Cancer Chemother Pharmacol (1999) 43: 348–352

SHORT COMMUNICATION

Anders Johnsson · Carina Strand · Gerrit Los

Expression of GADD153 in tumor cells and stromal cells from xenografted tumors in nude mice treated with cisplatin: correlations with cisplatin-DNA adducts

Received: 5 March 1998 / Accepted: 21 July 1998

Abstract *Purpose*: Cisplatin is a commonly used antineoplastic agent that acts by forming adducts with DNA, and causing a response to the cellular injury. One of the components of this cellular injury response is the activation of the "growth arrest and DNA damage gene" *GADD153*. The level of *GADD153* induction in tumor cells has been associated with the degree of cytotoxicity. The pupose of this study was to determine whether cisplatin activates *GADD153* also in nontumor cells and how GADD153 protein levels correlate with cisplatin-DNA adducts in different cell types. *Methods*: Nude mice with xenografted squamous cell carcinoma were treated with cisplatin 10 mg/kg. Tumors were removed at 0 h (untreated controls), 24 h, and 48 h and immunohistochemically stained for GADD153 protein and cisplatin-DNA adducts. The staining reaction was quantitated in tumor cells and nonmalignant stromal cells separately, using computerized image analysis. *Results*: The GADD153 level was 4.5 times higher in tumor cells than in stromal cells in untreated mice. At 24 h after cisplatin treatment the GADD153 level had increased by 50% and 72% in tumor cells and stromal cells, respectively. Analysis of the cisplatin-DNA adducts showed a reversed pattern, with six-fold higher levels in stromal cells than in tumor cells at 24 h after treatment. By combining these data, we estimated that approximately 25-fold more GADD153 per cisplatin-DNA adduct was induced in tumor cells than in stromal cells. *Conclusion*: Our data suggest that different cell types may respond differently to DNA damage caused by cisplatin.

A. Johnsson · C. Strand
Department of Oncology, University Hospital,
S-221 85 Lund, Sweden

A. Johnsson (✉) · G. Los
UCSD Cancer Center, 9500 Gilman Dr, La Jolla,
CA 92037-0058, USA
Tel.: +1-619-822-1120; Fax: +1-619-822-1111;
E-mail: Anders.Johnsson@onk.lu.se

Key words Cisplatin · Adducts · GADD153

Introduction

Cisplatin is a commonly used anticancer agent that is thought to act by forming adducts with cellular DNA [15] causing a cellular injury response (CIR) that ultimately results in the activation of the apoptotic pathway [4]. Cells injured by exposure to cisplatin undergo a CIR that shares characteristics with responses produced by many other DNA-damaging agents [4]. Although very little is known about the specifics of any of the signal transduction pathways activated by cellular injury, it is possible to identify the general nature of some components that must be present. One of the features is the activation of the "growth arrest and DNA damage gene" *GADD153* [1, 9]. *GADD153* is one of five GADD genes found to be coordinately induced by treatments that result in either growth arrest or DNA damage [11]. In tumor cells, the *GADD153* promotor has been found to be responsive to a broad spectrum of genotoxic agents, and has been shown to be activated transiently but to a high level as a direct response to DNA damage [3]. No information is yet available about the tissue specificity of *GADD153* activation during CIR, in other words whether nontumor cells undergo a similar activation of *GADD153* during a response to damage. The latter is an important question, since tumors are composed not only of tumor cells but also of nonmalignant stromal cells, e.g. fibroblasts, endothelial cells, and inflammatory cells, that may affect the expression of *GADD153* in tumors. To address this issue, we determined the GADD153 protein level in tumor cells and stromal cells in a head and neck cancer xenograft model and correlated the GADD153 protein level with the cisplatin-DNA adduct formation in these cells using quantitative immunohistochemistry. This methodological approach provided us with an estimation of the GADD153 protein level per cisplatin-DNA adduct in both cell types.

