Bone Marrow Transplantation (1987), **1**, 303–310

Detection of malignant cells in histologically normal bone marrow using culture techniques

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Summary: Cellular material retained on screens used to filter aspirated bone marrow for future autologous marrow transplantation was studied using long-term culture techniques in 20 consecutive patients. All patients had marrow aspirate and biopsy specimens that were normal histologically. Cultures from five patients grew malignant cells similar to those of the known underlying malignancy. Two types of culture methods were used in these studies. Method I consisted of a long-term bone marrow culture system which predominantly favors adherent cells, and Method II consisted of a suspension type culture system favoring expansion of mononuclear cell types. These findings suggest that tumor cells are reinfused more often at the time of autologous bone marrow transplantation than has been previously suspected. Although the clinical significance of these findings is not known, it is clear that culture techniques combined with special stains and molecular probing will allow the improved detection of occult tumor cells in bone marrow from patients undergoing autologous marrow transplantation. These observations emphasize the need for a comprehensive study of histologically normal autologous marrow using culture techniques to determine the frequency of occult involvement by viable malignant cells and the clinical implications of these findings.

use of autologous bone marrow plantation (ABMT) permits the administration of high-dose chemoradiotherapy to cancer patients by eliminating suppression as the dose limiting . High-dose therapy with ABMT has d in the complete responses of ancies which could not be eliminated usly by conventional therapy. Presently, some of these complete responses represent cures.[1,2]

be eligible for ABMT, the marrow of patients must be free of malignancy time of marrow harvest, so that viable gnant cells are not reinfused at the time transplantation. Although techniques for purging malignant cells from harvested autologous marrow are under development,[3] most patients with metastatic tumor in the bone marrow are not eligible for ABMT.

The standard method used to determine the presence of metastatic tumor cells in bone marrow for ABMT is the examination of bone marrow aspirate and biopsy specimens by light microscopy. This method is not always accurate; using chromosomal analysis, monoclonal antibody and culture techniques, histologically normal marrow has been found to harbor malignant cells.[4–10] Recently, we performed culture studies of bone marrow left on harvest filter screens to determine whether a significant number of stem cells were filtered out of the marrow. These techniques also permitted us to detect malignant cells in histologically normal marrow from patients with a variety of different tumors.

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20 August 1986; accepted 3 November 1986

Materials and methods

Bone marrow harvest

Normal bilateral iliac crest bone marrow aspirate and biopsy specimens are required of all patients at the University of Nebraska Medical Center (UNMC) prior to autologous bone marrow harvesting. If the bone marrow is free of metastatic tumor and the patient meets all other eligibility requirements, marrow is aspirated from the posterior iliac crests while the patient is under general anesthesia. Approximately 2×10^8 nucleated cells/kg of body weight are collected and placed in Hanks' balanced salt solution (BSS) to which heparin has been added. The entire marrow specimen is filtered sequentially through stainless steel mesh with 0.307 mm and 0.201 mm width openings to remove particulate material.

Cultural techniques

Cells, fat and particulate material collected during the filtering process were scraped from the screens and placed into Hanks' BSS. Particles were allowed to settle from this suspension for 5 min at room temperature. Material in suspension was layered onto lymphocyte separation medium (LSM) (Litton Bionetics, Kensington, MD) and centrifuged at 400*g* for 20 min. The settled particles or the cells collected by LSM separation were washed once by centrifugation (400*g* for 7 min) and then resuspended in Tris-buffered ammonium chloride (ACT) for 5 min to lyse mature red cells. Medium containing fetal calf and/or horse serum was added to each tube and the suspensions were washed again. Cultures were established from cells obtained from the harvest screen using two different methods. Method I, designed to maintain slowly growing adherent cells, was employed to assess the survival of marrow stem cells following the screening procedure. At the same time, we attempted to culture malignant cells from the marrow of patients with lymphoma, as reported by others,[4,5] using Method II, which includes frequent media changes and stimulated lymphocyte conditioned medium for the clonal expansion of lymphoma cells.

In Method I, we employed a modification of the protocol described by Coulombel *et al.*[11] using RPMI 1640 medium supplemented with 10% horse serum, 10% fetal calf serum, 2-mercaptoethanol (10^{-5} M), hydrocortisone sodium succinate (10^{-6} M), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). Flasks containing 2×10^7 cells were incubated for 7 days at 37°C in 5% CO_2 in air, at which time the flasks were transferred to an incubator at 33°C for the remainder of the study.

