Does chemotherapy-induced oxidative stress improve the survival rates of breast cancer patients?

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Abstract

Anti-neoplastic agents induce oxidative stress leading to lipid, carbohydrate, protein and DNA damage. We sought to explore the role of drug-induced oxidative stress on breast cancer patient's survival. We observed that neoadjuvant patients presented a marked raise in DNA damage and protein carbonyl levels after chemotherapy, while post-chemotherapy DNA repair activity of the KU86 enzyme and total antioxidant capacity of the plasma were higher in adjuvant group. With respect to patient's survival, we observed that increasing levels of KU86 and antioxidant capacity of the plasma during chemotherapy significantly influenced the survival rates of the patients, protecting from disease recurrence and death. Our results suggest that chemotherapy induces a certain level of systemic oxidative stress, which is maintained along successive clinical interventions and could influence the clinical outcome of the patients.

Introduction

A wide variety of cytotoxic agents are used in the treatment of breast cancer, often achieving significant palliation of the illness (1). However, adverse consequences of their action on normal tissue tend to occur, as antitumour drugs are indiscriminate, and may produce severe toxicity unless the dose is limited, in which case the chemotherapy efficiency may be reduced (10,13,16). With respect to these adverse consequences, the majority of antineoplastic drugs are known to induce the generation of free radicals (3). The cumulative production of free radicals leads to oxidative stress, which has been detected in cancer cells. This phenomenon is related to oncogenic stimulation, provoking damage in cellular components, promoting DNA mutation and altering crucial cellular processes such as enzymatic catalysis or signal transduction (21). Moreover, the activation of enzymatic and non-enzymatic antioxidants contributes to generating a particular microenvironment that notably influences tumour behaviour, tumour response to chemotherapy and thereby the cancer patient's clinical outcome (11,21). Nevertheless, to date few researchers have addressed the question of the effect of chemotherapy on the oxidative status of cancer patients, although antineoplastic agents induce oxidative stress in biological systems (3) and the influence of this physiological imbalance on both carcinogenesis and disease progression is well known (20,25).

Effects of chemotherapy on oxidative damage and antioxidant markers in blood plasma

It has been reported that breast cancer patients suffer increased DNA damage, either independently or because (and this is more likely) breast cancer patients have impaired DNA repair mechanisms, particularly those specialized in the removal of oxidative damage (12), and increased serum concentrations of carbonyls (19). Under our experimental conditions, neoadjuvant chemotherapy increased DNA and protein damage, whereas these markers remained unchanged in the adjuvant group after chemotherapy. From these results, it is not possible to discern why adjuvant patients do not present greater DNA and protein damage after chemotherapy, but an association was observed between these data, the activation of DNA repair enzyme KU86 and the increased total antioxidant capacity after chemotherapy in the adjuvant group. It is well established that solid tumours cannot grow beyond 2 mm if angiogenesis is not activated. Oxidative stress due to tumour size-induced hypoxia is thought to play an important role in cancer angiogenesis and progression (9,25). Indeed, although the results were not statistically significant, we observed a direct correlation between tumour size and DNA damage before chemotherapy (data not shown), which could contribute to the differences in the levels of oxidative damage markers observed between the groups. On the other hand, the adjuvant patients were subjected to surgery before chemotherapy, which may have modified their oxidative status prior to systemic therapy, given that free radicals are known to be produced by macrophages and neutrophils during healing (18). Potischman et al. (15) contributed to the very scarce data regarding the effects of surgery and chemotherapy on the oxidative status of breast cancer patients, in a study that reported a significant increase in the plasma levels of major antioxidants, such as α-tocopherol and retinol, both in patients subjected to

surgery alone and in those treated surgically and with chemotherapy. Accordingly, it seems plausible that, under our experimental conditions, activated antioxidant defence may inhibit the increased presence of oxidative stress markers in the adjuvant group, although basal levels of oxidative damage with respect to chemotherapy administration remain high in comparison with those of the neoadjuvant group.