Material and methods

Animals and treatment

Nude Balb/c mice with the cisplatin-sensitive xenografted squamous cell carcinoma line AB [20] were treated i.p. with 10 mg/kg of cisplatin. The animals were killed at 24 h (six mice) and at 48 h (seven mice) posttreatment. Two mice served as untreated controls. The choice of these time-points was based on our previous experience. The GADD153 mRNA level has been shown to peak at 24 h after cisplatin exposure in cultured cells and at 48 h in xenografts of nude mice [3], whereas hardly any induction of GADD153 is seen at 8 h after treatment of cultured cells. The tumors were removed quickly and frozen at −70 °C. Multiple parallel cryosections were prepared on poly-L-lysine-coated slides, 10 μm thick for the cisplatin-DNA adduct staining and 8 μm for the GADD153 staining.

Cisplatin-DNA adduct immunostaining

A previously described [6, 17] immunohistochemical staining technique for visualizing cisplatin-DNA interaction products was performed, using the NKI-A59 antiserum (gift from Dr den Engelse, the Netherlands Cancer Institute, Amsterdam), diluted 1:1500. A brown nuclear staining reaction was developed by peroxidase-antiperoxidase complex and diaminobenzidine. Methyl green was used as nuclear counterstain. All samples were stained in one batch.

GADD153 immunostaining

We utilized the polyclonal antiserum GADD153 (R-20; Santa Cruz Biotechnology, Santa Cruz, Calif.), which reacts with GADD153 of mouse, rat and human origin. An avidin-biotin technique was used, with diaminobenzidine as developing agent. The primary antibody was diluted 1:100. Methyl green was used as nuclear counterstain. All samples were stained in one batch.

Image analysis

The staining reaction was quantitated with the computerized image analyzer CAS 200 (Cell Analysis System, Elmhurst, Ill.) using the quantitative nuclear antigen (QNA) software package, as previously described for cisplatin-DNA adducts [6]. The same principles were applied for analysis of the GADD153 slides. With a 40× objective lens, there were 30–100 cells per microscopic field and for each sample 10 to 25 fields were measured. On the slides stained for cisplatin-adducts as well as for GADD153, the staining reaction was determined in tumor cells and stromal cells separately. The two tissue types were identified visually by the observer, based on nuclear appearance and growth pattern. The tumor cells had large and irregular nuclei, usually gathered in nodules, while the stromal cells typically had smaller, elongated nuclei and formed easily distinguishable streaks between and surrounding the tumor nodules. The QNA has a scene segmentation function for interactive exclusion of undesired parts of the microscopic field. This function was frequently used to measure narrow streaks of stromal cells by cutting out neighboring tumor cells from the digitized image, thus allowing analysis of stromal cells only.

The results of the measurements are presented as "percentage positive nuclear area" (PNA). On the adduct slides, the nonspecific nuclear staining was corrected for by subtracting the PNA values of the untreated control animals from the PNAs of the remaining slides. On the slides stained for GADD153 the background was corrected for by subtracting the PNA values of two slides stained without the primary antiserum.

Model for analyzing correlations between cisplatin-DNA adducts and GADD153

To estimate the relation between the GADD153 induction and the amount of cisplatin-DNA adducts, the following equations were used:

$$\bar{Y}_c = \frac{\sum_1^n \left[\frac{g_{24jc} - \bar{g}_{0c}}{a_{24jc} - \bar{a}_{0c}}\right]}{n} \qquad \bar{Z} = \frac{\sum_1^n \left[\frac{y_{tj}}{y_{sj}}\right]}{n}$$

where y represents the increase in GADD153 (g) per level of cisplatin-DNA adducts (a) in cell type c, be it tumor (t) or stroma (s), from 0 h to 24 h in the jth sample (1,2,…,n), and z represents the ratio between tumor cells and stromal cells with regard to the level of "GADD153 induced per adduct".

Results

Immunohistochemistry of Cisplatin-DNA adducts

The staining pattern of cisplatin-DNA adducts, including the intratumoral variation in PNA levels, was similar to that which has been described in several previous reports [5–7]. The nuclear staining in untreated control animals was very low (PNA < 1). This nonspecific staining was subtracted from the remaining samples in Fig. 2. There was a relatively weak staining reaction in tumor cells from cisplatin-treated animals. The staining intensity in stromal cells was clearly stronger than in tumor cells (Fig. 1), with an average of six-fold higher PNA values at 24 h and eight-fold higher at 48 h posttreatment (Fig. 2).

