

Effect of peg-filgrastim-supported dose-dense adjuvant chemotherapy on the peripheral blood leukocyte phenotype in breast cancer patients

ELENA COLLOVÀ, BIANCA ROVATI, DONATELLA GRASSO, KATIA BENCARDINO,
MARIANGELA MANZONI and MARCO DANOVA

Flow Cytometry and Cell Therapy Unit, Medical Oncology Fondazione IRCCS San Matteo, Pavia, Italy

Received July 8, 2008; Accepted October 8, 2008

DOI: 10.3892/mmrr_00000066

Abstract. The aim of this study was to evaluate the effect of dose-dense adjuvant chemotherapy regimens with peg-filgrastim support on the phenotype of peripheral blood leukocytes in breast cancer patients. We evaluated the leukocyte phenotype of 14 patients aged 46-67 years undergoing 4 courses of chemotherapy with either epirubicin/cyclophosphamide (n=7) or 5-fluorouracil/epirubicin/cyclophosphamide (n=7) followed by 4 courses of taxol supported by peg-filgrastim (6 mg) administered 72 h after each chemotherapy course. The overall leukocyte number significantly increased from the first treatment course, while total lymphocytes tended to decrease with a negative peak following the 6th course ($p=0.03$). B (CD19⁺, CD20⁺) and early B lymphocyte subsets (CD20⁺/CD38⁺) significantly decreased during treatment ($p<0.05$), while T lymphocyte subsets did not show significant changes, except a decrease in T helper (CD4⁺) cells. Immature T lymphocytes (CD4⁺/CD8⁺ subset), dendritic cells (CD11c⁺) and NK cells (CD56⁺) increased with respect to the baseline. Our results suggest that dose-dense chemotherapy programs with the support of peg-filgrastim did not significantly impair the immune system of breast cancer patients and allowed for a rapid restoration of most immune competent cells. These observations may have important clinical implications with a view to vaccination or other immunotherapeutic approaches to solid tumours.

Introduction

Very complex interactions occur between tumours and the immune system. The immune system plays an important role in the control of tumour growth, and its direct anti-tumour

activity seems to have a critical impact on the prognosis of cancer patients. It has been reported that cell-mediated immunity to tumour-associated antigen may predict survival better than tumour stage, grade and lymph node status (1). However, chemotherapy (CT) is considered to be a major cause of immune deficiency in cancer patients. T-cell deficiency has been reported following cytotoxic antineoplastic therapy leading to frequent opportunistic infections (2). The immunosuppressive effects of antineoplastic agents such as alkylating agents, fludarabine, melphalan, thiotepa and cyclophosphamide are well established. Little information is available concerning the actual impact on the immune system of other widely used antineoplastic drugs, such as platinum derivatives, anthracyclines, etoposide and 5-fluorouracil.

Dose-dense CT regimens have shown very promising results in the adjuvant treatment of primary breast cancer (3), with less toxicity as compared to dose-escalation regimens. Haematological support with granulocyte-colony stimulating factor (G-CSF), either natural or pegylated, was demonstrated to reduce the duration and severity of CT-induced neutropenia, and thus infectious complications and hospitalizations. G-CSF also has an impact on the immune system; it has been shown to influence cytokine production and to modify T and B lymphocyte subsets (4-6).

Dendritic cells are a subpopulation of leukocytes, identifiable in fresh blood only through cytofluorography. Their potential applications in the immunotherapeutic management of cancer have been investigated (7).

We previously investigated the *in vivo* effect of topotecan on the peripheral blood leukocyte (PBL) phenotype and found that the drug did not negatively influence the lymphocyte subsets of either CT-naïve or pre-treated ovarian cancer patients (8). The aim of this study was to evaluate the effect of dose-dense adjuvant CT regimens with G-CSF support on the phenotype of PBLs, including dendritic cells, in patients with breast cancer.

