Survival of Tumor Cells in Stem Cell Preparations and Bone Marrow of Patients with High-Risk or Metastatic Breast Cancer after Receiving Dose-intensive or High-Dose Chemotherapy[1]

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ABSTRACT

Purpose: **We evaluated whether dose-intensive or high-dose chemotherapy can eliminate micrometastases in high-risk breast cancer patients.**

Experimental Design: **We monitored cytokeratin (CK)/17-1A positive cells in the bone marrow (BM) and peripheral blood stem cells (PBSC) and studied Her-2/neu serum levels of patients with locally advanced ($n = 13$; group 1) and metastatic breast cancer ($n = 30$; group 2) using immunomagnetic separation, immunocytochemistry, and ELISA.**

Results: **CK+ cells were found in the BM of 3 of 13 (23%) group 1 patients before but not after chemotherapy, resulting in an overall survival (OS) of 92% after a median follow-up of 33 months. Contamination of PBSC in 2 of 9 (22%) patients was not associated with decreased survival. In group 2 patients, the CK+ rate was 60% (18 of 30 patients) before and 40% (4 of 10 patients) after therapy with an OS rate of 43% after 29 months. PBSC samples were positive in 7 of 24 (29%) patients. CK+ BM and PBSC led to a rapid progress and short OS, whereas tumor cell-free BM and PBSC resulted in a mean OS of 30 months. The antigen 17-1A was detected on most CK+ cells in both patient groups before therapy, on all of CK+ PBSC, and on CK+ cells in group 2 patients after therapy. Increased Her-2/neu levels were found in group 2 patients before chemotherapy.**

***Conclusion*: Micrometastatic cells are present in PBSC grafts and can survive even high-dose chemotherapy. The presence of immunotherapeutic target antigens supports the idea that a combined chemoimmunotherapy might be successful in eliminating minimal residual disease.**

INTRODUCTION

The usefulness of HD[3] chemotherapy with autologous stem cell support has been under evaluation in several treatment trials of patients with locally advanced or metastatic breast cancer, and the final results of ongoing trials have to be awaited (1–4). In most treatment regimens, PBSCs are mobilized by conventional-dose chemotherapy plus granulocyte colony-stimulating factors (5, 6). These new strategies for cancer therapy have evoked a need for the detection of tumor cell contamination of blood or BM missed by conventional tumor staging procedures, because a significant correlation between tumor cell detection in BM and poor prognosis factors as well as decreased disease-free and overall survival have already been described for breast cancer patients (7–15). It is also well known that most of these cells rest in the G_0 phase of the cell cycle (16), and it has been demonstrated recently (17) that micrometastatic breast cancer cells show a considerable heterogeneity in the expression of carcinoma-associated cell-surface molecules including Her-2/neu, CO17–1A, MUC-1, and Lewis^Y. In addition to BM studies, enhanced serum levels of Her-2/neu were found in some patients with Her-2/neu positive tumors, and this subset of patients had a worse prognosis than individuals not expressing Her-2/neu in the primary tumor (18–20). Taking into account the reduced efficacy of chemotherapeutic agents in nonproliferating cells and the considerable heterogeneity of neoplastic cells, these data suggest that a combined therapeutic approach, including immunotherapy and purging of PBSC, might improve the survival. This approach was supported in clinical trials targeting Her-2/neu or the 17-1A antigen in breast (21, 22) and colorectal cancer (23). Furthermore, purging of PBSC grafts from patients with breast cancer resulted in effective elimination of tumor cells (24, 25).

In contrast to investigations with BM, the actual risk of tumor cell contamination of PBSC collections and its clinical significance have not been extensively investigated. Whereas Sharp *et al.* (26) showed that tumor cell contamination of PBSC conferred a worse prognosis, Stadtmauer *et al.* (27) demon-

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[3] The abbreviations used are: HD, high-dose; BM, bone marrow; CK, cytokeratin; IMS, immunomagnetic separation; Mab, monoclonal antibody; MNC, mononuclear cell; OS, overall survival; PBSC, peripheral blood stem cell; IC, immunocytochemistry; G-CSF, granulocyte colony-stimulating factor.

strated that contamination of PBSC was not correlated with decreased survival.

At present, IC using CKs as epithelial marker proteins is the most common method for tumor cell detection with a sensitivity of one tumor cell among 10^6 marrow cells (28). Furthermore, IMS techniques have been developed for enrichment of epithelial cells from larger samples of mononuclear cells with subsequent detection of tumor cells by IC (29–32).