In Method II, we employed a modification of the technique described by Philip *et al.*[5] using RPMI 1640 medium supplemented with 20% fetal calf serum, 1% lymphocyte conditioned medium, penicillin (100 U/ml) and streptomycin (100 μg/ml). Flasks containing 5×10^6 cells were cultured at 37°C in 5% CO_2 in air. The cultured cells were used for further analyses.

Marrow colony-forming unit–GEMM assay

Adherent cells from long-term marrow cultures established using Method I were removed using trypsin–EDTA and washed. The cells (6×10^5 per plate) were cultured in GEMM medium with 0.3% Bacto-agar in the presence of two units of erythropoietin. The GEMM medium was composed of McCoy's 5A medium supplemented with amino acids, 1% phytohemagglutinin-stimulated lymphocyte conditioned medium, and 10% fetal calf serum. Plates were cultured at 37°C in 5% CO_2 in air for 14 days. Colonies and clusters were examined and enumerated using an inverted microscope. Abnormal-appearing cells or unusual-appearing colonies were noted in the GEMM (granulocyte, erythroid, macrophage, megakaryocyte) assays of two patients, and cell preparations were made for cytologic analyses.

Cytologic and immunocytochemical techniques

Cells obtained directly from the harvest screens and cells harvested from the long-term cultures established by Method I

were spun onto precleaned glass slides using a cytospin centrifuge (Shandon Southern, London, UK). The slides were air-dried and stained with Wright's stain. In one case (case 1), slides were also fixed for 2 min in ice-cold acetone and stained with an avidin-biotin immunoperoxidase technique using antibodies to α-lactalbumin and epithelial membrane antigen (antibodies No. A579 and No. M613, Dako Corporation, Santa Barbara, CA). In another case (case 2), the cells were stained for melanin using the Fontana-Masson stain.

Molecular probing techniques

Bone marrow cells from cases 3–5, cultured using Method II, were subjected to molecular probing for immunoglobulin heavy chain (J_H) and T-cell receptor (CT_β) gene rearrangements[12,13] DNA was extracted using guanidinium isothiocyanate, followed by extraction with phenol:chloroform and then chloroform alone. Extracted DNA was precipitated with 80% cold ethanol, and dissolved in Tris buffer and quantitated. The restriction enzymes EcoRI and Hind III were used to digest 10 μg DNA. Enzyme-digested DNA was electrophoresed in 0.75% agarose with Tris-borate buffer, pH 8.0, at 40 V for 16–18 h, and then stained and denatured. The DNA was transferred to nylon membranes and hybridized with appropriate radiolabeled probes. The J_H probe was obtained from Philip Leder,[14] and the CT_β probes were obtained from Tak Mak[15] and Jeffrey Sklar.[16] The blots were hybridized to the respective nick translated cloned DNA probes at 42°C in 50% formamide, 5x SSC (0.75 M sodium chloride and 75 mM sodium citrate), 3× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA. The hybridized blots were washed at 52°C in 0.2× SSC and 0.1% SDS. Autoradiography was performed using Kodak X-O-Mat AR-5 X-ray film at −70°C. Raji cell DNA was used as the positive control for the immunoglobulin gene rearrangement studies, and CEM cell DNA[17] was used as the positive control for T-cell receptor gene rearrangement analysis.

Results

Culture techniques were used to study harvested bone marrow from 20 consecutive patients with various types of malignancies. In all patients, the bone marrow specimen was histologically normal. The 20 patients included seven with solid tumors (three breast carcinoma, two melanoma, one rhabdomyosarcoma and one poorly differentiated sarcoma), eight with non-Hodgkin's lymphoma (NHL) and five with Hodgkin's disease. Culture of the marrow retained on the filter screens for colony formation in the CFU-GEMM assay demonstrated that <0.4% of the stem cells were removed by the screening procedure. Unexpectedly, malignant cells were also detected in five of 20 patients. These five patients included one with breast carcinoma, one with melanoma (both detected by culture Method I), and three with NHL (detected by culture Method II). The five case summaries are as follows.

Case 1. In December 1985, a 37-year-old female had a breast biopsy which showed infiltrating ductal carcinoma. A modified radical mastectomy was performed, and 11 of 12 axillary lymph nodes showed metastatic carcinoma. No other metastatic disease was found, and she was referred to UNMC for the harvest and cryopreservation of autologous bone marrow for possible ABMT at a later time. Bilateral bone marrow aspirate and biopsy specimens taken from the posterior iliac crests at the time of marrow harvest were normal by light microscopy. However, adherent layer cells from cultures after 4 weeks, using Method I, grew abnormal-appearing colonies in the GEMM assay. Stained cytospin preparations of the cultured cells revealed the presence of malignant cells (Figure 1). A breast origin for the cultured tumor cells was confirmed by immunocytochemical stains for α-lactalbumin and epithelial membrane antigen.