Patients and methods

Patients. We evaluated the PBL phenotype of 14 patients, aged 46-67 years with histologically confirmed breast cancer, participating in a multicentric multinational Phase III adjuvant CT clinical trial. Patients were treated with 4 courses of either epirubicin/cyclophosphamide (EC; n=7) or fluorouracil/epi-

Correspondence to: Dr Elena Collovà, *Present address:* Medical Oncology Unit, A.O. Ospedale Civile di Legnano, Via Candiani 2, I-20025 Legnano (Milano), Italy
E-mail: elena.collova@ao-legnano.it

Key words: peg-filgrastim, dose-dense chemotherapy, breast cancer, leukocyte phenotype

Table I. Patient characteristics.

Characteristic	No. of patients
Median age	52 years (range 46-67)
Histology	
Ductal carcinoma	10
Lobular carcinoma	4
Grading	
G1	1
G2	10
G3	3
Estrogen/progesteron receptor	
Positive	11
Negative	3
C-erb B2	
Negative	12
Positive	2
Lymph node involvement	4 (range 1-22)
Surgery	
Radical mastectomy	10
QUART	4

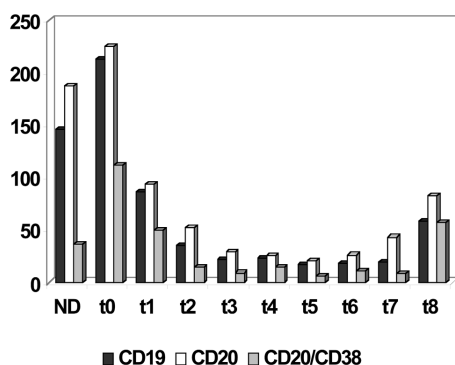


Figure 1. B and early B lymphocyte subsets from normal donors and breast cancer patients at baseline after 7 courses of chemotherapy and one month after the end of treatment. CD19⁺: t1-t7 and t8 vs t0, $p < 0.05$; CD20⁺: t1-t7 and t8 vs t0, $p < 0.05$; CD20⁺/CD38⁺: t1-t7 and t8 vs t0, $p < 0.05$. ND, normal donors; t0, baseline; t1-t7, time points of the immunological study after each course of chemotherapy; t8, time point at follow-up, one month after the last course of chemotherapy.

rubucin/cyclophosphamide (FEC; $n=7$), followed by 4 courses of taxol plus peg-filgrastim (6 mg) administered at every course 72 h after CT. Patient characteristics are summarised in Table I. Twenty healthy subjects, aged 18-60 years, were used as controls.

Flow cytometry. The PBL phenotype was analysed by flow cytometry before the start of treatment and immediately before each CT course planned at a 14-day interval. A 3-ml sample of whole blood was obtained by venupuncture from all patients

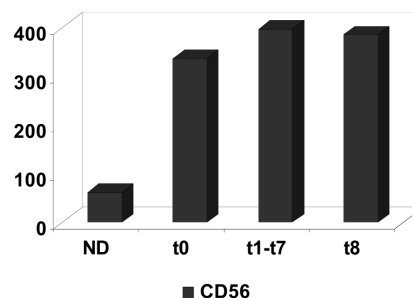


Figure 2. CD56 (NK subset) from normal donors and breast cancer patients at baseline. CD56⁺: t0 and t1-t7 vs ND, $p < 0.05$. ND, normal donors; t0, baseline; t1-t7, time points of the immunological study after each course of chemotherapy; t8, time point at follow-up, one month after the last course of chemotherapy.

