JPP Journal of Pharmacy and Pharmacology

JPP 2010, 62: 931–934

Received December 4, 2009
Accepted March 30, 2010
DOI 10.1211/jpp.62.07.0015
ISSN 0022-3573

Research Paper

Systemic chemotherapy-induced microsatellite instability in the mononuclear cell fraction of women with breast cancer can be reproduced *in vitro* and abrogated by amifostine

Jorge L.F. Pinto[a,b], Fernando L.A. Fonseca[a,c], Sarah R. Marsicano[a], Pamela O. Delgado[a], Aleksandra V.L. Sant'Anna[a], Patrícia G. Coelho[a], Patríca Maeda[a,c] and Auro Del Giglio[a,b,c]

[a]ABC Foundations School of Medicine, Santo André, Brazil, [b]São Paulo University Medical School, São Paulo, Brazil and [c]Oncology Department, Albert Einstein Jewish Hospital, São Paulo, Brazil

Abstract

Objectives Microsatellite instability (MSI) induction by alkylating agent-based chemotherapy (ACHT) may underlie both tumor resistance to chemotherapy and secondary leukaemias in cancer patients. We investigated if ACHT could induce MSI in tumor-derived plasma-circulating DNA (pfDNA) and in normal peripheral blood mononuclear (PBMN) cells. We also evaluated if amifostine could interfere with this process in an in-vitro model.
Methods MSI was determined in pfDNA, PBMN cells and urine cell-free DNA (ufDNA) of 33 breast cancer patients before and after ACHT. MCF-7 cells and PBMN from normal donors were exposed *in vitro* to melphalan, with or without amifostine.
Results We observed at least one MSI event in PBMN cells, pfDNA or ufDNA of 87, 80 and 80% of patients, respectively. *In vitro*, melphalan induced MSI in both MCF-7 and normal PBMN cells. In PBMN cells, ACHT-induced MSI occurred together with a significant decrease in the expression of the DNA mismatch repair gene hMSH2. Amifostine decreased hMSH2 expression and also prevented MSI induction only in normal PBMN cells.
Conclusions ACHT induced MSI in PBMN cells and in tumour-derived pfDNA. Because of its protective effect against ACHT induction of MSI in normal PBMN cells *in vitro*, amifostine may be a potential agent for preventing secondary leukaemias in patients exposed to ACHT.
Keywords amifostine; breast cancer; chemotherapy; microsatellite instability

Introduction

Microsatellites are small DNA sequences of two, three or four nucleotides repeated in a row along the DNA molecule and found in hundreds of locations in human DNA. Changes in the number of microsatellite repeats, known as microsatellite instability (MSI), lead to genetic instability together with a predisposition to various carcinomas, such as colon, endometrium or ureter.[1] MSI is caused by deficiencies in the DNA repair system resulting from either a hereditary mutation or somatic epigenetic silencing of DNA repair genes, such as hMSH2, hMLH1 and PMS2, by promoter methylation. The adequate functioning of the DNA repair system, which is responsible for correcting nucleotide base mismatches and insertions and deletions that may arise during DNA duplication,[1] depends on the coordinated interplay between several proteins encoded by such genes.[1,2]

Interestingly, the incidence of MSI is higher in secondary leukaemias than in de-novo leukaemias.[3,4] In fact, Worrillow *et al*. have demonstrated that the frequency of the hMSH2–6 exon 13 variant (C) allele was significantly higher in patients with secondary leukaemias who had been exposed to ACHT.[5] It is possible that alkylating chemotherapy (ACHT) with agents such as cyclophosphamide and melphalan could induce alterations in the expression of DNA repair genes and MSI, especially in chemotherapy-resistant normal hematopoietic cells that survive ACHT.[3] We have also shown that ACHT induces MSI in peripheral blood mononuclear (PBMN) cells of breast cancer patients in parallel with a decrease in the immunohistochemical expression of the hMSH2 protein in these cells.[6]

Correspondence: Auro Del Giglio, Hematology and Oncology, ABC Foundation School of Medicine, Albert Einstein Jewish Hospital, Rua Mariana Correia 369, CEP 01444–000 São Paulo, SP, Brazil.
E-mail: fon_fonseca@yahoo.com.br