In this study, we evaluated whether dose-intensive or HD chemotherapy can eliminate micrometastases in patients with locally advanced or metastatic breast cancer. For that purpose, BM and PBSCs were studied for CK+/17-1A+ cells at primary diagnosis during and after therapy using IMS followed by IC. In addition, it was elucidated whether the measurement of the extracellular domain of Her-2/neu in serum samples is able to detect minimal residual disease before and after chemotherapy.

PATIENTS AND METHODS

Patients and Treatment

Between June 1997 and June 1999, we studied 43 patients with locally advanced ($n = 13$) and metastatic breast cancer ($n = 30$) who were referred to the Department of Internal Medicine (Cancer Research), University Hospital of Essen. Patient characteristics at the time of entry into the study are summarized in Table 1 and Table 2. All of the patients gave written informed consent for the investigations, including the BM aspirations.

Table 1 Adjuvant therapy: characteristics of group 1 patients

Characteristics	No. of patients
Total	13
Age [mean (range)]	48 (46–54)
Clinical staging	
II	5
III A	4
III B	4
Grading	
G_2	8
G_3	4
n.d.[a]	1
Histological type	
Infiltrating ductal	8
Infiltrating lobular	4
n.d.	1
No. of positive axillary lymph nodes	
Mean	17/24
Range	6/9–29/38
Estrogen/progesterone receptor status	
ER+/PR+	7
ER−/PR−	1
ER+/PR−	4
ER−/PR+	1
Menopausal status	
Pre	4
Peri	4
Post	5
Type of initial surgery	
Mastectomy	10
Breast-conserving surgery	3
Treatment	
High dose	8
Overall survival rate	8/8
Relapse	4/8
Standard therapy	5
Overall survival rate	4/5
Relapse	1/5

[a] n.d., not determined.

Pretreatment Staging

Staging procedures included computed tomography of head, chest, abdomen, and pelvis, radionuclide bone scan, and iliac crest bone biopsy (optional) plus BM aspiration and tumor marker evaluation. Additional radiological tests or biopsies of suspicious lesions were done individually according to the findings of the staging examinations.

Mobilization Chemotherapy

The patients described in this report were treated according to three different protocols for high-risk (group 1) and metastatic breast cancer (group 2), implying three different mobilization regimens. Patients treated in an adjuvant setting and those with resectable lesions not pretreated with anthracyclines received three cycles of epirubicin (45 mg/m^2; day 1 and 2) and cyclophosphamide (600 mg/m^2; day 1 and 2) plus G-CSF (filgrastim at 5 μg/kg of body weight or lenograstim at 150 μg/m^2) starting on day 5 until recovery of WBCs or until completion of stem cell collection. Treatment was repeated every 2 weeks, and PBSCs were collected after the second course of chemotherapy. Patients with metastatic disease who were pretreated with anthracyclines in an adjuvant setting received three courses of Taxol (175 mg/m^2) and cisplatin (50 mg/m^2) plus G-CSF every 2 weeks. Subsequently, cyclophosphamide (2 g/m^2) and G-CSF were given for stem cell mobilization. After stem cell collection, those patients with oligotopic recurrence were restaged and were assigned for either surgery or irradiation of the involved lesions. Within 24 h of surgery or in parallel with radiotherapy, folinic acid (500 mg/m^2 for 2 h) and 5-fluorouracil (2 g/m^2 for 24 h) were applied on a weekly-times-six schedule before the patients received HD chemotherapy.

Patients with advanced recurrent disease received three courses of doxorubicin (50 mg/m^2) and docetaxel (75 mg/m^2) plus G-CSF starting on day 3. Stem cells were collected after the second and, if necessary, third cycle of doxorubicin/docetaxel (German Breast Cancer Dose Intensity Study (GEBDIS) protocol; Table 2).

Stem Cell Collection and Flow Cytometry

PBSC collection was performed with a Spectra blood cell separator (COBE BCT, Lakewood, CO). Flow cytometry analysis was done on a Coulter Epics XL (Coulter Corporation, Hialeah, FL). Details of the procedures have been described elsewhere (33).

