Elimination of Malignant Clonogenic Breast Cancer Cells from Human Bone Marrow


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ABSTRACT

Autologous bone marrow transplantation is a promising approach to the treatment of breast cancer but is at present limited to patients without bone marrow metastases. To eliminate malignant clonogenic breast cancer cells from normal human bone marrow, immunomagnetic separation has been combined with chemoseparation using 4-hydroperoxycyclophosphamide. Breast cancer cell lines have been mixed with a 10-fold excess of irradiated human bone marrow from normal donors. Mixtures have been incubated with a combination of five different monoclonal antibodies which bind to epithelial cell surface antigens of M, 42,000, 55,000, 72,000, 200,000, and >200,000. Antiglobulin coated microspheres which contained magnetite were added, and tumor cells were trapped in a magnetic field. Elimination of tumor cells from the decanted marrow was measured in a limiting dilution assay. Two treatments with antibody and microspheres permitted elimination of 2–4 logs of clonogenic breast cancer cells, depending upon the cell line studied. Similar treatment of nonirradiated normal marrow failed to affect levels of colony forming units–granulocyte-macrophage significantly. Use of immunomagnetic purging in combination with 4-hydroperoxycyclophosphamide eliminated up to 5 logs of tumor cells but reduced the recovery of colony forming units–granulocyte-macrophage. If prompt engraftment is observed following reinfusion of similarly treated marrow in phase I trials, these techniques should permit extension of autologous bone marrow transplantation to a larger population of breast cancer patients.

INTRODUCTION

For many cancers, an increase in the dose of chemotherapy administered is associated with an increase in the rate of tumor response. For some malignancies, the higher the dose of chemotherapy administered, the greater the likelihood of cure. Maximally tolerated doses of some drugs are limited primarily by toxicity to normal bone marrow cells. For these agents, potentially lethal myelotoxicity can be overcome by an infusion of bone marrow following the administration of chemotherapy in high dosage (1–3). Over the past decade, high dose chemotherapy followed by autologous bone marrow transplantation has become the treatment of choice for certain patients with acute leukemia, Hodgkin’s disease, non-Hodgkin’s lymphoma, neuroblastoma, and Wilms’ tumor (4). More recently, encouraging results have been reported using high doses of chemotherapy followed by ABMT in the treatment of small cell lung cancer, testicular cancer, and breast cancer (5–8).

One potential limitation of ABMT, however, is the possibility that clonogenic metastatic tumor cells might contaminate the bone marrow which is reinfused following chemotherapy. Despite the absence of morphologically identifiable tumor on microscopic examination of the marrow, residual neoplastic cells might be collected and returned to the patient with normal hematopoietic stem cells, assuring the recurrence of tumor (9). A number of techniques have been developed to eliminate malignant cells ex vivo before returning the marrow to the patient. Many studies of high dose chemotherapy followed by reinfausation of “cleansed” or “purged” marrow have been performed in animals and humans with adequate, consistent reconstitution of all marrow elements (10).

Among the solid tumors seen in adults, breast cancer is one of the most responsive to chemotherapy. A steep dose-response curve is exhibited by many of the agents used to treat this disease (11–14) making it an attractive target for high dose chemotherapy with ABMT. Involvement of the bone marrow with tumor, however, is a major consideration in the application of ABMT to the treatment of breast cancer. In recent studies, 17–28% of women with newly diagnosed breast cancer and no histological evidence of metastases had tumor cells detected in their bone marrow by immunohistochemical methods which detect breast cancer-associated antigens (15, 16). Overt metastatic disease will develop in 50% of women with breast cancer within 5 years of initial diagnosis, and at first relapse, 13–57% will have bone marrow metastases (17, 18).

Several techniques are currently under investigation to eliminate breast cancer cells from bone marrow, including lectin separation (19) and immunoseparation with monoclonal antibodies linked to plant toxins (immunotoxins) (20). Recent studies with leukemias and lymphomas suggest that a combination of purging methods utilizing both immunoseparation and chemoseparation can prove superior to either method used individually (21–24). In this report, we present evidence that a combination of immunoseparation and chemoseparation is also more effective than either individual technique for the selective and complete elimination of breast cancer cells from human bone marrow.

MATERIALS AND METHODS

Breast Cancer Cell Lines. The CAMA-1 cell line used for these studies was derived from the malignant pleural effusion of a postmenopausal woman with adenocarcinoma of the breast (25). Other breast cancer cell lines used included BT-20 (26), SK-BR-3 (27), BT-483 (28), and MCF7 (29). Culture media were obtained from Hazelton Research Products (Lanexa, KS). Culture conditions were optimized for each cell line used. Cells were grown in T-150 flasks as monolayer cultures in RPMI 1640 (SK-BR-3, BT-483 and BT-20) or Dulbecco’s modified minimal essential medium (CAMA-1 and MCF7), supplemented with 4.5 g/liter glucose, 5–15% fetal bovine serum (GBCO Laboratories, Inc., Grand Island, NY) and 1% /-glutamine (Hazelton).