Case 2. A 25-year-old female had a malignant melanoma (Clark's level IV, Breslow depth 1.9 mm) removed from the skin of her breast. Bilateral mastectomies

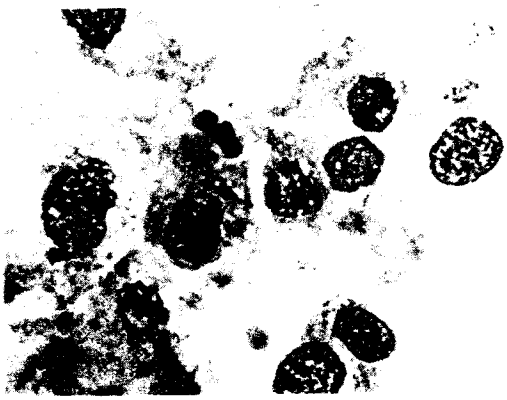


Figure 1 Cytocentrifuge preparation from a bone marrow culture showing large markedly-atypical breast cancer cells (Wright's stain ×320)

and axillary lymph node dissections showed no evidence of residual or metastatic melanoma. Forty-one months later, an inguinal lymph node biopsy specimen showed metastatic melanoma. Computerized tomography demonstrated a mass in the left adrenal gland. Brain metastases, abdominal adenopathy, and enlargement of the adrenal mass were found one month later, despite cimetidine therapy. After receiving *n*-phosphonoacetyl-L-aspartate disodium (PALA) and brain irradiation, she was referred to UNMC for high-dose therapy with ABMT. Pulmonary and hepatic metastases were also found.

Bilateral posterior iliac crest bone marrow aspirate and biopsy specimens taken at the time of marrow harvest showed no evidence of metastatic tumor by light microscopy. The time interval between initial marrow biopsy and the actual marrow harvest was 7 days. She received high-dose cyclophosphamide and *cis*-platinum followed by ABMT. Bone marrow recovery occurred promptly following aplasia. No further progression of her disease was seen, and a decrease in tumor size was noted in some areas by post-transplantation day 75. After one week in culture using Method I type culture system, cells recovered from the filter screens grew black pigmented colonies (Figure 2a). The presence of melanoma cells was demonstrated in cytospin preparations of the

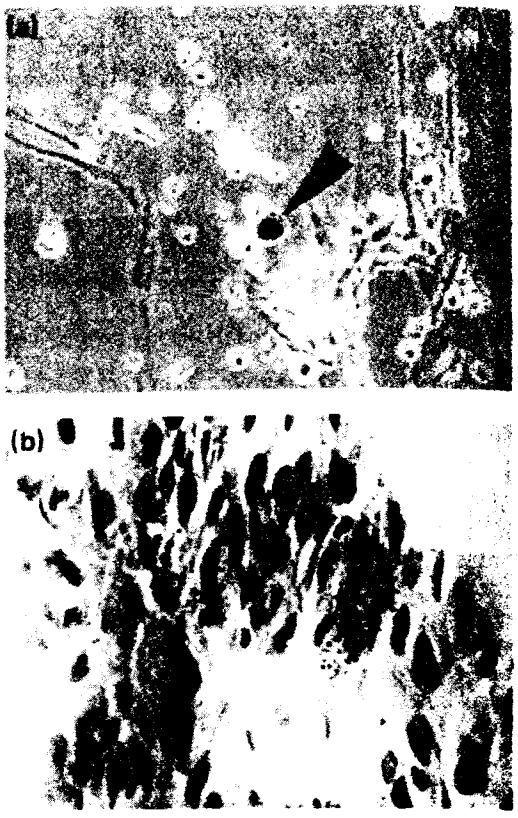


Figure 2 a Pigmented melanoma cell in a long-term bone marrow culture of histologically normal marrow, b smear preparation showing a group of spindle-shaped melanoma cells, note the punctate melanin pigment (Wright's stain ×250)

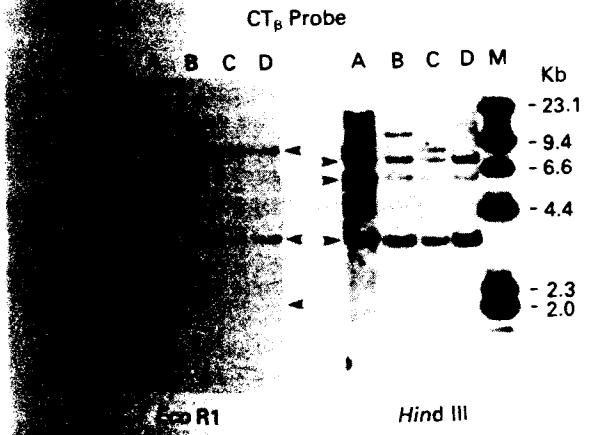
cultures (Figure 2b), and confirmed by the Fontana-Masson stain for melanin.