Cryopreservation and Thawing of PBSC

At the end of each stem cell apheresis, the collected cells were aliquoted to provide an equal amount of CD34+ cells for each course of HD chemotherapy, and the volume was brought to 44 ml/bag either by centrifugation and removal of excess plasma or by adding PBS without Ca^{2+} and Mg^{2+} (Department of Pharmacy, University of Essen). The minimum number of

Table 2 Metastatic disease: characteristics of group 2 patients

Characteristics	No. of patients
Total	30
Age [mean (range)]	43 (26–57)
Clinical staging at first diagnosis	
I	6
II	11
III	5
III B	1
IV	4
n.d.[a]	3
Grading	
G_1	2
G_2	18
G_3	8
n.d.	2
Histological type	
Infiltrating ductal	26
Infiltrating lobular	4
Site of metastasis	
Bone	12
Lung	8
Liver	10
Lymph nodes	13
Other (skin/ovary)	5
No. of metastatic sites/patient	
1	1
2	3
3	2
>3	24
Estrogen/progesterone receptor status	
ER+/PR+	19
ER−/PR−	9
ER+/PR−	1
ER−/PR+	1
Menopausal status	
Pre	19
Peri	4
Post	7
Type of initial surgery	
Mastectomy	16
Breast conserving surgery	13
n.a.	1
Treatment	
GEBDIS Protocol	17
Overall survival rate	8/17
Complete remission	1
Partial response	7
No change	3
Progressive disease	6
Metastatic resectable disease	13
Overall survival rate	7/13
Complete remission	6
Partial response	1
No change	2
Progressive disease	2

[a] n.d., not detected; n.a., not applicable.

CD34+ cells/kg body weight and course of HD chemotherapy was set to 1.5 to 2.0 $\times$ 10^6 with an additional back-up bag that was infused after the last course of HD chemotherapy. The procedure has been described in detail before (33).

Thawing in a 37°C water bath and transfusion of the PBSCs were done at the patients' bedside after i.v. premedication with antihistamines, cimetidine, and prednisone. Cells were transfused by i.v. push with a 50-ml syringe after taking samples for sterility tests.

HD Chemotherapy

All of the patients received two courses of HD chemotherapy. For those treated in the adjuvant protocol or for resectable disease, the first treatment consisted of cyclophosphamide (2 g/m^2 for 1 h; day 1 to 3) and carboplatin (500 mg/m^2 for 1 h; day 1 to 3). The second course consisted of thiotepa (200 mg/m^2 for 1 h; day 1 to 3) and mitoxantrone (20 mg/m^2 for 1 h; day 1 to 3). Stem cells were infused on day 5 of each course, and G-CSF (filgrastim at 5 μg/kg of body weight or lenograstim at 150 μg/m^2) was started the same day until recovery of WBCs.

Patients with unresectable advanced disease received at first etoposide (500 mg/m^2 for 4 h; day 1 to 3), ifosfamide (4 g/m^2 for 18 h; day 1 to 3), and carboplatin (500 mg/m^2 for 18 h; day 1 to 3), and then thiotepa (200 mg/m^2 for 24 h; day 1 to 4) and cyclophosphamide (1500 mg/m^2 for 24 h; day 1 to 4). Stem cells were reinfused on day 5 and 7, respectively, and G-CSF was started the same day until recovery of WBCs (GEBDIS protocol; Table 2).

Treatment after HD Chemotherapy

Radiation Therapy. Patients with stage III breast cancer received radiotherapy after completion of HD chemotherapy. External beam radiation consisted of 50 Gy to the chest wall and supraclavicular and infraclavicular lymph nodes. Parasternal lymph nodes were irradiated with 50 Gy in case of central or medial tumor localization. Patients undergoing lumpectomy received a boost to the primary breast site depending on the extension of the surgery and the results (tumor-free sites) of the pathological examination. All of the other patients received radiation therapy individually depending on the sites of involvement.

Hormonal Therapy. Premenopausal patients with positive hormonal receptors who were treated in an adjuvant setting received luteinizing hormone-releasing hormone agonists for 2 years. Postmenopausal patients (based on patients' history or follicle-stimulating hormone serum level > 10 units/liter) received tamoxifen for 5 years.

Patients with advanced recurrent disease received tamoxifen for 5 years or until progression irrespective of their receptor status. Those who had been pretreated with hormonal therapy were switched to a different hormonal treatment. All of the other patients were treated individually based on the hospital oncologists' decision.