Amifostine is an aminothiol that needs to be dephosphorylated at the tissue level to form its active metabolite, which inactivates electrophilic substances and can also scavenge free radicals. It is a selective, radio-protective drug used in both chemotherapy and radiotherapy to reduce normal tissue toxicity.[7] Amifostine protects normal cells from DNA strand breaks produced by the alkylating agent melphalan.[8]

Since plasma DNA derives mainly from tumour cells,[9,10] the objective of the present study was to investigate whether ACHT induces MSI in tumour-derived plasma-circulating DNA. The presence of free DNA was also analysed in the urine of study participants, since tumour DNA may be filtered at the glomerular level and secreted into urine together with DNA from normal exfoliated urinary bladder cells.[11] In addition, we evaluated if the generation of MSI by alkylating agents could be reproduced *in vitro* in MCF-7 breast cancer cells and normal PBMN cells exposed to melphalan. Finally, we tested if the cytoprotective drug amifostine[12,13] could prevent the induction of MSI by melphalan in this model.

Materials and Methods

Patients with a histological diagnosis of breast cancer who had never received systemic chemotherapy or previous hormone therapy were enrolled. The study protocol was approved by our Institutional Review Board and all patients signed an informed consent form before inclusion.

DNA from PBMN cells, plasma and urine of breast cancer patients was extracted as previously described.[6,14] Blood (pfDNA and PBMN) and urine (ufDNA) samples were analysed by PCR at 0, 3 and 6 months after the start of ACHT for six MSI markers (BAT40, BAT26, MR2, TP53 PCR15.1, APC and ALU).[6,15] MCF-7 (Universidade Federal do Rio de Janeiro Cell Bank, Brazil) and PBMN cells from seven normal volunteers were incubated *in vitro* with melphalan (GlaxoSmithKline) at a dose of 0.7 mg/ml as recommended by Mougenot *et al*[16] for 30 min with and without amifostine (Schering Plough) at 20% of the melphalan dose. After washing the exposed cells, they were resuspended in PBS. Cell viability at 48 h was determined by Trypan blue assay.

For all samples, PCR products were denatured and subjected to electrophoresis in Gene Gel Clean 15/24 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) for 90 min at 600 V and 8°C, and then silver-stained using a Hoefer Automed Gel Stainer (Amershan Pharmacia Biotech AB). MSI was defined as the appearance of a new band. Loss of heterozygosity (LOH) was defined as the disappearance of previously visualised bands for each marker.[15] The presence of MSI was determined by visual inspection of silver stained gels by two investigators (J.L.P.F. and F.L.A.F.).

Evaluation of promoter methylation of hMLH1 and hMSH2 genes was conducted with BioChain's DNA Methylation Detection Kit.[17] Unmethylated cytosines of genomic DNA can be converted to uracil by sodium bisulfite treatment. Methylated cytosines, on the other hand, cannot be converted by this treatment. After sodium bisulfite treatment, methylated and unmethylated DNA can be distinguished by PCR with specially designed primers that produce a 354 bp-long fragment for hMLH1 and a 107 bp gene product for hMSH2.

Statistical analysis

Correlations between categorical variables were analysed using the χ^2 or Fisher's exact test. Analysis of variance was used to study correlations between continuous and categorical variables, and simple regression to analyse correlations between continuous variables. Statistical calculations were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Thirty-three previously untreated breast cancer patients (90% infiltrative ductal carcinoma, 6% lobular carcinoma and 4% colloid carcinoma) with a mean age of 51 years received ACHT (16 ACT; 3 FAC; 2 TAC; 1 FEC; 10 AC) as adjuvant (16), neoadjuvant (11) and palliative (4) therapy. Samples from three additional patients receiving fulvestrant (AstraZeneca Pharmaceuticals) only as neoadjuvant therapy as a part of another ongoing clinical protocol were also included.

The comparison of baseline PBMN cell, ufDNA and pfDNA samples revealed only one MSI event in a single pfDNA sample. Taking baseline PBMN cell, pfDNA and ufDNA samples as controls for the evaluation of follow-up samples, we observed at least one microsatellite instability event or LOH in 87, 80 and 80%, respectively, of PBMN cell, pfDNA or ufDNA samples at 6 months, mainly in MSI markers BAT40 and BAT26. Patients receiving fulvestrant exclusively also exhibited both MSI and LOH in PBMN cells, pfDNA and ufDNA. The concordance between the rate of MSI alterations in DNA from different sources was low – only 14.74% between PBMN cells and pfDNA and 8.42% between pfDNA and ufDNA.

