Eur J Cancer, Vol. 26, No. 8, pp. 865–867, 1990.
Printed in Great Britain

0277–5379/90 $3.00 + 0.00

Papers

Use of Monoclonal Antibody MBr1 to Detect Micrometastases in Bone Marrow Specimens of Breast Cancer Patients

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Bone marrow specimens obtained from 121 breast cancer patients immediately after surgery were examined by an immunofluorescence method with monoclonal antibody MBr1 to detect tumour cells undetectable by other diagnostic procedures. 80 women were node-negative and 41 node-positive. In no case could conventional histology demonstrate tumour cells, whereas MBr1 was positive in 20 (16.5%) of the 121 cases. No difference was observed in MBr1 positivity between node-negative and node-positive cases (17% vs. 15%). With regard to clinical outcome (median follow-up 48 months) 27 women relapsed, including 6 of 20 MBr1-positive and 24 of 101 MBr1-negative patients. First distant metastases or death from progression of disease were taken as end-points. Multivariate analysis showed that the additional contribution of MBr1 positivity, after making allowance for other prognostic factors, was negligible.
Eur J Cancer, Vol. 26, No. 8, pp. 865–867, 1990.

INTRODUCTION

THE STATUS of axillary lymph nodes at diagnosis is the most important prognostic factor in women with operable breast cancer. Prognosis is worse in node-positive patients and correlates with the number of affected nodes. Nevertheless, a quarter of node-negative women have distant metastases within 10 years of diagnosis [1]. As a consequence, many other prognostic factors have been investigated to identify those node-negative women who would be at higher risk of relapse despite negative axillary nodes. The absence of hormone receptors and high ^{3}H-thymidine labelling indices also have prognostic implications; but it is not possible to identify with certainty node-negative patients who will eventually have distant metastases [2–4].

Since the skeleton is the most frequent site of metastases, attempts have been made to detect micrometastatic bone marrow lesions with monoclonal antibodies capable of recognising a specific keratin on breast carcinoma cells, a cytoplasmic keratin, on bone marrow samples from breast cancer patients. MBr1, from the National Cancer Institute, Milan, is unreactive on normal bone marrow cells and has been studied in 159 women operated on for breast cancer [5]. For 121 of these patients adequate clinical follow-up was available. We have assessed the prognostic significance of having MBr1-positive cells in bone marrow.

PATIENTS AND METHODS

Bone marrow specimens from 121 women with breast cancer, admitted to the Division of Surgical Oncology C, from 1982 to 1985, were evaluated. Patients' ages ranged from 31 to 77 (median 48). All patients presented with operable cancer of the breast, clinically and mammographically assessed as T_1, T_2, N_0, N_{1a} without distant metastases (M_0). Pathological assessment was pT_1 in 95 cases and pT_2 in 25 (1 undetermined). The type of operation depended upon primary tumour size: T_1 cases underwent conservative procedures (quadrantectomy, axillary dissection and radiotherapy on the residual breast), whereas for T_2 tumours traditional modified radical mastectomy was done. Immediately after the operation, a sample of bone marrow was obtained by a Yamshidi needle biopsy on the anterior-upper iliac spine. One biopsy was considered adequate. Possible dissemination of cancer cells due to surgical manoeuvres was felt to be excluded on the basis of a small series of cases previously studied [5] for which two biopsy specimens had been obtained before and after the operation with similar results. Patients with axillary nodal involvement received adjuvant chemotherapy or hormone therapy.

Bone marrow samples in tubes containing Hank's fluid were processed in the laboratory of the Division of Experimental Oncology E for immunofluorescence. A part of the specimen was sent to the Division of Anatomic Pathology and Cytopathology for conventional histology.

The pathological diagnosis was invasive cancer for all patients. No nodal involvement was observed in 80 patients and nodal metastases were found in 41: 1 affected node in 19 women, 2–3 in 13 and more than 3 in 9.

The median duration of follow-up was 48 months. The patients had clinical examinations twice a year and annual chest X-ray, bone scan and mammography. First distant metastases or death from progression were taken as end-points. Time to each of these events was measured from the date of surgery. Relapse-free survival in patients with distant metastases was analysed by the Kaplan–Meier product-limit method [6].