Case 3. A 40-year-old female was found to have an abdominal mass due to diffuse large cell lymphoma. Bilateral posterior iliac crest bone marrow aspirate and biopsy specimens were normal by light microscopy. The patient was referred to UNMC for the harvest and cryopreservation of autologous bone marrow prior to the institution of conventional therapy. The harvest marrow was also free of tumor according to light microscopy. The time interval between initial marrow biopsy and actual marrow harvest was 8 days. However, after 2 weeks

culture, cultured cells obtained from the screens using Method II were shown to have a clonal rearrangement of genes for the cell receptor (CT_β) (Figure 3, lane B).

4. A 59-year-old woman presented pleural tumor masses due to peripheral lymphoma. She was treated with ation chemotherapy which resulted complete response. Over the next 18 , the disease relapsed three times, onded completely each time to therapy. The patient was referred in relapse to UNMC for high-dose therapy with ABMT. Bilateral posterior iliac crest bone marrow aspirate and biopsy specimens taken just prior to and at the time of marrow harvest were normal. The time interval between initial marrow biopsy and actual marrow harvest was 3 days. Following marrow harvesting and cryopreation, the patient was treated with dose chemotherapy and total body tion, followed by ABMT. She died of on day 33 after transplantation. At autopsy, evidence of marrow engraftment was present and no residual lymphoma was found. After 4 weeks in culture, the culture cells obtained from the filter screens using Method II showed a clonal rearrangement of genes for the T-cell receptor (CT_β) (Figure 3, lane C).

Case 5. A 33-year-old male presented with chest pain, dyspnea and night sweats. A biopsy specimen of a mediastinal mass revealed a diffuse large cell lymphoma of B-cell lineage. No malignancy was found below the diaphragm or in the bone marrow. The patient received eight cycles of combination chemotherapy, which resulted in an 80–90% reduction in the size of the mediastinal mass. He then received radiation therapy to the mediastinum and neck, resulting in a complete response. However, the mediastinal mass recurred one year later, and he was referred to UNMC for high-dose therapy with ABMT. Bilateral posterior iliac crest bone marrow aspirate and biopsy specimens taken just prior to the marrow harvest showed no evidence of



Rearrangements of T cell β-chain (CT_β) DNA fragments in cells from the histologically normal bone Cases 3 and 4 had clonally rearDNA for the T cell receptor. Lane A is DNA from CEM cells (positive lanes B and C are rearranged DNA 3 and 4, respectively; lane D is a pattern from placental DNA; lane M DNA. Arrows indicate the germpattern

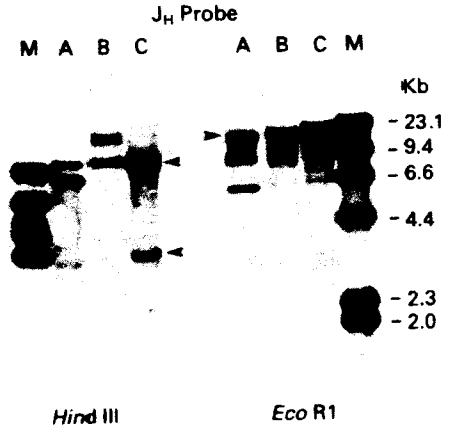


Figure 4 Rearrangement of immunoglobulin heavy chain (J_H) DNA fragments in cells cultured from histologically normal bone marrow. Case 5 had clonally rearranged DNA for the immunoglobulin heavy chain gene. Lane A is DNA from Raji cells (positive control); lane B is rearranged DNA from case 5; lane C is a germ-line pattern from placental DNA; lane M is marker DNA. Arrows indicate the germ-line DNA pattern

lymphoma. The time interval between initial marrow harvest and the actual marrow harvest was one year. The harvested marrow, which was also free of lymphoma, was cryopreserved and then returned to the patient after the administration of high-dose chemotherapy. A complete tumor response resulted. However, after 3 weeks in culture, the culture cells obtained from the filter screens using Method II showed a clonal rearrangement of the immunoglobulin heavy chain (J_H) genes (Figure 4, lane B).