The incubation of MCF-7 and PBMN cells from volunteers with melphalan with and without amifostine revealed that melphalan cytotoxicity was not affected by the addition of amifostine (data not shown) in MCF-7 cells. Amifostine prevented MSI in normal PBMN cells, but not in MCF-7 cells. In the absence of amifostine, MSI was induced in both MCF-7 cells and normal PBMN cells. Furthermore, in PBMN cells from six of seven normal donors treated with melphalan without amifostine, we observed MSI at 48 h (four in BAT40, one in APC and one in BAT26), coinciding with a significant decrease in the percentage of cells expressing hMSH2 as compared to cells treated with melphalan plus amifostine (43 versus 74%, Mann–Whitney $P = 0.0175$) (Figure 1). There was no evidence of methylation of the hMSH2 promoter to account for the lower expression of hMSH2 induced by melphalan (Figure 2), and the hMLH1 promoter was hypermethylated at all time points in the PBMN cells of all seven volunteers (data not shown).

Discussion

In line with our previous work,[6] the present results provide evidence that ACHT induces MSI in pfDNA, which is mainly of tumour origin,[9,10] and in ufDNA,[18] which represents a mixture of free-circulating tumour DNA that is secreted into urine along with the DNA from normal exfoliated urinary bladder cells.[19] It is possible that induction of MSI by ACHT in tumour and normal PBMN cells may underlie both tumour

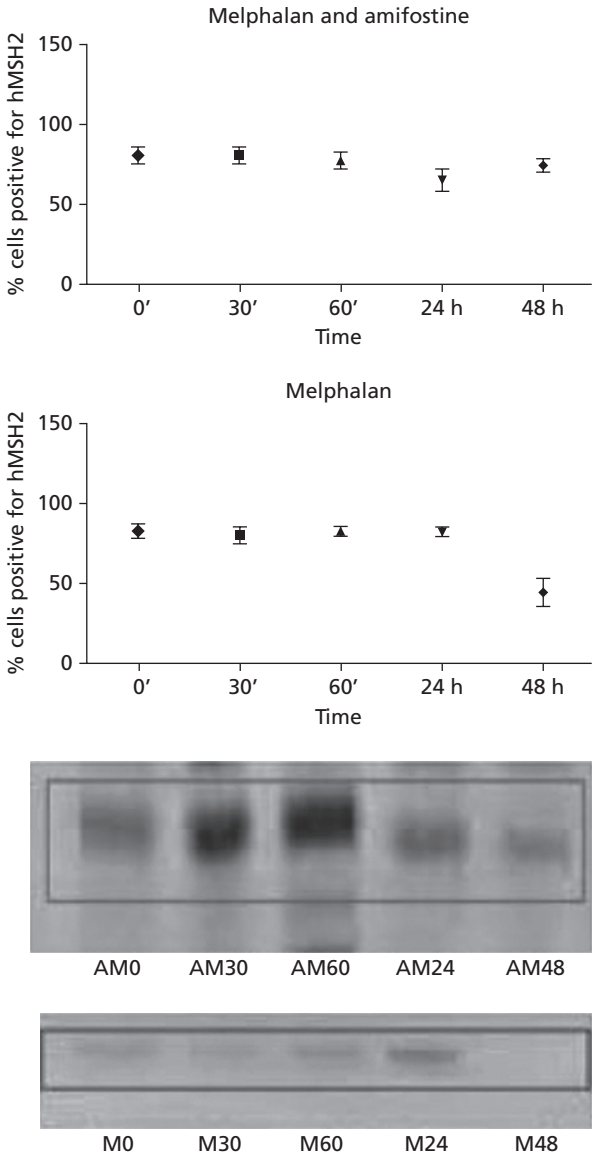

Figure 1 hMSH2-positive cells 30 min after incubation of peripheral blood mononuclear cells with melphalan or melphalan plus amifostine. The graphs show means and standard deviations for seven normal samples. Gels show loss of heterozygocity for BAT40 in a melphalan (M)-treated sample at 48 h (M48). AM, melphalan plus amifostine.

resistance to chemotherapy and the future development of secondary leukaemia and of other solid tumours in patients undergoing ACHT.