Sample Preparation. BM (10 ml) was aspirated under local anesthesia from the upper iliac crest from each patient by needle aspiration under the conditions of normal coagulation parameters. MNCs were isolated from heparinized BM (5000 units/ml bone marrow) by Ficoll-Hypaque density gradient centrifugation (density, 1.077 g/mol; Pharmacia, Freiburg, Germany) at 400 $\times$ *g* for 30 min. Interface cells were washed (400 $\times$ *g* for 15 min) and resuspended in PBS. Using a Hettich centrifuge (Tuttlingen, Germany), 2 $\times$ 10^6 cells (1 $\times$ 10^6/slide) were directly spun onto glass slides (400 $\times$ *g* for 5 min) coated with poly-L-lysine (Sigma Chemical Co., Deisenhofen, Germany).

Positive IMS. If enough MNCs were available, epithelial cells were isolated from 1 $\times$ 10^7 to 3 $\times$ 10^7 MNCs using Dynabeads Anti-Epithelial Cell uniform, magnetizable polystyrene beads (Dynal, Oslo, Norway), coated with a mouse IgG1 monoclonal antibody (Mab Ber-EP4) specific for two (M_r 34,000 and 39,000) glycopeptide membrane antigens expressed

on most normal and neoplastic human epithelial tissues, identical with the tumor-associated 17-1A antigen.

Samples of 1×10^7 to 3×10^7 MNCs or PBSCs were resuspended in 1 ml of separation medium, containing PBS with 1% BSA. Prewashed dynabeads (10×10^6; bead to target cell ratio, 4:1) were added to the cell suspension and incubated at 2–4°C on an apparatus that provides both gentle tilting and rotation for 30 min. Subsequently, the bead/cell suspension was placed on a magnet for 2 min. After discarding the supernatant, the rosetted cells were isolated by removing the vial from the magnet and resuspended in 2 ml of PBS/BSA. Cytospins were performed as described under sample preparation.

IC. After overnight air drying, staining for CK+ cells was performed as already described (17) using the Epimet kit (Micromet, Munich, Germany). The identification of epithelial cells by using this kit is based on the reactivity of the murine Mab A45-B/B3, directed against a common epitope of CK polypeptides. The kit uses Fab fragments of the pan-Mab complexed with alkaline phosphatase molecules. Briefly, the method includes: (*a*) permeabilization of the cells with a detergent (5 min); (*b*) fixation with a formaldehyde-based solution (10 min); (*c*) binding of the conjugate Mab A45-B/B3-alkaline phosphatase to cytoskeletal cytokeratins (45 min); and (*d*) formation of an insoluble red reaction product at the site of binding of the specific conjugate (15 min). Subsequently, the cells were counterstained with Mayer's hematoxylin for 1 min and finally mounted with aqueous permanent mounting medium containing 15 mM NaN_3 (Dako, Hamburg, Germany). A negative control antibody (conjugate of Fab-fragment; Micromet, Munich, Germany) served as a negative control. For each test, a positive control slide with the breast carcinoma cell line MCF-7 (American Type Culture Collection, Rockville, MD) was treated under the same conditions. The microscopic evaluation was carried out independently by two investigators.

Her-2/neu was determined using the Her-2/neu (c-erbB-2) sandwich enzyme immunoassay (Oncogene Science, Cambridge, MA), which uses a mouse Mab for capture and a different biotinylated mouse Mab for the detection of human neu protein. Both capture and detector reagents specifically bind to the extracellular domain of neu protein. The capture antibody has been immobilized on the interior surface of microplate wells. To perform the test, serum (dilution, 1:50 in dilution buffer), controls, and standards were incubated in the coated wells for 3 h at 37°C to allow binding of the antigen by the capture antibody. After a wash cycle, the immobilized antigen was mixed with the detector antiserum for 1 h at 37°C. After a second washing cycle, the amount of detector antibody bound to antigen was measured by binding with a streptavidin/horseradish peroxidase conjugate, which catalyzed the conversion of the chromogenic substrate *o*-phenylenediamine into a colored product. This colored product was quantitated by spectrophotometry and related to the amount of *neu* protein in the sample.

Evaluation of Data. Patients were evaluated as tumor cell positive if at least one CK+ cell was detected as analyzed by IC.

