SPANDIDOS PUBLICATIONS

Maintenance immunotherapy in metastatic breast cancer

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Abstract. Maintenance chemotherapy provides only a modest survival advantage in metastatic breast cancer (MBC). We have previously shown that a maintenance immunotherapy (MI) regimen based on low-dose interleukin-2 (IL-2) and 13-*cis* retinoic acid (RA) improved the lymphocyte and natural killer cell (NK) counts, and $CD4^+/CD8^+$ ratio in patients with a clinical benefit from chemotherapy. With the aim of improving progression-free survival (PFS), 100 consecutive MBC patients with a clinical benefit from chemotherapy were treated with an MI. Patients with MBC were eligible if they had no evidence of progression after 6-8 courses of epirubicin-paclitaxel induction chemotherapy. Treatment consisted of low-dose IL-2 and oral RA given until progression. The primary endpoint was progression-free survival (PFS); secondary endpoints were toxicity, overall survival (OS), and changes in immunological parameters. From 04/1997 to 04/2002, 100 patients with MBC were enrolled. After a median follow-up of 49 months, median PFS and OS were 37.1 and 57.5 months, respectively. No WHO grade 3 or 4 toxicity was observed; grade 2 cutaneous toxicity and autoimmune reactions occurred in 19 and 16% of patients, respectively. A sustained improvement in lymphocytes, NKs, and in the $CD4^+/CD8^+$ ratio was observed, with respect to baseline values. In conclusion, MI with IL-2 and RA in MBC patients who do not progress after 6-8 courses of chemotherapy is well-tolerated, improves lymphocyte, NK, $CD4^+/CD8^+$ ratio, and appears to delay disease recurrence. A randomized trial is warranted.

Introduction

The prognosis of metastatic breast cancer (MBC) remains severe, despite great progress in clinical oncology. This disease is rarely cured, and the treatment goal is to prolong survival while preserving a satisfactory quality of life. Chemotherapy is indicated in patients with hormone receptor - negative, rapidly progressing, visceral disease and with disease that has become resistant to endocrine therapy. In the last two decades, the prognosis of MBC patients has improved with the use of modern chemotherapeutic agents (1). However, although high response rates may be obtained initially, the median survival rarely exceeds 26 months (1). Maintenance chemotherapy, that may provide a strategy for prolonging time to progression, offers a modest survival advantage to patients with MBC (2). The reason why such limited efficacy is observed is that chemotherapy is active on non-tumorigenic, terminally differentiated cells with restricted proliferative potential, that compose the bulk of the tumor (3,4). The cause of disease relapse, even in patients with a complete clinical response, is the presence of 'minimal residual disease' (MRD) (5). MRD is commonly composed of breast cancer stem cells that are intrinsically resistant to chemotherapy because they overexpress the multidrug-resistant transport (MDR1) gene (3,4). These cells, after pertinent stimuli, proliferate and cause tumor relapse (6). An impaired immune function, often associated with advanced breast cancer, is one of the conditions that facilitate the proliferation of MRD (7). Such a dysfunction may appear early in the course of disease and may worsen after chemotherapy (8,9). For this reason, an immunological approach to maintenance therapy is particularly appealing. The mechanism of action of the biological agents used and their toxicity are generally non-cross-reactive with those of chemotherapy, and more importantly, these agents can increase immune surveillance.

Interleukin-2 (IL-2) may be the ideal cytokine to be administered because it improves T-cell proliferation, increases the generation of cytotoxic T lymphocytes, and promotes the tumoricidal activity (10) of natural killer cells (NK). Maintenance of self tolerance through the termination of T-cell responses is also a recognized role of IL-2 (11). Another important function of IL-2 is to inhibit angiogenic activity mediated by the secretion of α-interferon and the p-10 protein (12). Bulky solid tumors that are refractory to chemotherapy have responded to high-dose intravenous IL-2 immuno-therapy, at the price of considerable toxicity (13). To better exploit the properties of IL-2 and to decrease its