Bone Marrow. Using protocols approved by the Duke University Medical Center Institutional Review Board, bone marrow was obtained from healthy volunteers and diluted 15-fold with Hanks’ balanced salt solution without magnesium or calcium. Diluted marrow was layered over Ficoll-diatrizoate (Biometrics Laboratory Products, Kensington, MD) and centrifuged at 1300 rpm for 15 min. The mononuclear layer of cells at the interface was washed and resuspended in Hanks’ balanced

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3The abbreviations used are: ABMT, autologous bone marrow transplantation; 4-HC, 4-hydroperoxycyclophosphamide; CFU-GM, colony forming units–granulocyte-macrophage.
salt solution. Bone marrow cells were irradiated with 5000 cGy at 926 cGy/min. For assays of normal progenitor cells, the marrow was separated and washed, but not irradiated.

Monoclonal Antibodies. A panel of five murine monoclonal antibodies was used for immunoseparation of breast cancer cells from human bone marrow. These antibodies were selected from a group of 123 murine monoclonal reagents (Cetus Corporation) which reacted with breast cancer (30). Antibodies were chosen which lacked reactivity to normal human bone marrow, as judged by indirect immunofluorescence and flow cytometry. Five IgG antibodies were then chosen which exhibited additive binding to breast cancer cell lines by indirect immunofluorescence and flow cytometry. These antibodies also demonstrated additive binding to sections of human breast cancer using the biotin-avidin immunoperoxidase technique (31). Among cancers from 13 patients, the combination of antibodies bound intensely to >95% of cells in 10 of 13 patients and intensely to a majority of cells in all 13 patients (31). The final panel included 2G3, 741F8, 9C6, 260F9, and 317G5, which recognized a high molecular weight mucin-like glycoprotein, a M, 200,000 protein, a M, 72,000 protein, a M, 55,000 protein, and a M, 42,000 protein, respectively.

Magnetospheres. Monosized porous polymer particles were rendered magnetizable by in situ precipitation of γ-Fe2O3, magnetite, inside the uniform spheres. The pores were covered with polymeric material to give a smooth surface, which allowed physical adsorption of proteins. A sheep anti-mouse immunoglobulins antibody was adsorbed to the particle surface after affinity purification and removal of antibodies reacting with human immunoglobulins by solid phase adsorption (32). These particles are now available as Dynabeads M-450 with sheep anti-mouse immunoglobulins from Dynal, Inc. (Great Neck, NY).

Immunoseparation with Magnetospheres and Monoclonal Antibodies. Experiments were performed with a mixture of 1 × 10⁶ CAMA-1 breast cancer cells and 1 × 10⁷ irradiated bone marrow within a volume of 1 ml contained in a 12- x 75-mm polypropylene tube. Cell suspensions were incubated with the five murine monoclonal antibodies for 1 h at 4°C, on a rocker. Magnetospheres were then added for 1 h longer at 4°C. The suspension was then placed on an array of small, permanent samarium-cobalt magnets, each 16.5 x 0.5 in. The treated cell suspension was held on the magnetic device for 60 s. Nonadherent cells were decanted and assayed for residual clonogenic breast cancer cells in a limiting dilution assay or for progenitor cell recovery in a methylcellulose tissue culture assay.

Chemoseparation with 4-HC. 4-HC was kindly supplied by Dr. M. Colvin of the Johns Hopkins Oncology Center. Mixtures of human bone marrow and tumor cells were incubated with 4-HC and tissue culture medium (TC-199) for 30 min in a 37°C water bath, at a final concentration of 10⁻⁴ M 4-HC, on a rocker. Magnetospheres were then added for 1 h longer at 4°C. The suspension was then placed on an array of small, permanent samarium-cobalt magnets, each 16.5 x 0.5 in. The treated cell suspension was held on the magnetic device for 60 s. Nonadherent cells were decanted and assayed for residual clonogenic breast cancer cells in a limiting dilution assay or for progenitor cell recovery in a methylcellulose tissue culture assay.

RESULTS

Optimal Antibody Concentration for Immunoseparation. CAMA-1 cells were mixed with a 10-fold excess of irradiated bone marrow. These suspensions were incubated with various concentrations of the five murine monoclonal antibodies for 1 h at 4°C. Using a single treatment, between 1.5 and 2.0 logs of clonogenic breast cancer cells were eliminated, with maximal antitumor activity observed between 0.2 and 20 μg/ml of each antibody (Fig. 1).