RESULTS

We studied CK+/17-1A+ cells in the BM and PBSCs of 43 patients with locally advanced ($n = 13$; group 1) or metastatic breast cancer ($n = 30$; group 2). The patient characteristics and clinical outcome are evaluated in Tables 1 and 2.

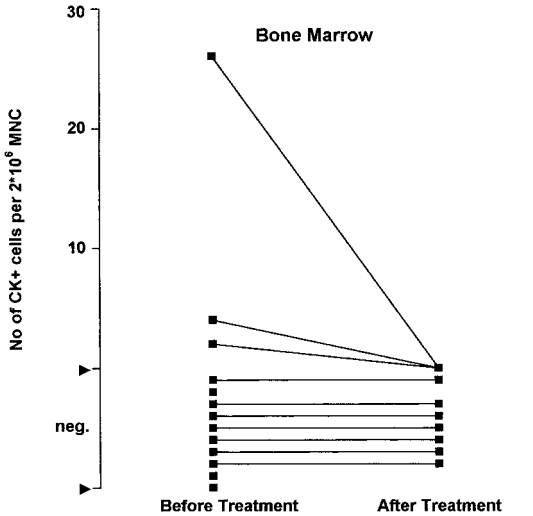

Fig. 1 Monitoring of CK+ cells in group 1 patients before and after treatment. ■, the number of CK+ cells/2×10^6 MNCs. The table under the figure summarizes the results for Her-2/neu before and after treatment; neg, negative; NR, normal range.

The results for micrometastatic cells in the BM and in PBSCs in group 1 patients before and after chemotherapy are shown in Fig. 1 and Table 3. With a median follow-up of 33 months, the OS rate was 92% with relapse free survival of 91%. Immunocytochemical BM involvement before chemotherapy could be demonstrated in 3 of 13 (23%) patients. A complete follow-up could be obtained in 9 of 13 patients with no micrometastatic cells found after chemotherapy in any patient analyzed.

Table 3 shows the detailed results for tumor cell detection in each group 1 patient. PBSC grafts were found to be positive in 2 of 9 (22%) patients. Interestingly, patient 3 showed a high frequency of CK+/17-1A+ cells in PBSCs and in the BM before chemotherapy but no CK+ cells after therapy. Furthermore, patient 12 had a high number of circulating CK+/17-1A+ cells in PBSCs but was negative for these cells in the BM before chemotherapy. None of these patients, including patients 8 and 9 with CK+ cells in the BM before chemotherapy, had a relapse up to now. A recurrence was only demonstrated for patient 2 after 12 months and patient 11 after 6 months, respectively. The epithelial cell surface antigen 17-1A was found on CK+ cells in 2 of 3 patients before chemotherapy and in all of the patients with CK+ PBSC grafts. Interestingly, enhanced Her-2/neu levels could only be demonstrated for patient 7 before chemotherapy.

The evaluation of micrometastatic cells and the results for soluble Her-2/neu for patients with metastatic disease are summarized in Fig. 2 and Table 4. With a median follow-up of 29 months, the OS rate was 43% with relapse free survival of 15%. At the time of diagnosis, 18 of 30 (60%) patients were found to

Table 3 Adjuvant therapy: monitoring of group 1 patients

	CK+ cells/ 2×10^6 MNC BM[a]	Her-2/neu	CK+ cells/ ml PBSC	CK+ cells/ 2×10^6 MNC BM[b]	TTP[c] (mo)	OS (mo)
1	0		n.d.	0	36+	42+
2	0		0	n.d.	12	42+
3	26#		105#	0	31+	39+
4	0		0	0	23+	35+
5	0		0	0	26+	33+
6	0		0	0	17+	34+
7	0	Positive	0	0	25+	32+
8	2#		0	0	19+	29+
9	4		0	0	22+	28+
10	0		n.d.	n.d.	17+	25+
11	0		n.d.	0	6	15
12	0		110#	n.d.	26+	32+
13	0		n.d.	n.d.	LFU	LFU

[a] Before therapy.
[b] After therapy.
[c] TTP, time to progression; #, and 17-1A positive; LFU, lost to follow-up; n.d., not determined.

have CK+ cells in the BM. A complete follow-up was obtained in 10 of 30 patients with 4 of 10 (40%) patients remaining CK+ after chemotherapy. Markedly high levels were evaluated for Her-2/neu before chemotherapy.

