DETECTION OF OCCULT TUMOUR CELLS IN BONE MARROW AND BLOOD IN BREAST CANCER PATIENTS

Methods and clinical significance

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Immunocytochemistry using tumour-associated monoclonal antibodies has led to improvements in the ability to detect occult breast cancer cells in bone marrow aspirates and peripheral blood. Nevertheless, the immunocytochemistry method needs to be further developed before it can be used routinely in the clinic. Reverse transcription polymerase chain reaction assays (RT-PCR) that screen for carcinoma-specific expression of mRNA in bone marrow and blood have been developed. However, it is not yet clear whether the most frequently employed RT-PCR assay for cytokeratin 19 has the specificity required to be safely used in the clinic. In spite of many unsolved standardization problems with micrometastatic detection methods, recent data show that the presence of occult tumour cells in the bone marrow at diagnosis and in the reinfused autograft after high-dose therapy appears to increase the rate of recurrence in the patients.

The majority of breast carcinomas are localized to the breast and axilla when first diagnosed, and are considered potentially curable. However, about 50% of these patients will develop recurrent disease within the subsequent few years. Even in patients without tumour involvement in axillary lymph nodes (LNs) at surgery, there is a death-rate of 25% over a decade (1). Previous reports have demonstrated that adjuvant chemotherapy improves the outcome of both lymph node positive (LN+) and negative (LN−) breast cancer patients (3, 4). About 70% of LN− breast cancer patients are cured by primary surgery without adjuvant therapy. Therefore, there is no justification for treating this group of LN− patients with adjuvant chemotherapy. Prognostic factors such as tumour size and histological grade have recently been reported to be of clinical value in selecting LN− patients with poor prognosis (5, 6). However, when these factors are used, there will still be a high proportion of LN− patients belonging to a low-risk group who will experience recurrences of their disease. This emphasizes the need for additional prognostic factors.

The presence of occult breast cancer cells in bone marrow at diagnosis has been reported to correlate with relapse-free survival (7–11). In spite of these promising results few cancer centres are exploring the use of these techniques for micrometastatic detection in the clinic.

New treatment modalities for breast cancer patients are being developed. High-dose chemotherapy with autologous haematopoietic progenitor cell support is being used with increasing frequency to treat breast cancer patients with poor prognosis. The most common reason for the ultimate failure of an autologous transplant is not lack of engraftment, toxicity of therapy, or infection, but rather relapse of disease. There is evidence that reinfusion of autografts containing tumour cells can contribute to relapse and influence patient outcome after high-dose treatment (12). Tumour cell contamination can be observed in histologically normal bone marrow autografts in patients undergoing high-dose treatment using sensitive immunocytochemical techniques. Peripheral blood progenitor cells (PBPC) autografts are increasingly used in the belief that

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these products will have a low probability of containing tumour cells. Recent findings confirm that although tumour cell involvement is less extensive in PBSC autografts than in bone marrow, it is still frequently found in PBPC collections from breast cancer patients (13, 14). Consequently, detection methods for minimal residual disease in the autografts, together with techniques to remove tumour cells are of growing importance in patients receiving high-dose therapy with stem cell support.

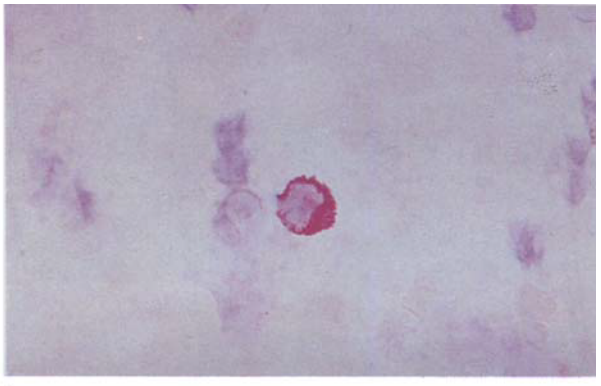
Immunocytochemistry employing monoclonal antibodies (mAbs) is the most frequently used method to detect tumour cells in bone marrow and blood. Such a method should be specific and the mAbs used should not cross-react with normal cells. Since the mAbs employed are tumour associated and not specific against breast cancer cells, these methods have their limitations. As will be outlined in this article, there is a need for standardization and improvement of immunocytochemical methods before they can be recommended for routine use in the clinic. Likewise, other sensitive methods such as reverse transcriptase polymerase chain reactions (RT-PCR) and enrichment of tumour cells followed by immunodetection need to be further developed before the clinical usefulness is established.