In this study, we were able to reproduce the induction of MSI in normal PBMN cells *in vitro* using the alkylating agent melphalan. Interestingly, this phenomenon was temporally associated with a significant decrease in the frequency of hMSH2-positive cells. It is possible that ACHT induces a decrease in hMSH2 expression, facilitating MSI induction. Further studies should focus on elucidating the mechanism by which ACHT decreases the expression of hMSH2 in normal PBMN cells, since this decrease was not due to methylation.

The concordance we found between the rate of MSI alterations in DNA from different sources was low. This

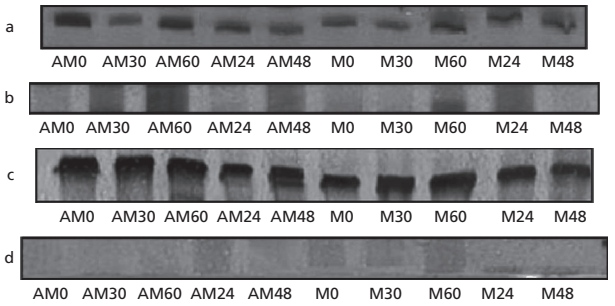

Figure 2 hMSH2 methylation assays of MCF-7 and of normal peripheral blood mononuclear cells at different times. a,b: hMSH2 methylation assay of MCF-7 cells with melphalan (M) or melphalan plus amifostine (AM) at 0, 30 and 60 min, 24 and 48 h with bisulfite (a) and without bisulfite (b); c,d: methylation assay of normal peripheral blood mononuclear cell samples with melphalan (M) or melphalan plus amifostine (AM) without bisulfite (c) and with bisulfite (d). There is no evidence of methylation.

suggests that susceptibility to ACHT-induced MSI varies in different tissues in the body and between normal and tumour cells. Patients receiving fulvestrant exclusively also exhibited both MSI and LOH in PBMN cells, pfDNA and ufDNA, in agreement with previous results showing the genotoxicity of another (structurally unrelated) estrogen antagonist, tamoxifen;[20] therefore breast cancer patients on fulvestrant and tamoxifen, like those receiving ACHT, may also have MSI induced in both pfDNA and normal PBMN cells.

When we compared time 0 PBMN cell samples with time 0 ufDNA and pfDNA samples, we observed only one MSI event in pfDNA at diagnosis. These findings are in agreement with the low incidence of MSI reported in the literature in breast cancer at diagnosis.[21,22]

In our in-vitro model, the cytoprotective agent amifostine prevented MSI induction by the alkylating agent melphalan in six out of seven cases. This may be a useful clue for further clinical studies aiming at preventing secondary leukaemia in patients exposed to ACHT.[3–5] The fact that amifostine did not prevent MSI induction in MCF-7 cancer cells suggests that this effect may be restricted to normal cells. Further studies involving other cell lines are needed to confirm this apparent selectivity of amifostine in preventing ACHT induction of MSI in normal cells only.

The mechanisms of the decreased expression of hMHS2 associated with MSI induction by ACHT in normal and malignant cells are unknown. We observed no evidence of hMSH2 hypermethylation induction by ACHT (Figure 2) to explain the lower expression of hMSH2 after ACHT treatment of normal cells in the absence of amifostine. Further investigations focused on the mechanisms that are responsible for the induction of MSI by ACHT and on the protective effects of amifostine are needed.

Conclusions

Since we showed that ACHT can induce MSI in tumour-derived pfDNA, it is possible that MSI induced by systemic treatment for breast cancer may underlie the emergence of chemotherapy-resistant tumour cells.[23,24] Our in-vitro data

do not suggest that amifostine interferes with melphalan cytotoxicity in tumour-derived MCF-7 cells, but it may protect normal blood cells from MSI induction by melphalan. It is possible then that potential genotoxic effects on normal cells that could underlie drug-induced myelodysplasia and/or leukaemia could be prevented by amifostine without undue decrease of the antineoplastic effects of these drugs. It should be stressed that we only studied MCF-7 tumour cells, and that, in order to generalise these conclusions, other tumour cell lines should also be evaluated. Nevertheless, we believe that further experimental and clinical studies aimed at preventing MSI induction in normal blood cells during systemic cancer treatment for breast cancer with ACHT should be conducted in an attempt to decrease the later appearance of secondary haematologic tumours in these patients.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

Financial support was provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (Grant 05/00651–5).