The detailed data for patients with metastatic disease are shown in Table 4 and are divided into data for patients with complete follow-up and patients with incomplete follow-up. Evaluation was performed as indicated for patients with locally advanced breast cancer. In total, tumor cell contamination of PBSCs by immunocytochemical staining was found in 7 of 24 (29%) patients and correlated with decreased OS in 5 of 7 patients. The 17-1A antigen was present on CK+ cells of 12 of 18 (66%) patients before therapy (not determined in six cases), on all of the CK+ PBSCs, and on 2 of 4 CK+ cells after therapy. Although the number of completely monitored patients before and after chemotherapy was quite small, it is obvious that those patients (patients 5–7) with tumor cell contamination of BM and/or the PBSCs as well as enhanced serum Her-2 levels only had a partial response or no change to chemotherapy. On the other hand, those patients with no or a low number of micrometastatic cells in the BM before and after chemotherapy and no contamination of PBSCs (patients 1–4) could achieve a complete remission. Twenty of 30 patients were not completely analyzed for micrometastatic cells in the BM because of progressive disease, no application of a second course of HD chemotherapy, or refusal of marrow aspiration. As apparent from patients 14, 26, and 28–30, markedly enhanced numbers of CK+ cells in the BM and in PBSCs were associated with a rapid progress and short OS. On the other hand, tumor cell-free BM and PBSCs, as well as low Her-2 serum values, resulted in a mean overall survival of 30 months (patients 11, 16–18, and 23).

Serum analysis in this study group demonstrated enhanced levels of Her-2/neu in 6 of 30 patients (20%), resulting in a partial response or progressive disease and a short overall survival in most of the patients.

DISCUSSION

Various immunocytochemical assays for epithelial tumor cells, including our own, have been shown to detect as few as

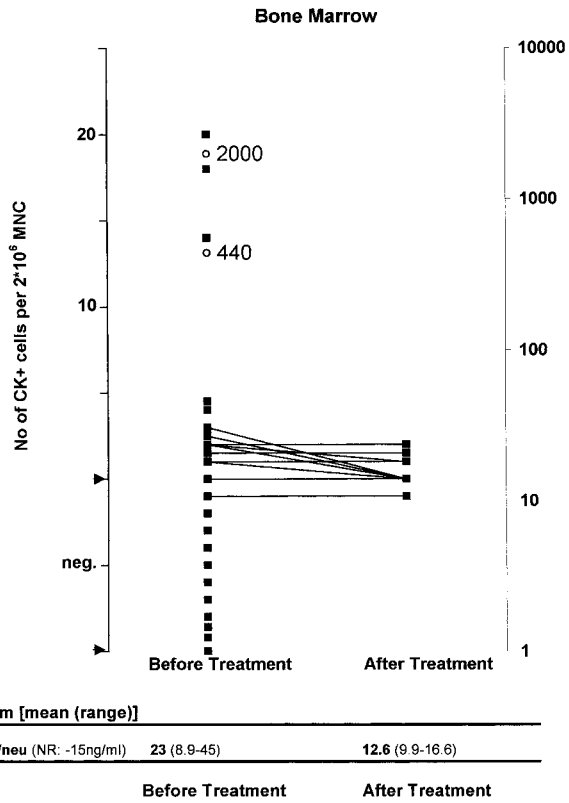

Fig. 2 Monitoring of CK+ cells in group 2 patients before and after treatment. ■, the number of CK+ cells/2 × 10^6 MNCs. ○ values refer to the second scale (1–10,000). The table under the figure summarizes the results for Her-2/neu before and after treatment; neg, negative; NR, normal range.

one tumor cell among 1 × 10^6 hematopoietic cells. IC using CKs, abundantly expressed as stable proteins in the majority of epithelial tumors, is currently the standard method for early detection of occult tumor cells in patients with solid tumors (28). Although it is still unclear whether this level of sensitivity is adequate, enrichment techniques can increase the sensitivity of tumor cell detection (29, 31, 32).

We combined IMS with IC and detected CK+ cells in 23% of group 1 patients and in 60% of group 2 patients before chemotherapy. Whereas no CK+ cells were found in patients of group 1 after chemotherapy, 4 of 10 (40%) patients of group 2 remained CK+ and 4 other patients who were positive before chemotherapy had negative aspirates afterward. After a median follow-up of 33 and 29 months, the OS rate was 92% in group 1 patients and 43% in group 2 patients, respectively. Increased numbers of CK+ cells in the BM and in PBSC collections were associated with a rapid progress and short overall survival, whereas patients with tumor cell-free BM and PBSCs, as well as low Her-2/neu serum values, had a mean overall survival of 30 months.