Material and Methods

Immunocytochemistry

Sensitive immunocytochemical methods using tumour-associated monoclonal antibodies and alkaline phosphatase anti-alkaline phosphatase (APAAP)-staining techniques to detect occult micrometastatic tumour cells in blood and bone marrow have been developed (15). In most reports cytospins containing a total number of $0.5-1 \times 10^5$ mononuclear bone marrow cells were tested. Recently, a new cytocentrifuge method has been developed making it possible to test 0.5×10^6 cells on each slide.

We perform routinely immunocytochemical evaluation of a minimal 4 slides giving a total number of 2×10^6 mononuclear cells from the blood or bone marrow of each patient (16). The slides are air-dried overnight and fixed for 10 min in acetone. The slides are then incubated for 30 min in a moist chamber with a 1:20 dilution of the anticytokeratin primary antibodies AE1 and AE3 (Signet Laboratories, Dedham, MA, USA) followed by two washings with Tris-HCL. As a second step, a polyclonal rabbit anti-mouse antibody (Dako, Glostrup, Denmark) is added and after incubation for 30 min the cells are washed twice with Tris-HCL. Finally, preformed complexes consisting of alkaline phosphatase monoclonal mouse and anti-alkaline phosphatase (Dako, Glostrup, Denmark) are added for 30 min. After washing twice with Tris-HCL, the colour reaction of antibody binding cells is achieved after a 10 min incubation with 0.26% New Fuchsin solution (Aldrich Chemical Company, Milwaukee, Wisconsin, USA). In addition, all slides are counterstained with haematoxylin in order to study the cellular morphology. As a negative control one slide is incubated first with isotype-matched mouse myeloma immunoglobulins (Bionectins, Maryland, USA) and thereafter with the APAAP technique as described above. The stained slides are mounted in glycerin-gelatine and examined under a light microscope by an experienced pathologist. Only cells that have the antibody-binding colour reaction and contain morphology as epithelial cells are scored as breast cancer cells (see Fig. 1A).



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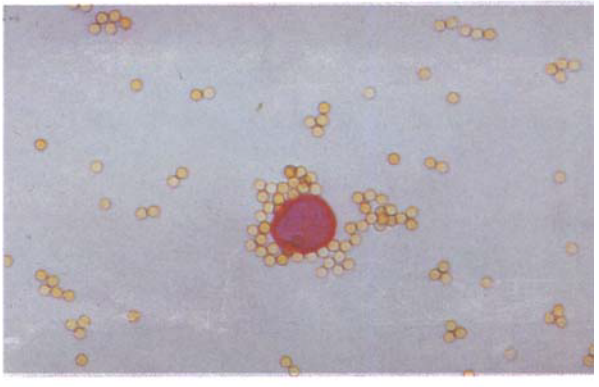

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*Fig. 1.*A Micrometastatic breast cancer cell detected in peripheral blood progenitor cells collected from a high risk stage II breast cancer patient. B: Immunomagnetic selected breast cancer cell from bone marrow (16).