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toxicity profile, IL-2 was administered subcutaneously in a phase 1B study and was combined with 0.5 mg/kg oral 13-*cis* retinoic acid (RA) for 5 days/week (14). In this study, the optimal biological dose of IL-2 was as low as $1.8x10^6$ IU. Such dosing was well tolerated, and improved the total lymphocyte count and the $CD4^+/CD8^+$ ratio in patients with several tumor types who had a tumor response or stabilization after standard chemotherapy (15-19). These results prompted us to begin a larger phase II study of low-dose IL-2 maintenance-therapy that could be self-administered by subcutaneous injection, and oral RA in patients with MBC who experienced a clinical benefit following induction chemotherapy.

Patients and methods

Patient selection. Patients were eligible if they had measurable and/or assessable metastatic disease, and no evidence of progression after six to eight courses of induction chemotherapy with epirubicin 90 mg/m^2 and paclitaxel 175 mg/m^2 (3-h infusion) administered on day 1 every 3 weeks. Other criteria included age between 18 and 75 years, Eastern Cooperative Oncology Group performance status ≤2, and a life expectancy of at least 12 weeks. Radiation therapy that did not involve the lesion chosen for measurement of response was allowed. A neutrophil count $>2x10^9$/l, platelet count $>100x10^9$/l, and normal kidney and cardiovascular function were required for entering the trial. Written informed consent, approved by the Institutional Review Board of each center, was obtained from all patients.

Treatment. One month after the last course of chemotherapy, eligible patients received self-administered subcutaneous IL-2 at a dose of $1.8x10^6$ IU daily at bedtime, 5 days/week, plus oral RA at a dose of 0.5 mg/kg of body weight, administered with meals for 5 days/week for three weeks of each month (14). After 1 week of rest, patients started a new 3-week course of therapy. Two months were considered to be a single cycle of therapy. After completing 1 year of treatment, responding patients continued to receive the same regimen as maintenance therapy for 2 weeks of each month. In the third year and thereafter, therapy was continued for 5 days of each month, according to the immune competence. Sites of injection were rotated daily, primarily using the lower abdomen and upper and lower extremities. An LH-RH analogue was administered to all pre- and perimenopausal patients with serum estradiol >40 pg/ml. Endocrine therapy with letrozole was administered to the 80 hormone receptor-positive patients until disease progression. The use of biphosphonates was possible in patients with bone loss and symptomatic bone lesions. Patients exhibiting evidence of disease progression were removed from the study and treated with a salvage chemotherapy, and were included in the analysis on an intention-to-treat principle.

Efficacy. Within 4 weeks before starting maintenance immunotherapy, the extent of metastatic disease was evaluated as an assessment of tumor response after induction chemotherapy, according to the RECIST criteria (20), with CT scan or magnetic resonance imaging scan. Thereafter, disease status was evaluated every 4 months. The primary study end point was progression-free survival (PFS). Secondary end points were overall survival (OS), evaluation of toxicity, rate of response conversion, and measurement of longitudinal changes in lymphocyte and NK cell counts.

Toxicity. Toxicity was classified by the WHO grading system; neurotoxicity grading was performed in accordance with the National Cancer Institute Common Toxicity Criteria, version 2.0. Cardiac dysfunction was graded according to the criteria of the New York Heart Association.