Correlation between oxidative stress and patients clinical outcome

Since the publication of the results from the National Surgical Adjuvant Breast and Bowel Project B-18 trial and the European Organization for Research and Treatment of Cancer Trial 10902, it is well-known that neoadjuvant and adjuvant chemotherapy are equivalent with respect to disease-free survival (DFS) and overall survival (OS) (8,22). These results were obtained after adjusting for tumour size, among other factors, this being the major criterion regarding pre-operative chemotherapy in order to allow breast conserving surgery. Thus, the results of our multivariate analysis show that greater tumour size significantly shortens time to recurrence and death. Therefore, the variable “Group”, dividing the patients into neoadjuvant and adjuvant settings, was eliminated from the final model, as tumour size is included. It is very important to take into account that factors other than tumour size may also influence survival rates and contribute to the clinical outcome. To the best of our knowledge, the present study is the first to report that changes in the activity of DNA repair enzyme KU86 and in the antioxidant capacity of the plasma during chemotherapy may influence DFS in breast cancer patients. As indicated by the results of the multivariate analysis, an increase in KU86 activity has a protective effect against disease recurrence and death, regardless of further systemic therapy. Moreover, higher levels of total antioxidant capacity of the plasma after chemotherapy increase survival rates of women who,

subsequent to chemotherapy and surgery (regardless of the sequence of these interventions), undergo hormone therapy. This latter interaction suggests that as oxidative damage becomes increasingly harmful with successive clinical interventions, higher levels of plasma antioxidants improve patients' survival rates, which may be highly significant since microenvironmentally high levels of oxidative stress have been related with the promotion of tumour cell migration, angiogenesis and metastasis, especially regarding cancer stem cells (CSCs), a specific tumour cell type with self-renewal capacity and multipotency, suspected of being responsible for carcinogenesis, disease recurrence and reduced survival rates (24,25) Indeed, it has been suggested that induced oxidative stress may play a role in treatment failure and contribute to the observed disease relapse despite tumour shrinkage (3,25). With respect to KU86 activity, whether CSCs or other tumour circulating cells use this molecular mechanism to survive in the blood stream and metastasize is still unknown, but previous data point to intracellular glutathione and antioxidant enzymes as the main sources of reducing power in CSCs (5). Nevertheless, it seems reasonable to conclude that increased DNA repair capacity in circulating cells in breast cancer patients subjected to systemic and surgical therapy may contribute to reducing treatment toxicity and cellular mutagenesis.

Concluding remarks and future directions

As a possible cause of treatment failure in breast cancer patients, changes in oxidative status and antioxidant response should be studied carefully in order to establish an effective strategy for decreasing systemic oxidative stress in patients receiving chemotherapy, because the antioxidant capacity of the plasma is a modifiable factor that could have therapeutic applications. In this respect, several studies have examined chemotherapy toxicity and efficiency, and antioxidant supplementation

(2,23). In general, these papers conclude that antioxidant supplementation does not affect chemotherapy efficiency or reduce dose-limiting toxicity, although a lack of adequate statistical power was a common limitation in these studies. Perhaps the question should not be whether antioxidant supplementation is beneficial or detrimental *per se* to a patient's clinical outcome, but rather whether antioxidant supplementation should be administered during the therapeutic schedule, as suggested by the results of the present study. Our data show that patients who undergo a second clinical intervention in possession of increased levels of systemic antioxidant capacity achieve better survival rates. Nevertheless, larger well-designed studies are needed to clarify the role of exogenous antioxidants in chemotherapy efficiency and toxicity. With respect to this conclusion, our study provides a deeper insight into the oxidative events accompanying chemotherapy in different treatment settings and represents a step forward in the study of the potential application of antioxidants in breast cancer treatment. Foreseeably, the next step should be to identify the plasma antioxidants that confer a survival advantage against breast cancer, and to study its protective activity in a lager cohort, followed by a well-designed randomized clinical trial. Indeed, our research group is currently working in this direction, on the basis of the conclusions of the exploratory work discussed in this article.

Innovation

In recent years, oxidative stress and antioxidant status have attracted increasing interest among basic and clinical researchers. Notable improvements in our knowledge about microenvironmental influence on gene expression and the impact of free radicals on key cancer-related processes, such as tumour cell spreading, angiogenesis and drug resistance, underlie novel scientific approaches to the study of cancer biology in the field of translational oncology. Nevertheless, pre-clinical or clinical data about the role of oxidative stress status in therapeutic response and survival remain extremely scarce. To the best of our knowledge, this paper is the first to report a significant and positive influence of a systemic antioxidant status marker, such as the antioxidant capacity of the plasma, and the activity of KU86, a DNA repair enzyme closely related to the damage inflicted by free radicals, on the survival rates of breast cancer patients subjected to chemotherapy. This is an interesting issue that warrants consideration to design new experiments in order to gain further insight into its potential therapeutic applications, given that systemic antioxidant status is an exogenously modifiable factor. The novel results described and discussed in this article may serve as a conceptual scaffold for designing experiments to better understand the influence of oxidative stress on the development of anti-neoplastic treatment resistance and its impact on patient survival rates.