Optimal Magnetosphere Concentration for Immunoseparation. Using a concentration of 2.0 μg/ml of each antibody, mixtures of CAMA-1 cells and irradiated bone marrow were treated with different concentrations of magnetospheres. Concentrations greater than 0.5 mg/ml, which provided a 7.5:1 ratio of beads:tumor cells, did not significantly increase elimination in a single treatment (Fig. 2).

Optimal Number of Treatments for Immunoseparation. The monoclonal antibody panel (2.0 μg/ml antibody) and magnetospheres (1.0 mg/ml) were used to compare single and multiple treatments. Additional aliquots of antibodies and magnetospheres were added for each treatment. While two treatments proved to be superior to a single treatment in eliminating clonogenic breast cancer cells, a third treatment did not significantly improve results. Under optimal conditions, two treatments removed 3.5 logs of clonogenic tumor cells (Fig. 3). Bone marrow progenitor cell recovery was shown to be greater than 85% using 2.0 μg/ml of each murine monoclonal antibody, 0.5 mg/ml of the magnetospheres, and two or even three separate incubations (Fig. 4).

Immunoseparation of Multiple Breast Cancer Cell Lines. To demonstrate that clonogenic elimination was not limited to the CAMA-1 tumor cell line, similar experiments were performed with four other breast cancer cell lines. From 2 to 5 logs of clonogenic CAMA-1 cells and irradiated bone marrow were treated with a 10-fold excess of irradiated human bone marrow, judged by visual scoring and [³H]thymidine uptake.
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Fig. 3. Effect of the number of treatments with antibody and magnetospheres on the immunomagnetic elimination of clonogenic CAMA-1 cancer cells from a 10-fold excess of undiluted human bone marrow, judged by visual scoring and \[^{3}H\]thymidine uptake.

Fig. 4. Effect of the number of immunomagnetic separations on recovery of normal CFU-GM from nonirradiated human bone marrow.

Fig. 5. Elimination of different breast cancer cell lines from a 10-fold excess of undiluted human bone marrow using two cycles of immunomagnetic separation.

tumor cells were eliminated, using immunomagnetic separation. The extent of elimination appeared to depend not only on tumor cell removal, but also on the clonogenic efficiency of the particular breast cancer cell line studied (Fig. 5). Residual tumor cells did remain, however, following immunoseparation of mixtures containing each of the cell lines. Consequently, other methods for purging were evaluated.

Chemoseparation with 4-HC. Incubation with different concentrations of 4-HC eliminated different fractions of clonogenic CAMA-1 cells. Concentrations of 20 \(\mu\)g/ml of 4-HC eliminated 1 log of clonogenic tumor cells, while 40 \(\mu\)g/ml reduced clonogenic tumor cells by 2 logs (Fig. 6). Greater than 20% of marrow progenitor cells could be recovered after treatment with 40 \(\mu\)g/ml of 4-HC (data not shown).

Chemoseparation and Immunoseparation. CAMA-1 breast cancer cells were treated sequentially with immunoseparation using the murine monoclonal antibodies and magnetospheres, followed by chemoseparation with 40 \(\mu\)g/ml 4-HC. Chemoseparation and immunoseparation were also performed in the reverse order. With the combined treatments, regardless of the sequence of incubations, 4.5 logs of clonogenic tumor cells could be eliminated (Fig. 7). The tumor cell elimination was greater with the combination of chemoseparation and immunoseparation than with either individual treatment (Fig. 7). Similar results were obtained with each of four breast cancer cell lines (Fig. 8). Progenitor cell recovery was better when the 4-HC incubation was performed first, but in either case recovery was greater than 15% (Fig. 9). Consequently, with a combination of chemoseparation and immunoseparation, more than 4 logs of clonogenic breast cancer cells could be eliminated with
loss of less than 1 log of CFU-GM. Similar results were obtained when blast forming units-erythrocyte were measured (data not shown).

DISCUSSION

A clonogenic assay has been developed which permits detection of up to 5 logs of clonogenic breast cancer cells in a 10-fold excess of human marrow. Using this assay, a combination of immunoseparation and chemoseparation has proven superior to either technique alone for eliminating breast cancer from bone marrow. Although this principle has been established in the past for leukemias and lymphomas, to our knowledge this is the first instance in which a combination of chemoseparation and immunoseparation has been used to eliminate solid tumor cells from human bone marrow.