Immunohistochemistry of GADD153

By subjective evaluation most of the tumor cells were positively stained for GADD153, whereas a majority of stromal cells were negative (Fig. 1). Staining was mainly seen in the nuclei and very little in the cytoplasm. No nuclear staining was seen in control slides stained without primary antibody, indicating that the nonspecific background was negligible.

The variation in GADD153 expression was determined both within and between samples. The intertumoral variation, expressed as coefficient of variation (CV) between the mean PNA values for each tumor, was 18.9% and 32.5% for tumor cells at 24 h and 48 h, respectively. For stromal cells the intertumoral CV was 46.3% and 38.4% at 24 h and 48 h, respectively. The intratumoral variation, expressed as mean CV of PNA values of the 10 to 25 fields measured in each tumor, was 33.8–41.3% in tumor cells and 63.4–65.6% in stromal cells.

Comparison of the GADD153 levels in the two cell types of the untreated controls, showed 4.5-fold higher PNA values in tumor cells than in stromal cells (Fig. 2). At 24 h after cisplatin treatment the difference in GADD153 level was 3.9-fold and the mean PNA values had increased from 6.0 to 10.3 in stromal cells and from 27.3 to 40.6 in tumor cells.

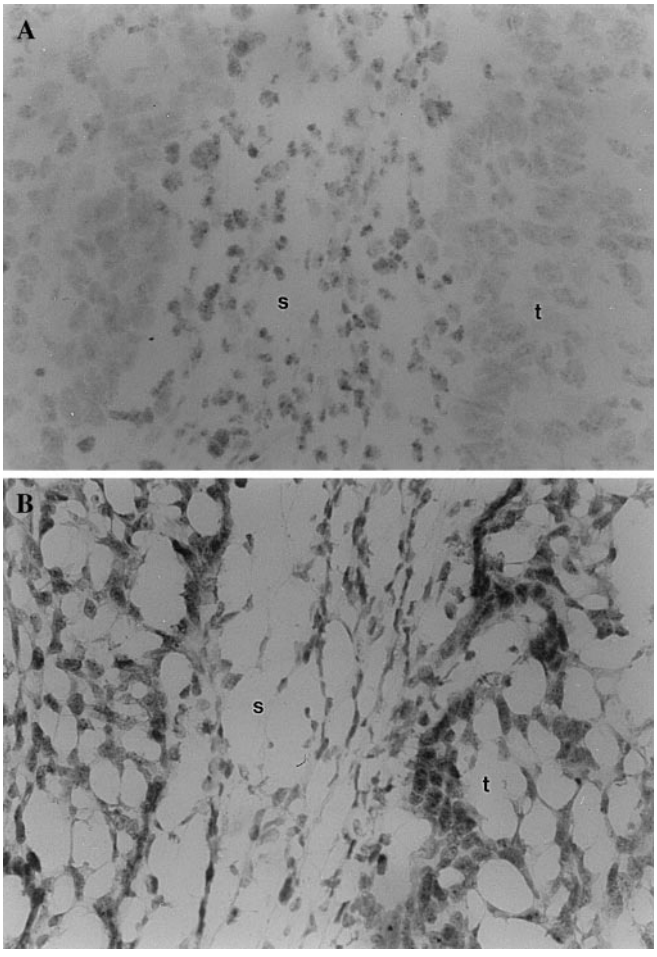

Fig. 1A,B Immunostaining for cisplatin-DNA adducts (**A**) and GADD153 protein (**B**) in tumor cells (*t*) and stromal cells (*s*) of the same topographic region of one tumor at 24 h after injection of 10 mg/kg cisplatin