Polymerase chain reaction

Polymerase chain reaction (PCR) is based on an in vitro enzymatic amplification of a specific target DNA segment, resulting in a highly specific, 10^5-10^6 fold enrichment of the sequence of interest. Cloning the breakpoints of specific translocations makes it possible to use amplification by PCR to detect tumour cells containing the translocation. One example of how PCR can be used in the clinic to detect micrometastatic disease is in patients with lymphomas. The t(14;18) translocation, resulting in the

juxtaposition of the proto-oncogene bcl-2 with the immunoglobulin heavy-chain locus on chromosome 14, appears in approximately 85% of patients with follicular lymphomas, and in 30% of high-grade diffuse non-Hodgkin's lymphomas. This PCR technique permits the detection of one lymphoma cell among 10^5 normal cells. In low-grade lymphomas, bone marrow infiltration is common at diagnosis. In spite of successful tumour reduction with disappearance of enlarged lymph nodes, residual bone marrow infiltration detected by PCR can be observed, virtually in all patients. Based on these observations, removal of contaminated lymphoma cells in autografts used to reconstitute haematopoiesis after high-dose therapy of follicular lymphomas appears to be of clinical value (17).

The use of PCR requires that the malignant cells carry a clonal somatic mutation in their genome that is absent in normal cells. Unfortunately, solid tumours such as breast cancer do not meet these requirements. In spite of this, several groups have developed reverse transcription polymerase chain-reaction assays (RT-PCR) that screen for carcinoma-specific expression of mRNA in mesenchymal organs such as bone marrow and blood. RT-PCR assays for cytokeratin 19 have been reported to be specific for detection of breast cancer cells in bone marrow (18,19). However, some groups have experienced false positive results when this method was used for tumour cell detection in bone marrow and peripheral blood, since healthy volunteers also express cytokeratins when sensitive PCR methods were used (20, 21). A new RT-PCR assay targeting Carcinoembryonic Antigen (CEA) expression in CEA-positive carcinomas has been developed (22). The use of this RT-PCR has been reported to be specific, but it is known that CEA is not expressed in all breast carcinomas.

Culture methods

Immunocytochemistry and PCR assays do not measure the viability or clonogenic capacity of the detected cells. For this purpose a culture technique must be used. Culturing fresh tumour cells from autografts is still under investigation, since the conditions required to expand tumour cells in vitro are not well defined for most types of cancers. In spite of these limitations, clonogenic tumour cells found in autografts from lymphomas and breast cancers suggest an unfavourable prognosis for the patients after high-dose treatment (23, 24).

Results and Discussion

Immunocytochemistry

Depending on the monoclonal antibodies used in conjunction with immunocytochemistry, the frequencies of bone marrow positive patients will differ. As indicated in Table 1, several studies show that 7.5–49% of node-negative breast cancers and 11.5–60% of node-positive cancers contain occult tumour cells in the bone marrow at diagnosis. Diel et al. (8), employing an antibreast mucin antibody (TAG-12) for micrometastatic detection in bone marrow, found that among a total of 406 patients tested 60% of LN+ patients had tumour cells present in the bone marrow while 30.5% of LN− patients were positive. In contrast, when an anticytokeratin 18 mAb was used only 11.9% of LN+ and 7.5% of LN− patients had tumour cells present in the bone marrow (9). Mansi et al. (7), employing a mAb that binds to the epithelian membrane antigen, show immunoreactivity in 33% of the LN+ and 19% in the LN− patients. In a more recent study Harbeck et al. (11) applied a cocktail of antibodies and found that 49% of LN− patients had occult tumour cells in the bone marrow.

There may be multiple explanations for these diverging results on micrometastatic disease in bone marrow in early breast cancer. Some antibodies used for detecting minimal residual disease in breast cancers cross-react with normal haematopoietic cells (15). This will lead to false positive

Table 1

Immunocytochemical detection of breast cancer cells in bone marrow

Marker Ag/mAb	No. of pat	Detection rate			References
		LN+	LN−	All	
EMA/anti-EMA	350	33%	19%	25%	Mansi et al. 1991 (7)
TAG12/2E11	406	60%	30.5%	45%	Diel et al. 1994 (8)
CK – 18/CK2	95	11.9%	7.5%	9.5%	Schlimok et al. 1987 (9)
C26,T16 CKIF/AE1	51	41%	27%	37%	Cote et al. 1991 (10)
EMA/Anti-EMA (E29) CK8, 18, 19/Anti-CK					
TAG12/12H12	100	30%	49%	38%	Harbeck et al. 1994 (11)
CKIF/AE1,AE3	58	36%	–	36%	Kvalheim et al. 1996 (26)