The fact that no CK+ cells could be detected in group 1 patients and in some group 2 patients after therapy are contradictory to the results by Braun *et al.* (14). Our findings might have different reasons. On the one hand, only unilateral BM

Table 4 Metastatic disease: monitoring of group 2 patients

	CK+ cells/2 × 10^6 MNC BM[a]	Her-2/neu	CK+ cells/ml PBSC	CK+ cells/2 × 10^6 MNC BM[b]	Response [TTP[c] (mo)]	OS (mo)
Complete follow up						
1	2		0	0	CR (4)	28+
2	2∼		0	0	CR (12)	28+
3	1#		0	0	CR (11)	37+
4	1#		0	1#	CR (14)	31
5	2	Positive	6#	1	PR (11)	33+
6	1#		95#	1#	PR (14)	38+
7	0	Positive	5#	0	NC (10)	24
8	0		0	0	P (6)	36+
9	2∼		0	0	P (8)	38
10	1∼		0	1	P (21)	27
Incomplete follow up						
11	0		0		CR (20+)	22+
12	0	Positive	0		CR (14)	20
13	14#		0		CR (8)	LFU
14	4#		16#		CR (12)	17
15	4#		n.d.		PR (15)	32
16	0		0		PR (15)	34+
17	0		0		PR (11)	29+
18	0		0		PR (22+)	33+
19	18#	Positive	0		PR (10)	22+
20	0		n.d.		PR (2)	20
21	0		n.d.		NC	29
22	1#		n.d.		NC (2)	12
23	0		n.d.		NC (10)	35+
24	0		0		P	12
25	2#		0		P	6
26	440#	Positive	1828#		P	3
27	0	Positive	n.d.		P	3
28	2		60#		P	7
29	20#		0		P	8
30	2000#		16#		P	10+

[a] Before therapy.
[b] After therapy.
[c] TTP, time to progression; #, and 17-1A positive; ∼, expression of the antigen 17-1A was not analyzed; CR, complete remission; LFU, lost to follow up; NC, no change; n.d., not determined; P, progressive disease; PR, partial response.

aspirates could be obtained from most of the patients, so that we might have some false-negative results. On the other hand, we cannot exclude that tumor load is below our sensitivity of tumor cell detection, especially in group 1 patients. Furthermore, although enrichment of tumor cells was performed in most cases, the results obtained in our group in cell culture models differ from those in clinical samples. This might be explained by the construction of the beads carrying only one antibody against one epitope on tumor cells, so that because of the antigenic heterogeneity of tumor cells, especially in breast cancer, tumor cells expressing other epitopes will not be caught by our technique. In the future, "cocktails" of beads, directed against a variety of antigens, may overcome this problem. Nevertheless, the presence of CK+ cells after therapy in group 2 patients indicates that tumor cells can survive HD chemotherapy.

In patients with metastatic disease, high numbers of CK+ cells were also shown in studies by Cooper *et al.* (34), who found 60–80% of microscopic tumor in histologically normal BM harvests. In this study, as well as in our setting, the majority of patients developed progressive disease in prior metastatic sites, which suggests that inadequate cytoreduction rather than seeding of new tumor sites was the reason for treatment failure. Thus, CK positivity of the BM after therapy may be suitable to predict response to systemic therapy.

Tumor cells still present after chemotherapy may as well be a result of reinfused PBSCs contaminated with tumor cells. To address this question, we also analyzed the PBSC samples for circulating CK+ cells. The overall detection rate in both patient groups was 27% (9 of 33 patients), with the highest probability being documented in patients with metastatic breast cancer. Similar detection rates for tumor cell contamination in PBSCs were found by three other groups studying CK+ cells in PBSCs (25, 27, 35). In contrast to studies with BM, the relationship between tumor cell contamination of PBSCs and its clinical significance has not been extensively investigated. Whereas Sharp *et al.* (26) showed that occult tumor cell contamination of PBSCs conferred a worse prognosis, Stadtmauer *et al.* (27) demonstrated that contamination of PBSCs was not correlated with decreased survival. The latter findings are in accordance with our data for group 1 patients with contaminated PBSC collections but no evidence of disease after a follow-up time of 39 and 32 months, respectively. In addition, the same tendency could be shown in two of seven group 2 patients who are still alive after a follow-up time of 33 and 38 months, respectively.