Notes

Patients and samples.

This study included patients diagnosed with breast carcinoma at the Complejo Hospitalario de Jaen (Jaen, Spain) between 2005 and 2007, for whom chemotherapy was scheduled as part of their treatment. All patients were initially staged based on physical examination, radiologic findings and pathologic examination of tumour biopsies. Finally, 70 patients were enrolled in this study after giving their signed informed consent. Of these patients, 38 received neoadjuvant chemotherapy and 32 received adjuvant chemotherapy, thus constituting the two experimental groups of this study. Chemotherapy consisted of anthracycline- and/or taxane-based regimens, as determined by the medical oncology team. The pathologic and clinical information was extracted from the medical reports produced in the Oncology Department Registry. Matched blood samples were collected from each patient before and after six cycles of chemotherapy. This study was conducted following the guidelines of the local Ethical Review Board and in accordance with Good Clinical Practices and the tenets of the Declaration of Helsinki.

Blood collection and processing. Matched blood samples were collected from each patient before and after six cycles of chemotherapy. Approximately 5 ml of blood was taken from each patient, by venous puncture, drawn into an EDTA-containing tube (Vacutainer®EDTA Tubes. BD, New Jersey, USA) and centrifuged at 1000 × *g* for 15 min. The plasma was kept in a separate tube and frozen at -80ºC. The white blood cells were removed, washed in 3 ml of RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA), carefully overlain on 5 ml of Histopaque 1077 (Sigma-Aldrich) and centrifuged at 700 × *g* for 30 min. The cells were kept until needed for analysis at –80ºC in a cryoprotectant

solution containing 90% foetal calf serum (Sigma-Aldrich) and 10% dimethyl sulphoxide (Sigma-Aldrich).

Alkaline single-cell gel electrophoresis (comet assay). DNA strand breaks were detected using the alkaline comet assay or single-cell gel electrophoresis, as described previously (6,7). The nucleoids stained with 4'6-diamidine-2-phenylindol dihydrochloride were scored using a Leica DMLS fluorescence microscope (Leica Microsystems, Wetzlar, Germany). One hundred comets from each gel (scored at random) were scored using computerized image analysis (Komet 3.0; Kinetic Imaging Ltd., Liverpool, United Kingdom) and the percentages of fluorescence in the comet tail (representing the fraction of DNA in the tail) and head (representing the fraction of DNA in the head) were measured.

Plasma protein carbonyl assay. The levels of plasma protein carbonyl groups were assessed using Protein Carbonyl Kit (Cayman Chemical Company, MI, USA), according to the manufacturer's instructions. Total protein concentration in the plasma samples was measured using Biuret Colorimetric Assay Kit (Spinreact S.A. Barcelona, Spain). The results were expressed as nmol of carbonyl proteins per mg of total proteins in the plasma.

Determination of the total antioxidant capacity of the plasma. Total antioxidant capacity was assessed using the method described by Re et al. (17). The resulting values were compared with a calibration curve constructed diluting the synthetic antioxidant Trolox (Fluka, Buchs, Switzerland) (concentration range 0-20 µM Trolox in the well). The results were expressed as µM Trolox.

DNA repair activity of enzymes RPA and KU86. The activity of the DNA repair enzymes Replication Protein A (RPA) (single strand breaks) and KU86 (double strand breaks), was assessed by an immunoenzymatic assay using the Active Motif RPA and

KU70/86 DNA Repair Kits (Active Motif, Tokyo, Japan), according to the manufacturer's instructions. Previously, the cell concentration in the samples was measured using the haematologic analyzer Sysmex KX21 (Sysmex Corporation Tokyo, Japan), and nuclear proteins of blood lymphocytes were extracted and quantified using the Nuclear Extract Kit (Active Motif) and the Bicinchoninic Acid Kit (Pierce Biotechnology, Illinois, USA), respectively.