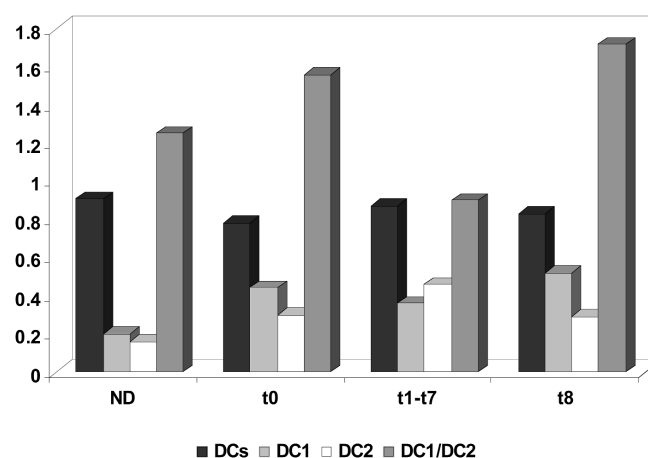


Figure 3. Mean percentages of dendritic cells (DCs) from normal donors and breast cancer patients at baseline, during chemotherapy and at follow-up. DC1: t0 vs ND, $p = 0.01$; DC2: t0 vs ND, $p = 0.02$; t1-t7 vs t0, $p = 0.01$. DC1/DC2: t1-t7 vs t0, $p = 0.002$. ND, normal donors; t0, baseline; t1-t7, time points of the immunological study after each course of chemotherapy; t8, time point at follow-up, one month after the last course of chemotherapy.

and controls, and EDTA was added. Each sample (100 μ l) was incubated with the respective antibody solution for 10 min at room temperature in the dark. Erythrocyte lysis and leukocyte fixation were obtained using the Immunoprep Reagent System (Beckman Coulter, USA). For analysis of the PBL phenotype, the following monoclonal antibodies or combinations of monoclonal antibodies were used: fluorescein isothiocyanate-conjugated (FITC) anti-CD2, phycoerythrin-conjugated (PE) anti-CD3, FITC anti-CD4, PE anti-CD4, FITC anti-CD8, FITC anti-CD10, PE anti-CD16, PE anti-CD19, FITC anti-CD20, FITC anti-CD23, PE anti-CD25, PE anti-CD38, PE anti-CD45RA, FITC anti-CD45RO, PE anti-CD56 and FITC anti-HLA-DR. Flow cytometric analysis was performed using Epics XL flow cytometer (Beckman Coulter) equipped with an argon ion laser working at 488 nm. The lymphocyte subsets were identified by forward and side scatter analyses. A total of 3000 events were analysed.

Circulating dendritic cells (DCs) were evaluated on peripheral blood mononuclear cells (PBMCs) separated from 8 ml of whole blood by density gradient at 1077 g/l (Ficoll-Paque, Pharmacia-Amersham, Sweden). DCs were identified as

Table II. Absolute number of peripheral blood leukocytes, lymphocytes, T, B and NK subsets in normal donors and early breast cancer patients before, during and post dose-dense CT + peg-filgrastim treatment.

	ND	t0	p-value	t1-t7	p-value	t8	p-value
WBC	6,097	7,327	NS	13,048	0.010	5,983	NS
ANL	1,172	1,439	NS	1,682	NS	1,625	NS
CD4	803	929	NS	684	0.040	542	0.010
CD8	405	598	NS	585	NS	713	NS
CD4CD8	16	34	NS	177	0.010	30	NS
CD4CD8r	2.04	1.92	NS	1.37	NS	1.07	0.040
CD19	219	212	NS	31	0.001	58	0.002
CD20	202	225	NS	41	0.001	83	0.002
CD20 CD38	72	112	NS	16	0.010	57	0.001
CD56	61	336	0.03	401	NS	385	NS
CD16	253	341	NS	278	NS	375	NS

ND, normal donors. t0, baseline; t1-t7, time points of the immunological study after each course of chemotherapy; t8, time point of follow-up one month after the last course of chemotherapy; WBC, white blood cells; ANL, absolute number of lymphocytes; NS, not significant.

HLA-DR-positive and CD3-, CD19-, CD20-, CD11b-, CD56-, CD16-, CD34- and CD14-FITC-negative. At least 2 peripheral blood DC subsets were described: myeloid-derived CD11c⁺ CD123-DCs (DC1) and lymphoid-derived CD11c-CD123⁺ DCs (DC2). Overall, 50,000 events were analysed.

Statistics. Statistical comparisons between absolute lymphocyte counts and subsets in patients and healthy controls were performed by the t-test for independent samples, while comparisons versus baseline values were carried out by the t-test for paired observations.