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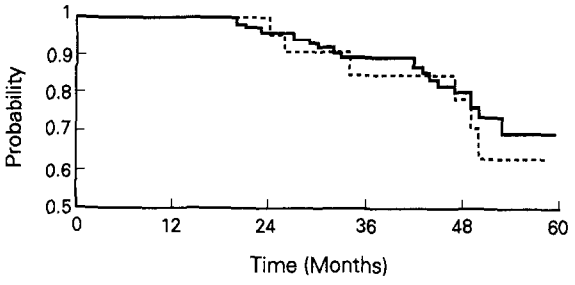

Fig. 1. Relapse-free survival in patients with distant metastases. —— = MBrl-negative and - - - - = MBrl-positive.

Preliminary graphical analysis suggested that the proportional hazard assumption was not tenable. On the other hand, the plots of log odds (probability of surviving/probability of dying) against log time for all the categories of variables showed parallel straight lines. Therefore the joint effect of factors widely accepted to be prognostic for breast cancer patients (age at surgery, type of surgery, pathological primary size and nodal status) and of MBrl was investigated by multiple log-logistic regression [7]. This model has been found to be suitable for fitting breast cancer data previously [8, 9].

The effect of each prognostic variable was tested by the Wald statistic. The effect of MBrl status on relapse-free survival in patients with distant metastases, after making allowance for the other prognostic variables, was evaluated by the likelihood ratio test by computing the difference between the log-likelihood of the complete model and that of the model without MBrl. This difference times minus 2 is approximately distributed as a χ^2 statistic with one degree of freedom.

The patient whose pT had not been assessed was not considered in the multivariate analysis.

RESULTS

Conventional histology did not show neoplastic cells in any of 121 bone marrow samples. In contrast immunoreactive tumour cells were detected by MBrl in 20 of the 121 women (16.5%). MBrl tests were positive in 14 of 80 node-negative cases (18%) and in 6 (15%) of 41 node-positive cases.

Follow-up showed 27 distant relapses. Relapses occurred in 6 of 20 MBrl-positive and in 21 of 101 MBrl-negative patients (Fig. 1). The overlap showed that, in terms of distant spread, MBrl-positive and MBrl-negative patients were similar. Nodal status had a significant prognostic impact (Table 1). But the additional contribution of MBrl positivity was negligible: the difference between the two log-likelihoods was not significant.

Table 1. *Log-logistic multiple regression model*

Variables	Odds ratio	Wald statistics	95% CI	*P*
Age (linear component)	—	1.37	—	0.24
Type of surgery (radical vs. conservative)	1.010	0.0002	0.74–1.38	0.98
pT (2 vs. 1)	1.220	1.43	0.87–1.73	0.24
Nodal status (+ vs. −)	1.320	4.19	1.00–1.75	0.04

−2 log likelihood of model with prognostic variables = 109.41 and of complete model (prognostic variables plus MBrl) = 108.95; difference = 0.46.

No difference was observed between MBrl-positive and MBrl-negative patients in terms of the site of distant metastases. 4 of 6 positive (67%) and 13 of 21 negative (62%) cases presented with bone metastases during follow-up.

DISCUSSION

Monoclonal antibodies have been used to detect micrometastatic lesions that are undetectable by conventional diagnostic procedures. MBrl, which recognises a breast carcinoma associated antigen but is negative on normal bone marrow cells, has been tested previously [5, 10, 11], to evaluate its sensitivity and specificity, which were considered adequate.

Similar studies have been done with different methods such as conventional antiserum directed against an epithelial membrane antigen [12], a pool of three mouse monoclonal antibodies that react with different epithelial antigens [13] and a pool of monoclonal antibodies directed against cytokeratine [14, 15]. These methods are superior in detecting tumour cells compared with conventional histology. In our series, immunofluorescence was positive in 16.5% of cases, whereas histology was not positive in any of the samples. This has also been observed by others [13–15]. Shlimok *et al.* [14] found 18% of cases positive, close to the 16.5% of our series. A higher number of positive cases was reported by Cote *et al.* [13] and Ellis [15], 35% and 33%, respectively. These higher rates could be due either to a more sensitive method or to a more advanced stage of the disease in the patients under study. We evaluated only patients clinically assessed as N_0, N_{1a}, whereas Cote *et al.* included patients with locally advanced tumours and Ellis *et al.* included some patients with metastases.