Statistical Analysis. Quantitative data were expressed as the mean ± standard error per group. The general linear model for repeated measures was used to determine significant differences between groups. Chemotherapy status (pre-chemotherapy and post-chemotherapy) was considered as an intra-group factor. Oxidative stress and antioxidant markers were considered as inter-group factors. Statistical significance was established at $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$. With respect to survival analysis, DFS was the primary end point of this study. DFS, in which relapse or death as a result of any cause is considered as an event, was calculated from the date of the first chemotherapy cycle to that of the first event, or to the date of censoring if eventless. OS was a secondary end point and was calculated from the date of the first chemotherapy cycle to that of death or that of censoring if alive. Both DFS and OS were analyzed by Kaplan and Meier's method, and the log-rank test was used to test for differences between the curves. Univariate and multivariate analyses were performed for the Cox regression model for survival, using DFS and OS as end points. As all oxidative stress and antioxidant markers were analyzed twice for each patient, before and after chemotherapy, paired data were available for each of these markers. An extended Cox regression model with time-dependent covariates and stratified by the nodal status (negative *vs* positive) was employed for univariate and multivariate analyses. Categorical variables such as group (adjuvant *vs* neoadjuvant), hormone therapy (yes *vs*

no), chemotherapy (anthracyclines and taxanes *vs* anthracyclines alone), Tastuzumab (yes *vs* no) and biological subtype (basal *vs* luminal and basal *vs* Her2), were added as non-time-dependent covariates to the Cox regression model. Potential prognostic factors were included in the multivariate model following both statistical and clinical-biological criteria. Factors with $P \leq 0.1$ in the univariate analysis were included in the multivariate analysis together with significant interactions, such as that between hormone therapy and total antioxidant capacity of the plasma, which was selected on the basis of previous data indicating that circulating sex hormone levels may have a significant impact on the total antioxidant capacity of the plasma and/or particular antioxidants (4,14). The stepwise backward elimination method with model removal set at $P \leq 0.05$ was used in the multivariate analysis to obtain the final model. All statistical analyses were carried out using SPSS 15.0 software.

Results

Patient population and clinical characteristics. The demographic and clinical characteristics of the patients included in this study are shown in Table 1. In brief, most of the patients were aged between 40 and 59 years old at diagnosis and there was a high proportion of women with ductal carcinoma, later-stage, high histological grade, oestrogen receptor-positive, progesterone receptor-positive and Her2 receptor-negative.

Oxidative damage markers.

Under our experimental conditions, DNA damage increased in the neoadjuvant group after chemotherapy. Concerning differences between groups for the same treatment period, the neoadjuvant group presented a lower percentage of DNA in tail before treatment with respect to the adjuvant group (Fig. 1A). A similar results profile to that presented for DNA oxidative damage was found in relation to plasma protein carbonyls (Fig. 1B).

DNA repair activity and total antioxidant capacity of the plasma.

KU86 activity (Fig. 2A) varied significantly between groups, being lower before chemotherapy and higher after chemotherapy in the adjuvant setting compared to the neoadjuvant setting. RPA activity (Fig. 2B) did not significantly change after treatment among any of the studied groups, nor did it vary between groups before or after the treatment. Total antioxidant capacity of the plasma (Fig. 3) showed increased values in the adjuvant group after chemotherapy and was significantly lower in the neoadjuvant group compared to the adjuvant group after chemotherapy treatment, although no differences between the groups were found before treatment.

Oxidative stress during chemotherapy and clinical outcome

DFS and OS were evaluated with a mean follow-up time of 50.1 months (range 9 to 58 months). The mean DFS times were 3.97 years and 4.51 years in the neoadjuvant

and adjuvant patients respectively, while the median OS was 4.21 years in the neoadjuvant group and 4.55 years in the adjuvant group. The log-rank test revealed statistically significant differences between the DFS rates of the two groups (P= 0.038) (Fig. 4). Tables 2 and 3 show the results of the univariate and multivariate analyses. In brief, KU86 activity and total antioxidant capacity of the plasma were associated with better DFS and OS, while higher tumour size was associated with shorter survival rates.

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Author Disclosure Statement

No competing financial interests exist.

Abbreviations

ABTS$^{\bullet+}$= 2,2’-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical

AJCC= American Joint Committee on Cancer

CI= confidence interval

CSCs= cancer stem cells

DFS= disease free survival

EDTA= ethylene diamine tetra-acetic acid

HR= hazard ratio

OS= overall survival

RPA= replication protein A

TEAC= trolox equivalent antioxidant capacity.

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