Although progenitor cell collections were not analyzed for clonogenic tumor cells, it appears likely that not all of the tumor cells detected by IC are able to grow *in vitro* (36). Furthermore, it is not known whether tumor cells in PBSCs can survive after cryopreservation in DMSO (10%). In addition, host immune factors may be important in eliminating small numbers of residual tumor cells.

In some other studies, the role of contaminated PBSC collections in stage IV breast cancer patients is discussed controversially. One study (37) supports the hypothesis that reinfused cells that were not exposed to HD chemotherapy and did not develop multidrug resistance may contribute to relapse. In contrast to these studies, Cooper *et al.* (34) postulated that the degree of tumor cell contamination may be rather a biological marker of residual tumor burden than contribute directly to relapse. Alternatively, this group postulated that there could be a threshold above which reinfused tumor cells confer a worse prognosis.

Data by Ross *et al.* (36) support the current opinion that PBSC collections may be preferred to BM as source of hematopoietic stem cells for autologous transplantation because of a lower degree of tumor cell contamination. This also holds true for our trials that identified 2 of 3 CK+ BM patients in group 1 and 9 of 15 CK+ BM patients in group 2 with no CK+ cells in their stem cell grafts. Only two patients without immunocytochemically detectable BM involvement (patient 12 in group 1 and patient 7 in group 2) had PBSC micrometastases. Thus, only in some instances, BM may provide a less contaminated source of hematopoietic stem cells.

The heterogeneity of solid tumors (different cell-cycle phase; mechanisms of resistance) poses a problem for all kinds of therapy and limits the chance of complete elimination of all of the residual tumor cells. The success of chemotherapy, aimed at proliferating cell populations, may be limited by the fact that many of the residual systemic tumor cells may be nonproliferative or dormant (16). In this context, Braun *et al.* (17) showed the heterogeneity of antigen expression in breast cancer, resulting in CK+/17-1A+ and CK+/Her-2/neu+ cells present in the BM before therapy. Furthermore, the persistence of isolated tumor cells in the BM after chemotherapy with taxanes and anthracyclines in high-risk breast cancer patients was shown to be an independent predictor for reduced overall survival in a multivariate analysis (14). Our study provides a first insight into the changes in the pool of micrometastatic cells during HD or intensified chemotherapy. We clearly demonstrate that micrometastatic cells survive even high doses of chemotherapy as demonstrated by the analysis of PBSCs and BM. Furthermore, most of the residual cells express the immunotherapeutic antigen 17-1A on their surface. Her-2/neu, as one further candidate for antibody-based immunotherapy, was not analyzed on these residual cells but has been demonstrated to be present on breast cancer cells before chemotherapy (16, 17). In addition, overexpression of Her-2/neu before chemotherapy was associated with partial response or progressive disease, and it has been suggested that blood-borne Her-2/neu-CK clustered cells are the possible precursors of distant metastases (38). In our study, the role of Her-2/neu could be demonstrated by serum analysis of Her-2/neu. Although enhanced levels could be demonstrated in patients with metastatic disease before chemotherapy, the measurement of Her-2/neu in serum samples is not able to detect residual disease after chemotherapy.

Thus far, however, the first encouraging results of Phase II trials with HD chemotherapy have not been confirmed in randomized studies. Whereas Stadtmauer *et al.* (3) and Rodenhuis *et al.* (4) could not show any benefit of HD chemotherapy as compared with conventional treatment in patients with metastatic disease, this issue seems to be not yet definitely resolved in patients with locally advanced disease, and the final results of ongoing trials must be awaited. Although the use of HD chemotherapy with PBSC support has been questioned recently, follow-up and prospective clinical trials will have to prove the clinical relevance of micrometastatic cells in PBSCs and BM even after HD chemotherapy. Nevertheless, in upcoming clinical trials, the characterization of residual cells might help to identify those patients who could benefit from additional cell cycle-independent treatment protocols. Among several proposals, antibody-based immunotherapy targeting the 17-1A antigen or Her-2/neu has been proposed recently as an effective treatment in breast (21, 22) and colorectal cancer (23). Furthermore, purging of stem cell grafts from breast cancer cells resulted in effective elimination of tumor cells (24, 25).

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