Statistical methods. The expected median PFS of MBC patients who achieve disease control after first-line chemotherapy was estimated to be 10 months. The minimal improvement, justifying the adoption of maintenance immunotherapy, was estimated to be at least 3 months (1). With 100 patients, the study had a power of 80% to detect a 60% improvement in median PFS (from 10 to 16 months), testing at the two-sided 0.05 significance level. All analyses were primarily performed on an intent-to-treat basis, including all patients. PFS was defined as the time from the start of maintenance immunotherapy until objective disease progression or death, whichever occurred first. Survival was calculated from the date when the immunotherapy treatment was started to the date of death from any cause. PFS and OS were calculated with the Kaplan-Meier lifetable method (21). The trial accrual was planned to be long enough to enrol enough patients, according to the historical accrual capacity of the centers. Subgroup analyses, according to the pre-specified prognostic factors, were performed to assess modifications of treatment effect in various subsets of patients. The results of the immunological parameters were expressed as the mean ± SD deviation of four determinations made in three different experiments, and the differences were determined using a repeated-measure analysis of variance. Post-hoc comparisons were performed by Tukey's honesty significant difference test. All analyses were performed using the SAS statistical package (release 8.00; SAS Institute, Cary, NC). All P-values were two-sided. Differences at $P<0.05$ were considered statistically significant.

Results

Patient characteristics. Between 04/1997 and 04/2002 a total of 118 patients received induction treatment with epirubicin plus paclitaxel as first-line chemotherapy for metastatic disease, with an accrual rate of 23 patients per year. The population of this study included many patients with metastatic disease at diagnosis because less advanced cases were treated in peripheral hospitals. Of these patients, 100 had a clinical benefit, while the remaining 18 did not undergo maintenance treatment due to disease progression (n=15) or refusal (n=3). The median disease-free interval was 23 months (range 1-48) for 55 patients, while 45 had metastatic disease at the time of first diagnosis. Infiltrating ductal carcinoma was the histological diagnosis in 90 patients, high-grade lobular infiltrating in 6 patients, and inflammatory in 4 patients. The initial stage at diagnosis was IIA for 8, IIB for 20, IIIA for 15, IIIB for 12, and IV for 45 patients. Modified radical

haracteristics of patients.

Characteristics	No.	%
No. of patients	118	100
Age, years		
Median	59	
Range	27-75	
Performance status (ECOG)		
0-1	107	90
2	11	10
Estrogen receptors		
ER^+ and/or PR^+	80	68
ER^- and/or PR^-	27	23
Unknown	11	9
Previous radiotherapy		
Yes	79	67
No	39	33
Stage at diagnosis		
IIA	9	8
IIB	24	20
IIIA	18	15
IIIB	14	12
IV	53	45
Metastatic sites		
Liver	28	18
Lung	35	23
Bone	58	38
Soft tissue	18	12
Brain	6	4
Skin	7	5
Response to chemotherapy		
Complete response	26	22
Partial response	37	31
Stable disease	37	31
Progressive disease	18	16

Twenty-eight patients had 2 or more metastatic sites.

mastectomy was performed in 52, bilateral mastectomy in 6, partial mastectomies in 35, and biopsies only in 7 patients. Seven stage IV patients received the protocol chemotherapy as primary treatment followed by palliative mastectomy. Forty-one patients had received tamoxifen as endocrine therapy and had progressed. Twenty-six pre- and perimenopausal patients with estrogen receptor-positive tumors (ER+) received an LH-RH analogue during all treatments. Adjuvant chemotherapy had been administered in 41 instances: cyclophosphamide, methotrexate, and 5-fluorouracil to 31 patients and anthracycline-based chemotherapy to 10 patients. Seventy patients received radiation therapy: 35 as adjuvant treatment in quadrantectomies, 20 after modified radical mastectomies with >5 positive axillary nodes, and 15 for palliation of metastases. Patient characteristics are listed in Table I.