The apparent efficacy of immunoseparation may relate to the use of multiple monoclonal antibodies. Expression of antigens by different breast cancers is quite heterogeneous (31). To compensate for this heterogeneity, a panel of murine monoclonal antibodies was used which recognized five distinct tumor associated determinants. These antibodies reacted strongly with multiple breast cancer tissue sections and cell lines but did not bind to human bone marrow cells by indirect immunofluorescence. Use of multiple monoclonal antibodies in the context of immunomagnetic purging eliminated up to 3.5 logs of clonogenic breast cancer cells. Failure of immunomagnetic separation to affect CFU-GM level is also consistent with the expectation that these monoclonal antibodies would lack reactivity with human bone marrow.

In early studies, monoclonal antibodies and complement were used to remove leukemia and lymphoma cells from bone marrow completely and selectively (33, 38-41). The immunomagnetic separation technique was developed because of increasing evidence that subpopulations of tumor cells can be resistant to complement-mediated lysis (42, 43). Many factors have been implicated in development of complement resistance, including the metabolic status of the target cell, membrane composition, time in culture, intracellular cyclic AMP levels, and anticomplementary factors elaborated by bone marrow (42, 43). Unlike complement-mediated purging, immunomagnetic separation does not require antibodies of a particular isotype, is not affected by anticomplementary factors associated with normal bone marrow cells, and can eliminate bone marrow cells expressing low levels of target antigen (44). As in the case of complement-mediated immunoseparation, multiple murine monoclonal antibodies can be utilized to compensate for the known heterogeneity of antigen expression among tumor cells (45, 46).

The immunomagnetic separation procedure has been used clinically to purge bone marrow contaminated with neuroblastoma (46) and Burkitt's and B-cell lymphomas (47), as well as to remove T-cells from allogeneic marrow in an attempt to prevent graft-versus-host disease (48). A standardized technique for immunomagnetic separation was developed for patients with stage IV neuroblastoma, 75% of whom have bone marrow metastases (49). Clinical results in neuroblastoma, using a murine spleen colony assay to determine the relative sensitivities of normal hematopoietic stem cells and clonogenic lymphoma cells to various doses of antitumor agents. These observations provide a rationale for the elimination of neoplastic cells from marrow suspensions by \textit{ex vivo} incubation with pharmacological agents. Initial preclinical studies revealed that incubation of mixtures of leukemic cells and normal bone marrow cells with 4-HC led to a dose-dependent antitumor effect without damaging the ability of bone marrow cells to reconstitute in the host animal following myeloablative therapy (55).

Clinical trials were performed with 4-HC marrow purging at the Johns Hopkins Oncology Center (56). In a trial of patients with acute myelogenous leukemia in second or third remission treated with high dose chemotherapy and 4-HC-purged autologous bone marrow, an actuarial disease-free survival of 43% and an actuarial relapse rate of 46% at 1 year were reported (57). Despite a dramatic reduction in the CFU-GM content of bone marrow purged with 100 µg/ml of 4-HC, consistent reconstitution was observed following high dose therapy (57). 4-HC has also been used to treat the bone marrow of patients with non-Hodgkin’s lymphoma and chronic myelogenous leukemia prior to ABMT (58, 59).

4-HC has several properties that make it an attractive candidate for use in chemoseparation of breast cancer cells from human bone marrow. The drug is easy to formulate and requires a short treatment time, an important consideration when using human bone marrow that must be harvested and frozen on the same day. Cyclophosphamide is a highly active agent against breast carcinoma both \textit{in vitro} and \textit{in vivo} (14, 60-62). From previously published clinical data, greater than 5% recovery of CFU-GM “post-purge” is adequate to permit effective marrow reconstitution (63).

Our preclinical studies suggest that the combination of immunological and pharmacological techniques is superior to either purging method individually for the eradication of breast cancer cells from human bone marrow. The clinical relevance of purging is still uncertain. Breast cancer may, however, provide a disease in which the importance of purging might eventually be tested.

In a metastatic breast cancer trial consisting of induction therapy with Adriamycin, 5-fluorouracil, and methotrexate, followed by intensification using high doses of cyclophosphamide, cisplatin, and 1,3-bis(chloroethyl)-1-nitrosourea with autologous marrow support, a 70% complete remission rate has been achieved (6). At 20 months, 53% of the entire group remained in complete remission without additional treatment (6). These preliminary results are encouraging but have been obtained in patients whose bone marrow was not contaminated with tumor cells. Until recently, any woman with tumor involvement in the bone marrow, or a significantly positive bone scan, was excluded from high dose chemotherapy protocols at our own institution, because of the risk of reinfecting previously collected tumor cells. With the development of an effective and safe purging technique, even those breast cancer patients with marrow involvement are candidates for treatment with high
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