Results

Breast cancer patients undergoing dose-dense CT followed by peg-filgrastim showed a significant increase in overall leukocyte number from the first CT course as compared to the baseline. Conversely, the total lymphocyte number tended to decrease with a negative peak following the 6th course of CT ($p=0.03$). B (CD19⁺, CD20⁺) and early B lymphocyte subsets (CD20⁺/CD38⁺) significantly decreased during the treatment period ($p<0.05$) (Fig. 1). T lymphocyte subsets (CD2, CD3, CD8) did not show significant changes, with the exception of the T-helper subset (CD4⁺), which significantly decreased from the 4th CT course with a subsequent decrease in the CD4/CD8 ratio ($p<0.05$). The CD4⁺/CD8⁺ subset, identifying immature T lymphocytes, increased compared to the baseline. With respect to the healthy controls, breast cancer patients showed a statistically significant increase in the number of natural killer (NK) cells (CD56⁺), which persisted throughout the treatment (Fig. 2).

The absolute number of lymphocytes (ANL), T and B subsets were not significantly altered in breast cancer patients with respect to the healthy controls. During treatment, the percentage of the DC2 subset was further increased; the

DC1/DC2 ratio consequently decreased. The mean percentage of DCs was not significantly altered in CT-naïve with respect to 20 normal donors (NDs) utilised as controls; the mean percentage of the DC subset was only significantly increased as compared to that of the NDs (Fig. 3).

After 1 month of follow-up, circulating leukocyte, lymphocyte and DC mean values were normalised. A slight increase in the DC1 number, the DC1/DC2 ratio and the NK cell subset was observed, together with a decrease in CD4⁺ cells and the CD4/CD8 ratio, although without statistical significance. Only B lymphocytes showed a persistent significant decrease at 1 month of follow-up ($p<0.05$) (Fig. 1). The median values of the absolute number of PBLs, lymphocytes and T, B and NK subsets are summarised in Table II.

Discussion

The impact of cytotoxic CT on the immune system has not as yet been fully elucidated. Mackall reported on the immunosuppressive effects of cytotoxic CT, such as a decrease in total T lymphocytes and the CD4⁺ subset following adjuvant CT and radiotherapy for breast cancer and the slow recovery of CD3/CD4⁺ lymphocytes in patients treated with intensive CT (2). Significant reductions in the absolute number of CD4⁺ lymphocytes were also reported by Sewell *et al* (9) in breast cancer patients treated with standard CT and by Sara *et al* (10) in patients with solid tumours treated with CT. Schroeder and colleagues (11) reported specific alterations of the T cell population in patients with breast cancer, such as a significant reduction in the absolute T cell number, but not in the CD4⁺/CD8⁺ ratio and a significant increase in CD3⁺ T cells. In contrast with such aberrations of PBL reported in patients submitted to cytotoxic CT, Melichar *et al* (12) observed only a few distinct PBL changes in a population of breast cancer patients and suggested the presence of T-cell

activation. Our group previously found little impact of a topotecan-based CT on lymphocyte subsets of either naïve or pre-treated ovarian cancer patients (8).

This study suggests that peg-filgrastim-supported dose-dense adjuvant anthracycline- and taxane-based CT does not significantly affect the immune system of breast cancer patients. In particular, T lymphocyte subsets did not show significant changes, except for a decrease in the number of T helper (CD4⁺) cells. Following the discontinuation of CT, the mean values of leukocytes, total lymphocytes and NK subsets tended to return to baseline values. The substantial preservation of NK and DCs during cytotoxic therapy and follow-up seems to indicate a preservation of the immune competence by these regimens of adjuvant CT. Only B (CD19⁺, CD20⁺) and early B (CD20⁺/CD38⁺) lymphocytes decreased significantly over the entire CT period, and the decrease persisted at follow-up.

It must be noted that our CT regimens consisted of taxol and were supported by peg-filgrastim. Taxanes have been previously reported to exert immunostimulatory effects, which were hypothesised to be implicated in anti-tumour activity (13). G-CSF itself was shown to have a number of effects on immune function. In preclinical studies, improvements in neutrophil count and antibacterial activity were reported, and also direct immunomodulatory properties were observed (6). Thus, taxol and peg-filgrastim might have played a role in the relatively small impact on the immune system observed in our cohort.