EMA = epithelian membrane antigen, TAG12 = tumour-associated glycoprotein 12 antigen, CK-18 = cytokeratin 18 antigen, CK8, 18, 19 = antibody-recognizing epitopes on cytokeratin 8, 18 and 19. C26, T16 = cell surface antigens, CKIF = cytoskeletal cytokeratin intermediate filaments antigens

Table 2

Prognostic significance of tumour cells in bone marrow detected by immunocytochemistry in primary breast cancer

References	Percent BM+ pat	Median follow-up (months)	Recurrence rate		P-value
			BM+ (%)	BM− (%)	
Mansi et al. 1991 (7)	25	76	48	25	<0.005
Diel et al. 1994 (8)	45	32	77	23	0.0001
Harbeck et al. 1994 (11)	38	34	39	15	0.0011

samples. Moreover, it has been reported that other antigens such as cytokeratin 18 antigen are down-regulated in micrometastatic adenocarcinoma cells. When using mAb to target this antigen on tumour cells, no binding will take place and false negative results might occur. To overcome the heterogeneous antigen expression on tumour cells, it seems necessary to use a combination of antibodies. However, to avoid reactivity against non-epithelial cells these antibodies should be carefully selected.

In the studies reported in Table 1 the breast cancer patients were stratified only according to lymph node involvement and not for other prognostic factors that influence the recurrence rate of the disease. It remains to be confirmed whether there is any correlation between micrometastasis and other bad prognostic factors.

We have studied a uniform cohort of 59 high-risk breast cancer patients, defined as patients with >8 positive lymph nodes. Of 2×10^6 mononuclear cells from each patient tested, 36% had micrometastases in the bone marrow at diagnosis using immunocytochemistry and anticytokeratin antibodies. The mean number of tumour cells detected was 4 with a range of 1–100 tumour cells in each patient (25).

Despite the need for immunocytochemistry methods to be improved and standardized, the presence of micrometastases in bone marrow at diagnosis has been associated with increased risk of systemic relapse. Table 2 shows that from three large studies (7, 8, 11) the presence of tumour cells in the bone marrow at diagnosis is a significant predictor for later recurrences. Still, there exists a relatively high percentage of bone marrow negative patients who relapse. Assuming that the presence of tumour cells in the bone marrow is the most prominent predictor of later relapse, these patients might have false negative bone marrow due to the limitations of the detection method used.

Monitoring micrometastases during therapy should possibly yield information about the efficacy of the treatment. In 28 patients with high-risk breast cancer we measured the presence of tumour cells in bone marrow and blood prior to therapy, and in granulocyte-colony stimulating factor (G-CSF) mobilized peripheral blood progenitor cell products (25). After 3 cycles of an efficient chemotherapy with 5-fluorouracil, epiadriamycin and cyclophophamide (FEC) 25% of the PBPC products still contained tumour cells (Table 3). As some studies indicate that tumour cells in the autografts might contribute to the relapse in the patients when reinfused, we have developed a purging procedure to remove the malignant cells from the autografts (26). Sclimok et al. (27) monitored the presence of tumour cells in bone marrow from patients treated adjuvantly with a mAb against colorectal cancer. In this study it was concluded that monitoring was only possible in patients with a high number of tumour cells in the bone marrow. Furthermore, this method was not feasible for the majority of patients since the number of tumour cells detected in the bone marrow was below 10 per 10^6 mononuclear cells.