With regard to the prognostic significance of MBrl positivity in our series, the risk of distant relapse was not associated with positivity, after making allowance for other well-known prognostic factors. On the contrary, the prognostic significance of nodal status was confirmed.

We realise that the observation period was short, given the long natural history of breast cancer, and our results should be considered with caution. Nevertheless, we think it premature to introduce MBrl into routine practice despite its ability to identify microfoci of neoplastic cells in bone marrow that are undectable by other procedures. Our series is still being followed up. A longer period of observation may provide more reliable and useful data on the real value of a promising diagnostic procedure.

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Eur J Cancer, Vol. 26, No. 8, pp. 867–871, 1990.
Printed in Great Britain

0277–5379/90 $3.00 + 0.00
Pergamon Press plc

Epidermal Growth Factor Receptor Expression and Suramin Cytotoxicity *in vitro*

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Twenty-five cell lines derived from nine different human cancers were tested for the cytotoxic activity of suramin. Two different initial cellular concentrations were used: C1 (800–2000 cells per well) and C2 (3000–7000 cells per well). Suramin concentrations ranged from 50 to 2500 μg/ml. Cytotoxicity was assessed by the MTT test. Epidermal growth factor receptors (EGFR) were assayed by competition analysis and Scatchard plots. In sixteen cell lines suramin had an unexpected growth stimulation effect at low concentration (50–125 μg/ml). IC_{50} varied from 21 μg/ml (osteosarcoma, OS2) to 1408 μg/ml (melanoma, CAL 24) and, within melanoma cell lines, it varied from 120 μg/ml (CAL 41) to 1408 μg/ml (CAL 24). The individual IC_{50} values were positively and significantly linked with the initial cellular density. Eighteen cell lines had measurable EGFR (six with two families of sites, twelve with one): K_d varied between 0.004 nmol/l for the highest affinity site (melanoma, CAL 7) to 1.852 nmol/l for the lowest affinity site (lung, CAL 12). There was no relation between presence or absence of EGF binding sites and distribution of IC_{50}, but for cells with measurable EGFR there was a weak but significant correlation between the number of EGF binding sites per cell and the corresponding IC_{50} ($r = -0.53$, $P = 0.021$).
Eur J Cancer, Vol. 26, No. 8, pp. 867–871, 1990.

INTRODUCTION

SURAMIN may reduce tumour growth. At a molecular level, suramin inhibits the activity of protein kinase C [1] and the cellular binding of growth factors such as transforming growth factor β and epidermal growth factor (EGF) [2]. Suramin inhibits the cellular proliferation of several human tumour cells *in vitro*, including glioma [3], prostate carcinoma [4], colon cancer [5] and non-small cell lung cancer cells [6]. Bergh [6] found a relation between the cytotoxic activity of suramin and cellular expression in EGF receptors (EGFR). Such experimental data led to tests of the activity of suramin as an anticancer drug [7]; partial responses were recorded in patients with malignancies refractory to conventional cytotoxic chemotherapy. Since *in vitro* data about the activity of suramin on tumour cells were obtained in a variety of experimental conditions, we have evaluated the cytotoxic effect of suramin on a large panel of human tumour cell lines and investigated the relation between drug efficacy and cellular EGFR content.

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MATERIALS AND METHODS

Chemicals

Suramin (Bayer, batch number 4240152) was donated by R. Bellon Laboratories (Neuilly sur Seine). A working solution was prepared before use at 100 μg/ml in Dulbecco's modification of Eagle's medium (DMEM). Human recombinant ^{125}I-EGF (ref. IM 196, specific activity 4514×10^{10} Bq/mmol, 92.5×10^4 Bq per 250 μl) and unlabelled human recombinant EGF (ref. ARN 5100) were from Amersham. DMEM, RPMI 1640, glutamine and fetal bovine serum (FBS) were from Gibco. Penicillin and streptomycin were from Meyrieux. Transferrin and insulin were from Flow. Bovine serum albumin (BSA), 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethylsulphoxide (DMSO) were from Sigma.

Cell cultures

Twenty-five cell lines derived from nine different human cancers were tested (Table 1). Cells were routinely cultured in a humidified incubator at 37°C in 8% CO_2 in air. Cells were grown