In conclusion, the outcome of our study regarding the changes in the PBL phenotype in a subset of breast cancer patients suggest that dose-dense CT programs with peg-filgrastim support do not significantly affect the immune system and allow for a rapid restoration of most immune competent cells. These findings may have important clinical implications for the improvement of immunotherapeutic approaches to cancer.

Acknowledgements

The present study was supported, in part, by the Fondazione IRCCS Policlinico San Matteo, Pavia (research grant to M.D.).

References

1. McCoy JL, Rucker R and Petros JA: Cell-mediated immunity to tumor associated antigens is a better predictor of survival in early stage breast cancer than stage, grade or lymph node status. *Breast Cancer Res Treat* 60: 227-234, 2000.
2. Mackall CL: T-cell immunodeficiency following cytotoxic antineoplastic therapy: a review. *Stem Cells* 18: 10-18, 2000.
3. Citron ML, Berry DA, Cirincione C, Hudis C, Winer EP, Gradishar WJ, Davidson NE, Martino S, Livingston R, Ingle JN, Perez EA, Carpenter J, Hurd D, Holland JF, Smith BL, Sartor CI, Leung EH, Abrams J, Schilsky RL, Muss HB and Norton L: Randomized trial of dose-dense versus conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node-positive primary breast cancer: first report of Intergroup Trial C9741/cer and Leukemia Group B Trial 9741. *J Clin Oncol* 21: 1431-1439, 2003.
4. Tayebi H, Kuttler F, Saas P, Lienard A, Petracca B, Lapiere V, Ferrand C, Fest T, Cahn J, Blaise D, Kuentz M, Herve P, Tiberghien P and Robinet E: Effect of granulocyte colony-stimulating factor mobilization on phenotypical and functional properties of immune cells. *Exp Hematol* 29: 458-470, 2001.
5. Valente JF, Alexander JW, Li BG, Noel JG, Custer DA, Ogle JD and Ogle CK: Effect of in vivo infusion of granulocyte colony stimulating factor on immune function. *Shock* 17: 23-29, 2002.
6. Rutella S, Pierelli L, Bonanno G, Sica S, Ameglio F, Capoluogo E, Mariotti A, Scambia G, D'Onofrio G and Leone G: Role for granulocyte colony stimulating factor in the generation of human T regulatory type 1 cells. *Blood* 100: 2562-2571, 2002.
7. Baggers J, Ratzinger G and Young JW: Dendritic cells as immunologic adjuvants for the treatment of cancer. *J Clin Oncol* 18: 3879-3882, 2000.
8. Ferrari S, Rovati B, Cucca L, Scarabelli C, Presti M, Beccaria C, Collovà E, Porta C and Danova M: Impact of topotecan-based chemotherapy on the immune system of advanced ovarian cancer patients: An immunophenotypic study. *Oncol Rep* 9: 1107-1113, 2002.
9. Sewell HF, Halbert CF, Robins RA, Galvin A, Chan S and Blamey RW: Chemotherapy induced differential changes in lymphocyte subsets and natural killer cell function in patients with advanced breast-cancer disease. *Int J Cancer* 55: 735-738, 1993.
10. Sara E, Kotsakis A, Soukalekos J, Kourousis C, Kakolyris S, Mavromanolakis E, Vlachonicolis J and Goergoulis V: Post-chemotherapy lymphopoiesis in patients with solid tumors is characterized by CD⁺ cell proliferation. *Anticancer Res* 19: 471-476, 1999.
11. Schroeder W, Vering A, Stegmüller M and Strohmeier R: Lymphocyte subsets in patients with ovarian and breast cancer. *Eur J Gynaecol Oncol* 18: 474-479, 1997.
12. Melichar B, Touskova M, Dvorak J, Jandik P and Kopecky O: The peripheral blood leukocyte phenotype in patients with breast cancer: effect of doxorubicin/paclitaxel combination chemotherapy. *Immunopharmacol Immunotoxicol* 23: 163-173, 2001.
13. Chan OT and Yang KX: The immunological effects of taxanes. *Cancer Immunol Immunother* 49: 181-185, 2000.