Table 3

Frequencies of immunocytochemical detection of tumour cells in 28 high-risk breast cancer patients in bone marrow and peripheral blood at diagnosis and in G-CSF mobilized peripheral blood progenitor stem cell product (PBPC) after 3 cycles of chemotherapy

Bone marrow n = pat. (%)	Peripheral blood n = pat. (%)	PBPC n = pat. (%)
13 (46%)	4 (14%)	7 (25%)

By performing double staining of individual tumour cells in the bone marrow, it has been shown that metastatic cells have a heterogeneous expression of antigens such as Major Histocompatibility Class I antigen and antigens against different proliferation-associated molecules (28, 29) In this study down-regulation of MHC class I antigens on tumour cells was observed. If this observation is a constant finding, the lack of MHC class I on tumour cells in vivo might allow them to escape from lysis by cytotoxic T-lymphocytes. Pantel et al. (28) studied the presence of proliferation antigens on tumour cells and their data indicate that many of the micrometastatic tumour cells present in the bone marrow are dormant. Until now a small number of patients and a limited number of tumour cells from these patients have been studied. Even if no firm conclusions can be drawn the double-staining method opens for further characterization of the tumour cells. It is hoped that this will bring us more knowledge about metastatic breast cancer cells.

Polymerase chain reaction

The results of RT-PCR examinations of bone marrow from patients with carcinomas should be interpreted with caution due to the risk of false positive results. Nevertheless,

Table 4

Detection of occult breast cancer cells in bone marrow by the reverse-transcriptase polymerase chain reaction for cytokeratin 19(CK19) in relation to clinical stage of the disease

Total No. pat.	No. of CK 19 LN+ pat. = 33	RT PCR+ pat. Metastatic pat. = 50	Reference
83	19 (57%)	41 (82%)	Fields et al. 1996 (19)

the use of CK RT-PCR to detect breast cancer cells in bone marrow has recently been reported to be of clinical significance (19). In Table 3 it can be seen that when RT-PCR for cytokeratin K19 was used, 57% in 33 LN+ patients had positive RT-PCR signals. Out of 50 breast cancer patients with metastatic disease, 82% were positive. In patients undergoing high-dose therapy the presence of CK19 RT-PCR positive cells in the bone marrow prior to treatment was a bad prognostic factor. This was, for unknown reasons, most prominent in patients with metastatic disease. There is a need to confirm these data in a larger cohort of patients before CK19 RT-PCR can be used routinely in the clinic.

Future perspectives

Usually among non-metastatic breast cancer patients only 1–10 tumour cells per 2×10^6 normal bone marrow cells are detected by immunocytochemistry. Therefore, to improve the sensibility and specificity of the detection methods there is a need to analyse a higher number of bone marrow cells from each patient. Standard immunocytochemistry methods are cumbersome if large numbers of cells are to be analysed. Based on our previous experience with purging of tumour cells and positive enrichment of haematopoietic progenitor cells (CD34+ cells) from bone marrow and blood (26, 30), immunomagnetic bead separation techniques for enrichment and detection of viable breast cancer cells in bone marrow and peripheral blood are being developed (16, 30). Briefly, immunobeads coated with mAbs that bind to carcinoma cells are added to mononuclear bone marrow cell suspension. After a 30 min incubation the bead/tumour cell complexes are formed. By placing a flat cobalt samarium magnet to the wall of the tubes the rosetted cells are fixed to the plastic wall, and unbound cells can be removed. After repeating this procedure twice in order to remove unbound cells efficiently, the bead/tumour cell complexes are centrifuged down on cytospin slides. To visualize the tumour cells, an immunocytochemistry method employing anticytokeratin antibodies and APAAP technique is used. Fig. 1B shows an immunobead-selected cancer cell from bone marrow from a breast cancer patient with non-metastatic disease. Enrichment of tumour cells by the immunomagnetic method is very efficient and reproducible, allowing us to have more tumour cells to study from each patient. This gives increased sensitivity and specificity. Together with additional techniques such as double staining, culturing methods or PCR, enrichment of tumour cells opens up the possibility for detailed studies of the selected cells.

In conclusion, detection of micrometastases appears to be a method that might bring new insight into the understanding of the metastatic process. Although improvements in detection methods are needed, convincing data exist showing that detection of occult breast cancer cells in bone marrow and blood could be of great clinical value.

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