Discussion

Our study was originally designed to determine whether stem cells (CFU-GEMM) were removed by the screening procedure performed on marrow harvested for transplantation. Our results showed that significant numbers of stem cells are not removed by this procedure.[18] The discovery of malignant cells in cultures of five of 20 histologically normal marrow harvests was unexpected. Although 40% of histologically normal bone marrow aspirates from patients with Burkitt's lymphoma have been shown to contain lymphoma cells using culture techniques,[5] the extent to which occult bone marrow involvement occurs in other lymphomas and solid tumors is presently unknown. We have cultured occult tumor cells from the marrow of three of eight patients with NHL, two of seven patients with solid tumors, and none with Hodgkin's disease.

The biological and clinical significance of tumor cells cultured from filter screens used to process autologous bone marrow is not clear. Our findings suggest, however, that tumor cells are reinfused more often at the time of ABMT than has been previously suspected. Two of our patients had their autologous marrow collected and cryopreserved, but not reinfused. The viability of tumor cells after the cryopreservation procedure is unknown. However, Vaughan *et al.*[19,20] have recently described five patients with NHL in whom the disease recurred rapidly in the bone marrow and blood after ABMT. Vaughan *et al.*[20] suggested that the relapses occurred as a result of reinfusion of small numbers of viable tu cells that were not detected by routine li microscopy. Further clinical studies needed to determine the significance occult tumor cells in autologous marro Three patients in the present study rece autologous marrow in which malignant were subsequently detected. Of these th patients, one with NHL had an early de due to sepsis. There was no evidence lymphoma at autopsy. A second pati with NHL achieved a complete tum response, but the clinical follow-up is sho (7+ months). The third patient has stab malignant melanoma with no evidence new lesions at 75 days after high-do therapy and ABMT. Undoubtedly, many other patients have undergone ABMT wi marrow unknowingly contaminated wi malignant cells.

Cytological examination of cell prepara-tions made directly from the harvest scree did not reveal the presence of tumor cells i our cases, presumably because the tumor cells were present in small numbers. Our detection of tumor cells in histologically normal marrow using culture systems could have occurred by two mechanisms. Firstly, the screens from which we established the cultures are used to filter the entire marrow harvest, which should increase the chance of detecting rare tumor cells. Secondly, our culture systems were designed to amplify the growth of specific cell types and are not very effective for maintaining terminally-differentiated cells. We believe that these are two important characteristics of our system, since they permit the selective amplification of rare contaminating tumor cells. Our observations suggest that this selective amplification increases the frequency at which tumor cells can be detected in histologically normal marrow. Also the plating efficiency of tumor cells obtained from bone marrow may be higher than that of primary tumors, possibly because some tumor cell selection has already occurred.

Although solid tumor cells can be identified in the background of normal hematopoietic and stromal cells in the GEMM colony assay, it is important to

obtain histochemical, immunocytochemical or molecular confirmation of malignancy since normal cells of unusual morphological appearance may also grow in these cultures. The application of molecular probing techniques and/or chromosomal analysis are essential to confirm the presence of lymphoma cells in culture, since lymphoma cells cannot be distinguished cytologically from non-malignant cells in our system. In two of three patients, gene rearrangement analysis confirmed previous immunohistochemical surface marker studies. However, clonal expansion of normal lymphocytes can occur in reaction to antigenic stimulation or stimulation by factors from functional T-cell clones.[21–23] Therefore, a monoclonal expansion of benign cells in our cultures from the third patient using Method II cannot be ruled out, although this appears unlikely since we did not see normal lymphocyte expansion in other bone marrow cultures treated in a similar way. Moreover, we have recently cultured tumor cells from the bone marrow of a patient with acute lymphoblastic leukemia.[24] At the end of 4 weeks of culture, only the malignant cells survived, as demonstrated by molecular probe and cytogenetic analyses, thus confirming the selective nature of our culture system for tumor cells.[24]

From this small series, we are unable to estimate accurately the frequency with which viable tumor cells are reinfused into patients undergoing ABMT, and the clinical significance of our findings is presently unclear. However, our observations do emphasize the need for a more comprehensive study of autologous marrow using culture techniques to determine the frequency with which occult malignant cells contaminate histologically normal marrow.

Acknowledgements

We wish to thank Mr Mark Arneson and the entire bone marrow transplantation team at the University of Nebraska Medical Center, Omaha, without whose help and cooperation these studies would not have been possible. We thank Ms Felicia Tilden for her technical assistance.

These studies were supported by a grant from the Nebraska Department of Health (86-50) and a UNMC seed grant.

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