Efficacy. A mean of 7.5 courses (range 6-8) of chemotherapy was administered to each of the 118 evaluable patients. Median follow-up was 49 months (minimum 60 months for living patients). At the time of protocol entry, the following responses to paclitaxel-epirubicin were recorded: complete response in 12 women (10.4, 95 CI 6-18%), partial response in 48 women (41.7, 95 CI 33-51%), stable disease in 40 women (34.75, 95 CI 26-4%), and progression in 15 women (13, 95 CI 7-21%). All patients were carefully staged before starting immunotherapy. After 487 cycles of immunotherapy, the median PFS (Fig. 1) was 37.1 months (range 2.8-120). Median OS was 57.5 months (range 11-120) (Fig. 2). Sixty-eight patients with ER^+ tumors had a response rate similar to 23 patients with estrogen receptor-negative (ER^-) tumors, but had significantly longer PFS (medians of 44.7 and 32.7 months, respectively) and OS (medians of 64.5 and 51.4). There was no statistically significant difference in OS between patients <40 or >40 years (65.4 and 49.5 months, respectively). A complete response was observed in twelve patients who initially had a partial response to chemotherapy. In particular conversion to a complete response was observed in the following sites: liver 4 times, lung twice, bone 5 times and soft tissue once. As of April 2007, 26 patients were alive (26%), and 19 were progression-free. Disease progression and deaths occurred with visceral disease in 52, bone and soft tissue in 18, brain in 3 patients, and 1 patient died of a second tumor (small cell lung cancer).

After 1 year of maintenance therapy, statistically significant improvements were observed in lymphocyte and NK cell counts and $CD4^+/CD8^+$ ratio in the 100 patients who had shown a clinical benefit. After 1 year, the 82 responding patients had further statistically significant improvements in the above-mentioned immunological parameters (Table II). In particular, lymphocyte numbers increased by 30% after 1 year of biological therapy; after 3 years, they increased by 45%. NK cell numbers raised by 60% after 1 year and by 64% after 3 year. $CD4^+/CD8^+$ ratio improved by 16% after 1 year and by 23 after 3 years.

No improvement in immunological parameters was observed in the 18 patients that had disease progression. In particular, after 1 year, the 16 remaining patients presented no statistically significant difference in lymphocyte and NK number and $CD4^+/CD8^+$ ratio with respect to the values recorded at the end of chemotherapy.

Toxicity. The adverse events resulting from the biologic therapy were acceptable (Table III) and compliance was optimal. Initially, low grade fever was observed in 8% of patients; this was avoided afterwards by administering IL-2 at night and preceding it with 500 mg acetaminophen. Grade 2 skin toxicity occurred in 19% of patients. Triglyceride elevation (twice baseline value) was observed in 6 patients. Sixteen patients manifested symptoms of autoimmune disease: thyroid abnormalities were observed in 8, urticaria in 6, and erythema nodosum in 2 patients. Hepatic toxicity was observed (low-grade abnormality of hepatic sonogram and

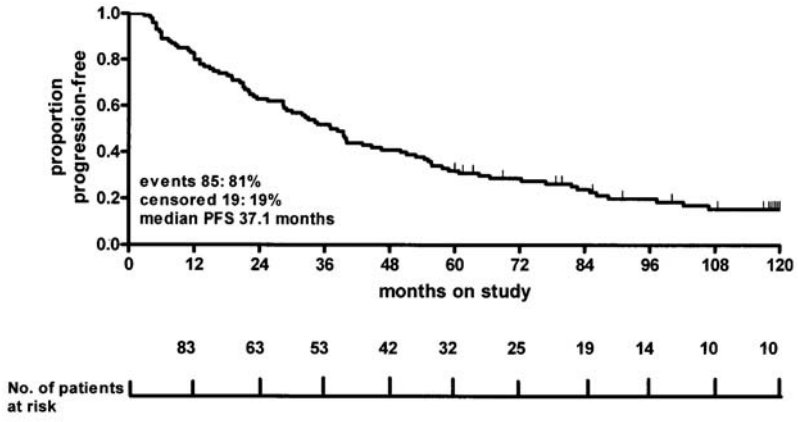

Figure 1. Progression-free survival (PFS): Median 37.1 months (range 2.8-120).