References

1. Kouraklis G, Misiakos EP. Hereditary nonpolyposis colorectal cancer (Lynch syndrome): criteria for identification and management. *Dig Dis Sci* 2005; 50: 336–344.
2. Hsieh P, Yamane K. DNA mismatch repair: molecular mechanism, cancer, and ageing. *Mech Ageing Dev* 2008; 129: 391–407.
3. Casorelli I *et al*. Drug treatment in the development of mismatch repair defective acute leukemia and myelodysplastic syndrome. *DNA Repair (Amst)* 2003; 2: 547–559.
4. Ben-Yehuda D *et al*. Microsatellite instability and p53 mutations in therapy-related leukemia suggest mutator phenotype. *Blood* 1996; 88: 4296–4303.
5. Worrillow LJ *et al*. An intron splice acceptor polymorphism in hMSH2 and risk of leukemia after treatment with chemotherapeutic alkylating agents. *Clin Cancer Res* 2003; 9: 3012–3020.
6. Fonseca FL *et al*. Systemic chemotherapy induces microsatellite instability in the peripheral blood mononuclear cells of breast cancer patients. *Breast Cancer Res* 2005; 7: R28–R32.
7. Marcu LG. The role of amifostine in the treatment of head and neck cancer with cisplatin-radiotherapy. *Eur J Cancer Care (Engl)* 2009; 18: 116–123.
8. Buschini A *et al*. Amifostine (WR-2721) selective protection against melphalan genotoxicity. *Leukemia* 2000; 14: 1642–1651.
9. Fleischhacker M, Schmidt B. Circulating nucleic acids (CNAs) and cancer – a survey. *Biochim Biophys Acta* 2007; 1775: 181–232.
10. Shao ZM, Nguyen M. Tumor-specific DNA in plasma of breast cancer patients. *Anticancer Drugs* 2002; 13: 353–357.
11. Melkonyan HS *et al*. Transrenal nucleic acids: from proof of principle to clinical tests. *Ann N Y Acad Sci* 2008; 1137: 73–81.
12. Orditura M *et al*. Amifostine: A selective cytoprotective agent of normal tissues from chemo-radiotherapy induced toxicity (Review). *Oncol Rep* 1999; 6: 1357–1362.
13. Capizzi RL. The preclinical basis for broad-spectrum selective cytoprotection of normal tissues from cytotoxic therapies by amifostine. *Semin Oncol* 1999; 26: 3–21.
14. Stemmer C *et al*. Use of magnetic beads for plasma cell-free DNA extraction: toward automation of plasma DNA analysis for molecular diagnostics. *Clin Chem* 2003; 49: 1953–1955.
15. Dietmaier W *et al*. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. *Cancer Res* 1997; 57: 4749–4756.
16. Mougenot P *et al*. In vitro cytotoxic effect of melphalan and pilot phase II study in hormone-refractory prostate cancer. *Anticancer Res* 2006; 26: 2197–2203.
17. Demokan S *et al*. Microsatellite instability and methylation of the DNA mismatch repair genes in head and neck cancer. *Ann Oncol* 2006; 17: 995–999.
18. Su YH *et al*. Detection of a K-ras mutation in urine of patients with colorectal cancer. *Cancer Biomark* 2005; 1: 177–182.
19. Szarvas T *et al*. Deletion analysis of tumor and urinary DNA to detect bladder cancer: urine supernatant versus urine sediment. *Oncol Rep* 2007; 18: 405–409.
20. Wozniak K *et al*. The DNA-damaging potential of tamoxifen in breast cancer and normal cells. *Arch Toxicol* 2007; 81: 519–527.
21. Adem C *et al*. Microsatellite instability in hereditary and sporadic breast cancers. *Int J Cancer* 2003; 107: 580–582.
22. Caldés T *et al*. Microsatellite instability correlates with negative expression of estrogen and progesterone receptors in sporadic breast cancer. *Teratog Carcinog Mutagen* 2000; 20: 283–291.
23. Watanabe Y *et al*. A change in microsatellite instability caused by cisplatin-based chemotherapy of ovarian cancer. *Br J Cancer* 2001; 85: 1064–1069.
24. Wild PJ *et al*. Microsatellite instability predicts poor short-term survival in patients with advanced breast cancer after high-dose chemotherapy and autologous stem-cell transplantation. *Clin Cancer Res* 2004; 10: 556–564.