFP were analyzed for GFP expression by fluorescence microscopy over a period of 72 hours. No GFP expression was detected at MOIs 0.1 and 1 at any time point after infection (data not shown). Collectively, the data presented above indicate that bone marrow cells are not permissive to infection by the viruses used in this study.

Purging bone marrow of breast cancer cells using JS1/34.5/47. Having established that JS1/34.5/47 infected and efficiently killed breast cancer cells without adverse affects on bone marrow cells, experiments to purge breast cancer cells from bone marrow were designed to exploit this difference. To this end, fluorescently labeled MDA-MB-231 cells were mixed with freshly isolated bone marrow cells, infected with JS1/34.5/47, and cultured in bone marrow culture medium (Myelocult) for 6 days. FACS analysis was then used to assess the effect of viral infection on both cell populations. Before purging bone marrow of breast cancer cells, we initially established that Myocult medium supplemented with cytokines did not affect proliferation of MDA-MB-231 breast cancer cells (data not shown). We also optimized the fluorescent labeling of breast cancer cells with CMFDA and found a concentration of 5 μmol/L CMFDA to be optimal over a period of 6 days, separating the breast cancer cells from the background autofluorescence emitted by bone marrow cells. Furthermore, concentrations of CMFDA ranging from 0.5 to 20 mmol/L did not seem to affect the growth rate of breast cancer cells (data not shown).

Efficiency of detection of CMFDA-labeled cells by FACS. In the clinical setting, the proportion of breast cancer cells in a bone marrow/peripheral blood progenitor cell graft is likely to be considerably <1% (28, 29). To determine the efficiency of detection of small numbers of fluorescent cells by FACS, known numbers of CMFDA-labeled MDA-MB-231 cells were mixed with a population of unlabeled MDA-MB-231 cells, and the proportion of fluorescent cells was estimated over a period of 5 days. The results shown in Fig. 7 show that using FACS analysis accurately and consistently detected as few as 1% labeled MDA-MB-231 cells against a background of 99% unlabeled cells.

Evaluation of cytotoxic effects of JS1/34.5/47 FACS analysis. To evaluate the biological effects of JS1/34.5/47 on bone marrow and MDA-MB-231 cells, independent cultures of bone marrow and MDA-MB-231 cells were treated with JS1/34.5/47, and FACS analysis was used to estimate the proportion of live and dead cells over a period of 6 days. The results presented in Fig. 8 show that the number of live bone marrow cells was maintained throughout the 6-day period, with the percentage of dead bone marrow cells remaining at under 20%, with no significant difference between the mock-infected control and the virus-infected samples. Three additional patient bone marrow samples were also tested with similar results (data not shown). In contrast, the percentage of PI-positive dead, for MDA-MB-231 cells, increased from 10% to 90% in the viral infected cultures with a corresponding decrease in the number of viable cells. In the mock-infected control, however, the percentage of dead MDA-MB-231 cells remained at 10%, whereas the live cells continued to proliferate. These results further substantiate the above data.
on the biological effects of JS1/34.5−/47− on breast cancer and bone marrow cells (Figs. 2 and 3, respectively).

**Evaluation of viral purging of bone marrow cells added to MDA-MB-231 cells.** To evaluate the ability of JS1/34.5−/47− to purge bone marrow samples of tumor epithelial cells, bone marrow cells were mixed with 10% CMFDA-labeled MDA-MB-231 cells and treated with JS1/34.5−/47− at MOI 0.1, and the numbers of live cells were analyzed by FACS at 0, 3, and 6 days after infection. The number of viable MDA-MB-231 cells remaining at days 3 and 6 were expressed relative to the number of viable MDA-MB-231 cells at day 0. The experiment was done using bone marrow cells from four patients to allow for the variability between individuals (Fig. 3). The results presented in Fig. 9 show that over the period of 6 days, mock-infected MDA-MB-231 cells exhibit substantial proliferation. However, infection with JS1/34.5−/47− results in a significant reduction in the number of viable MDA-MB-231 cells in comparison with the mock-infected controls. These results show that JS1/34.5−/47− at MOI 0.1 can purge MDA-MB-231 cells from bone marrow cocultures and effectively prevent proliferation of the epithelial cells in comparison with mock-infected controls. Most likely, increasing the MOI would result in total purging of the MDA-MB-231 cells.

**Effect of JS1/34.5−/47− on CK19 mRNA levels in bone marrow samples from breast cancer patient.** The ability to evaluate the presence of occult metastatic cells in bone marrow of breast cancer patients has been hampered by the fact that disseminated tumor cells are predominantly found in low numbers (28, 29). Improvements in the detection rates of disseminated tumor cells have been achieved by RT-PCR (29–32) and real-time PCR (33) for the tumor marker CK19, which is highly expressed in breast cancer cells.