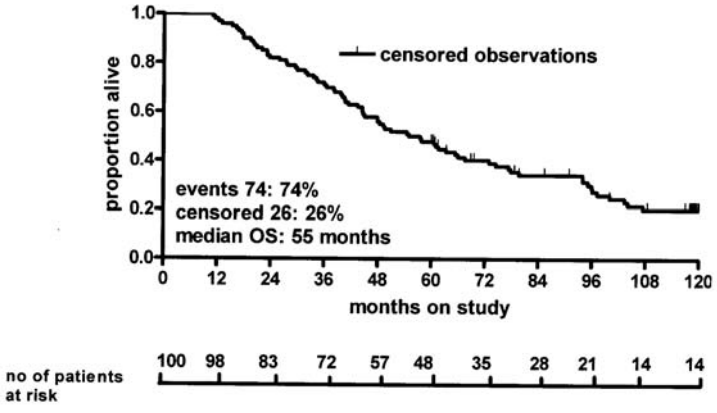

Figure 2. Overall survival (OS): Median 57.5 months (range 11-120).

Table II. Immunological parameters.

Patients' number	Baseline n. 100	1 year n. 82	3 years n.52
Lymphocyte/mm^3	1659±54	2158±64 (P<0.0001	2406±96 (P<0.0001)
Natural killer mm^3	310±18	497±24 (P<0.0001)	511±25 (P<0.0001)
CD4$^+$/CD8$^+$ ratio	1.60±0.06	1.86±0.07 (P<0.05)	1.98±0.1 (P<0.05)

liver enzyme elevation 3 times the baseline value) in 8 patients (16%). Liver biopsy, performed in two patients, showed fatty infiltration of 30 and 60% of hepatocytes, respectively, without abnormalities of the porto-biliary space, after 23 and 13 months of therapy, respectively. Both patients indulged in alcohol consumption. Retinoids were discontinued with no further problem.

Discussion

This report analyzes the results and the long-term follow-up of 100 consecutive patients with MBC treated with an anthracycline-taxane containing, standard induction chemotherapy followed with a maintenance immunotherapy alone or associated with hormonotherapy. The response rate of 63% reflects what can be expected from standard chemotherapy in this patient population (22-25).

A large retrospective study from MD Anderson Cancer Center, including 1581 patients with MBC, treated with anthracycline-based chemotherapy regimens, reported a 65% response rate with a median PFS and OS of 11.5 and 21.3 months, respectively (22).

Chemotherapy produces tumor shrinkage in advanced disease; the question is: how long this therapy should be continued without any interference with the quality of life? (2). To answer this question, several trials of maintenance chemotherapy have been conducted in the past. In a meta-analysis of 4 clinical trials that included 766 women (2), Coates *et al* indicated a statistically significant but modest survival advantage (23% longer median survival, P<0.01) for

Toxicity according to WHO grade criteria.

	No. 0 (%)	No. 1 (%)	No. 2 (%)	Total no. (%)
Hematologic				
Leucopenia	95 (95)	5 (5)	0 (0)	100 (100)
Neutropenia	99 (99)	1 (1)	0 (0)	100 (100)
Thrombocytopenia	94 (94)	6 (6)	0 (0)	100 (100)
Anemia	95 (95)	5 (5)	0 (0)	100 (100)
Gastrointestinal				
Diarrhea	95 (95)	5 (5)	0 (0)	100 (100)
Oral	90 (90)	10 (10)	0 (0)	100 (100)
Triglycerides	80 (80)	20 (20)	0 (0)	100 (100)
Cutaneous	30 (30)	51 (71)	19 (19)	100 (100)
Fever	67 (67)	17 (17)	16 (16)	100 (100)
Autoimmune reactions	66 (66)	20 (20)	14 (14)	100 (100)

women randomized to a longer duration of chemotherapy, without compromising the quality of life. The 'Manta' randomized study explored the possibility of improving PFS in patients exhibiting a clinical benefit from a taxane-anthracycline-based chemotherapy (24). With a response rate of 62.4%, the administration of additional courses of paclitaxel to patients who achieved a clinical benefit after six to eight courses of first-line chemotherapy failed to improve PFS, which was 8 months for the maintenance arm and 9 months for controls. Better results have been obtained with a pegylated liposomal doxorubicin (PLD)-based maintenance chemotherapy (25). Patients achieving a clinical benefit with sequential PLD and docetaxel were randomized to 6 further courses of PLD or observation. The toxicity profile was acceptable and the PFS of the treatment arm was 13.22 months, compared with 10.16 in the observation arm (p=0.0005).