To assess the ability of JS1/34.5−/47− in clearing breast cancer cells from bone marrow samples, we aimed to establish the relative levels of CK19 transcript in bone marrow samples of breast cancer patients infected ex vivo with JS1/34.5−/47−, compared with mock-infected controls, as a measure of tumor cell clearance. To do this, we employed real-time PCR to detect CK19 mRNA levels in bone marrow aspirates of 15 independent high-risk breast cancer patients, 6 days after infection with JS1/34.5−/47− at MOI 0.1, in triplicate. Real-time PCR results were normalized against the control gene GAPDH. The percentage reduction in CK19 mRNA levels was then determined from the average difference in PCR cycle number taken to reach threshold fluorescence in infected bone marrow samples in comparison with mock-infected control for each individual sample (Fig. 10). Microscopic observations of the bone marrow cultures treated with virus throughout the 6-day culture period did not reveal any deleterious affects, in comparison with mock-infected controls, supporting the results presented earlier.

Because our previous studies have shown specific tumor kill by JS1/34.5−/47− in vivo (26), we anticipated CK19 mRNA levels to be reduced in infected bone marrow samples in

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**Fig. 5.** Infection of primary human mammary tumor cells. Primary human mammary tumor cells were purified from fresh breast tumor specimens and infected with JS1/34.5−/47−/GFP at MOI 0.1. The cells were examined for GFP expression 24 hours after infection using fluorescent microscopy. In comparison with mock-infected control cells, the majority of the infected cells exhibit GFP expression. Similar results were observed at 48 and 72 hours after infection.

**Fig. 6.** Electron micrograph of virally infected bone marrow cells. Freshly prepared bone marrow cells were infected with JS1 WT and JS1/34.5−/47− at MOI 0.1 and observed by electron microscopy at the indicated time points after infection. No viral progeny or particles were found in the cells in the duration of this experiment. Furthermore, virally infected cells appear indistinguishable from the mock-infected controls over a period of 72 hours, indicating that these cells are not permissive to infection with the viruses used.
comparison with mock-infected controls. Comparison of relative levels of CK19 mRNA in infected and mock-infected samples at day 6 did reveal some significant differences. Specifically, 8 of the 15 samples tested showed a relative reduction in CK19 mRNA levels in infected samples compared with parallel mock-infected controls. The percentage reduction observed ranged from 42% to as much as 95% (Fig. 10). Hence, we conclude that the reduction in relative levels of CK19 observed in Fig. 10 strongly suggest that the tumor cells were purged from the patient samples.

Recently, concerns about the specificity of detection of CK19 expression by RT-PCR (34–37) and real-time PCR (33) have been expressed, based on the detection of the expression of this epithelial antigen in the peripheral blood mononuclear fraction and bone marrow of normal controls and patients with hematologic malignancies. In this context, our data show that bone marrow cells are not permissive to JS1/34.5−/47− infection, and that JS1/34.5−/47− infection does not have a deleterious effect on the proliferation of bone marrow cells. Additionally, we show that in culture, JS1/34.5−/47− infects primary human mammary tumor cells efficiently. Hence, in this study, CK19 mRNA in the patient samples after JS1/34.5−/47− infection most likely emanates from mononuclear hematopoietic cells rather than persistent tumor cells.

Discussion

Autologous stem cell transplantation is used to rescue cancer patients from myelosuppression caused by high-dose, multiagent chemotherapy. However, the possible presence of occult tumor
cells in autologous bone marrow or peripheral blood progenitor cell transplants remains a great concern in cancer therapy (38–40). Although the clinical value of purging in autologous bone marrow transplants remains somewhat controversial, the development of novel tools to obtain bone marrow/peripheral blood progenitor cell preparations free of cancer cells remains a desirable objective.

Using an oncolytic HSV virus, we have previously shown enhanced tumor kill in mouse models (26). In this study, we have examined the biological effects of a novel recombinant HSV-1 vector (JS1/34.5/-47) constructed in our laboratories. JS1/34.5/-47 was derived from a new clinical isolate (JS1) deleted for both ICP34.5- and ICP47, providing tumor-selective replication and leading to the immediate-early expression of the US11 gene. Expression of US11 blocks phosphorylation of PKR, thus enhancing replication of ICP34.5-deleted viruses in tumor cells, without compromising safety (41, 42). Phase I and II clinical trials are currently ongoing with this virus by intratumoral injection in a range of tumor types.

In examining the biological effects of JS1/34.5/-47, we have found that this virus can efficiently infect a spectrum of human breast cancer cell lines, irrespective of their estrogen receptor status. Furthermore, viral infection seems to result in cell death at MOIs ranging from 1 to 0.01 in all the cell lines tested, showing the potent cytotoxicity of this virus in epithelial cells. In this context, the cytotoxic effects of the virus were apparent as early as 24 hours after infection at MOI 0.1. At this time point, viral progeny were already present in the nucleus, cytoplasm, and intercellular space causing lytic, necrotic death as early as 48 hours after infection. Interestingly, the filamentous structure of cytokeratin proteins was also lost at an early time point in the infected breast cancer cells, as judged by electron microscopy. However, we were able to detect CK19 protein in the infected cells throughout the assay, using antibodies specific to this protein (data not shown). These data suggest that, whereas the filamentous ultrastructure of the cytoskeletal proteins seems to be very quickly disrupted in virally infected cells, protein fragments bearing epitopes of antibodies specific to these proteins persist.

Importantly, JS1/34.5/-47 was also able to efficiently infect purified primary breast tumor cells in vitro, as judged by the expression of GFP in JS1/34.5/GFP transduced cells, showing that this virus has the capacity to target breast tumors. In contrast, JS1/34.5/-47 had no significant effect on the biology of human bone marrow cells, as judged by clonogenicity studies, suggesting that these cells are not permissive to JS1/34.5/-47 infection. However, the possibility exists that JS1/34.5/-47 is able to infect bone marrow cells, but the cells do not support replication of this virus. In this context, using electron microscopy, we were unable to detect viral progeny within bone marrow cells over a period of 72 hours, indicating that human bone marrow cells are not permissive to JS1/34.5/-47 infection, most likely due to absence of viral receptor.

Collectively, these data show that JS1/34.5/-47 is able to selectively infect and kill human breast cancer cells, sparing bone marrow cells. Hence, JS1/34.5/-47 is potentially a novel tool for the ex vivo purging of hematopoietic cells in

Fig. 9. Evaluation of efficiency of purging bone marrow cells of MDA-MB-231 cells. Freshly prepared bone marrow cells from four patients were mixed with 10% CMFDA-labeled MDA-MB-231 cells and infected with JS1/34.5/-47 at MOI 0.1. The numbers of live MDA-MB-231 cells were analyzed by FACS at 0, 3, and 6 days after infection in both infected and mock infected control populations of cells. The number of viable MDA-MB-231 cells at each time point was then expressed as a relative proportion to the number of viable MDA-MB-231 cells at day 0.
preparation for autologous cell transplantation. In this light, we have employed FACS analysis to evaluate the efficacy of JS1/34.5/47 as an ex vivo hematopoietic cell-purging agent. Our data show that FACS analysis can be used to accurately detect a minority population of cells present only at 1%. Furthermore, using FACS analysis to measure the numbers of live bone marrow and tumor cells in cocultures of MDA-MB-231 with freshly prepared bone marrow cells, we were able to show that in JS1/34.5/47 infected cultures, the proliferation of tumor cells was significantly inhibited. In contrast, in control uninfected cultures, tumor cells were able to proliferate ~7- to 8-fold, resulting in a dramatic decrease in the numbers of bone marrow cells. The slight increase in the numbers of MDA-MB-231 cells in the infected cultures is most likely due to the fact that JS1/34.5/47 was used at an MOI of 0.1 for the total coculture population. Therefore, a fraction of the cancer cells would have remained uninfected at the start of the experiment and hence continued to proliferate. Despite this proliferation, the decrease in the proportion of viable MDA-MB-231 cells in the virally infected cultures compared with the control remains highly significant. The possibility exists that all the MDA-MB-231 cells would have been killed at higher MOIs. Additionally, this study shows effective virus induced kill in a broader range of breast cancer cell lines than previously reported. Furthermore, the virus was found effective at reducing CK19 mRNA levels in 53% of bone marrow samples from breast cancer patients, suggesting that with suitable further development, this virus may be of use in purging of breast cancer cell in the clinical setting. With such a development, more effective purging strategies may become an integral part of novel therapies for a spectrum of malignancies in the future.

Fig. 10. Effect of JS1/34.5/47 on CK19 mRNA expression in the bone marrow of breast cancer patients. Bone marrow from breast cancer patients was infected with JS1/34.5/47 MOI 0.1 ex vivo. Expression of CK19 mRNA was examined by real-time RT-PCR 6 days after infection, and the results were normalized against GAPDH. CK19 mRNA levels were then expressed as percentage reduction in transcript number relative to the mock-infected controls for each sample.

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