Our trial was designed to verify if chemotherapy used as debulking strategy followed by biological agents able of improving the immune response may be effective in prolonging PFS in MBC. Due to tumor heterogeneity (26), chemotherapy is capable of eradicating non-tumorigenic, terminally differentiated cells. Nevertheless, disease may relapse in the presence of chemotherapy-resistant, slowly proliferating cells (MRD) identified as breast cancer stem cells, which are resistant to chemotherapy because of their intrinsic properties. The genetic instability of tumor cells and the clonal expansion of breast cancer stem cells may cause tumor relapse. In particular, a deficit in immune function may facilitate the growth of breast cancer stem cells. NK and T-cell function decreases following adjuvant therapy for early breast cancer (9). In addition, patients with advanced breast cancer exhibit markedly reduced blood dendritic cell (DC) counts at diagnosis. Circulating DC may be compromised by loco-regional and systemic cancer treatments, nevertheless, they respond vigorously to *ex vivo* conditioning, thus enhancing their immunostimulatory capacity and potential for immunotherapy (27,28). The induction of endogenous LAK cell activity and the production of tumor inhibitory cytokines (10) may constitute the primary cytotoxic activity of IL-2. The administration of low-dose IL-2 to patients who achieved clinical benefit from chemotherapy may have the same effect as *in vitro* experiments in which lymphocytes are incubated with tumor cells. Following injection of IL-2 into patients, the host immune effector cells may act as lymphocyte-activated killer cells in the presence of MRD. Preventing autoimmunity and limiting inflammation is performed through the amplification of T-regulatory cells (Treg) (11). While expression of FoxP3 in mice, the marker of Treg function, is strictly correlated with regulatory activity, there is increasing evidence suggesting that in humans, activated T-effector cells may also express FoxP3 (28). A decrease of Treg cells with the chronic administration of IL-2 is demonstrated by 16% of our patients developing autoimmune disease. In addition, the treatment with LH-RH analogues in pre- and perimenopausal patients produced a sustained decrease in circulating estrogens that have been described to be responsible for promoting tolerance by expanding the regulatory T-cell compartment (29). Although this was not statistically significant, one possible effect of this therapy was the trend toward a longer median survival (65 months) in our younger patients with respects to their older counterparts (49.5 months); this is the opposite of what is described in the literature (30). The combination of IL-2 and RA was chosen for its immunological properties, that led to the improved results obtained by our group in the immunotherapeutic maintenance therapy of several tumor types (15-19). In addition, retinoids have been described to induce a series of effects on breast cancer cell

lines. They enhance the secretion of transforming growth factor ß, which inhibits the growth of most epithelial cells (31). They exhibit proapoptotic effects on ER^+ and ER^- breast cancer cell lines (32). Products of retinoic acid metabolism blocking agents induce breast cancer cell differentiation (increased expression of cytokeratin and ER) and inhibit breast cancer cell growth *in vitro* and *in vivo* (33,34). Moreover, it has been shown that a novel retinoic acid metabolism blocking agent may reverse the resistance that develops with prolonged exposure to aromatase inhibitors (35). Synergism between retinoids and IL-2 has been demonstrated; RA increases the number of IL-2 receptors and the percentage of peripheral blood lymphoid cells expressing the surface markers of T-helper cells (36). An important function of retinoids is to facilitate the differentiation of immature myeloid suppressor cells ($Gr\text{-}1^+CD115^+$), which are responsible for the development of tumor-induced T-cell anergy in tumor-bearing hosts (37) with an improvement in the immune response (38,39).

In conclusion, the maintenance therapy mainly consisting of IL-2 and RA we used is a safe and feasible treatment. However, although generating a reliable working hypotheses, our results need to be confirmed through a randomized trial.

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