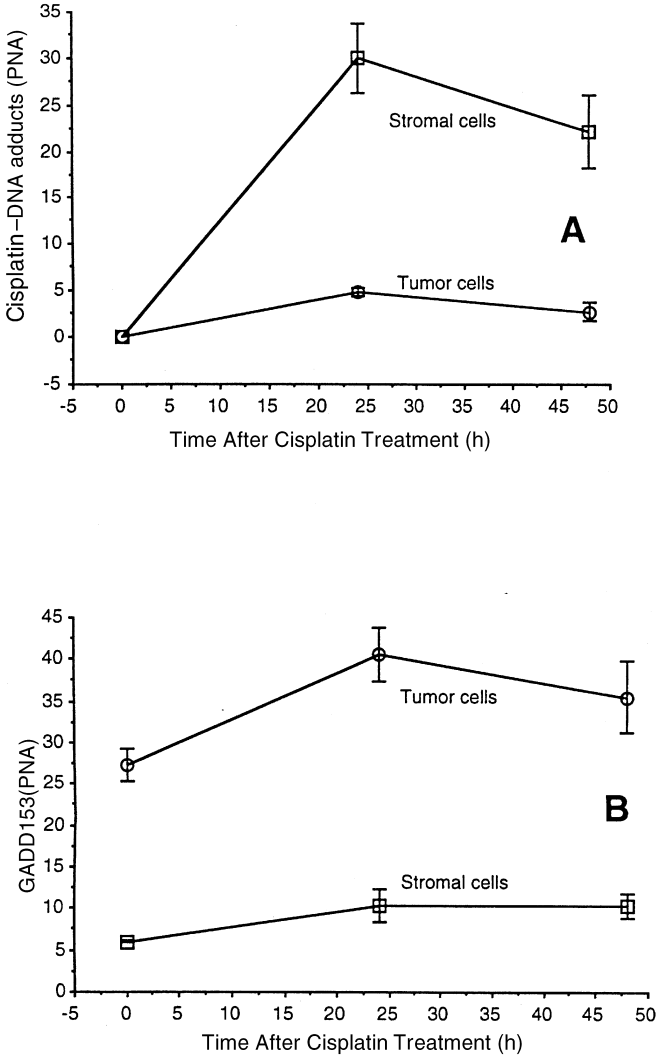

Fig. 2A,B Immunostaining levels of cisplatin-DNA adducts (**A**) and GADD153 protein (**B**), expressed as PNA (percentage positive nuclear area) in tumor cells and stromal cells from nude mouse tumor xenografts at 24 h and 48 h after treatment with 10 mg/kg cisplatin. The 0-h animals were untreated. *Bars* indicate means ± SE. The differences between the two cell types were statistically significant ($P < 0.01$, Mann-Witney *U*-test) at all time-points in both **A** and **B**

Correlations between cisplatin-DNA adducts and GADD153

The mean level of "GADD153 per cisplatin-DNA adduct" induced at 24 h was 2.76 in tumor cells and 0.12 in stromal cells, indicating that the increase in GADD153 per adduct was on average 23-fold higher in tumor cells than in stromal cells.

Discussion

Expression of *GADD153* after exposure to DNA-damaging agents has mainly been studied in terms of mRNA levels in homogenized tumor samples, while reports on the distribution in different cell types are lacking. This study shows that comparative analysis of GADD153 protein expression in different cell types is feasible with an immunohistochemical approach, by addition of methyl green to the original staining protocol, thus allowing quantitative studies with the CAS 200 image analyzer. This technique, which previously has been used successfully for analysis of cisplatin-DNA adducts [5–7] has now been applied to study the GADD153 protein levels in frozen sections of xenografted tumors. As in all immunohistochemical assays, the stoichiometric relations are incompletely known, and the analyses of the GADD153 stainings should therefore be regarded as semiquantitative rather than quantitative.

The intratumoral variation in GADD153 levels, expressed as CV, was approximately 30–40% in tumor cells and 65% in stromal cells. The reason for this difference in variation between the cell types was probably the fact that the staining intensity was higher in tumor cells than in stromal cells. We have previously found for cisplatin-DNA staining that the "relative variation" (CV) increases with decreasing staining level, whereas

the "absolute variation" expressed as standard deviation (SD) usually remains fairly independent of the staining level [5, 6]. In a previous investigation [10] the intratumoral variation of *GADD153 mRNA* between multiple biopsies of the same tumors has been shown to range from 20% to 29%, i.e. the same order of magnitude as the 30–40% intratumoral variation of GADD153 protein level found in tumor cells in the present study.

The baseline expression of GADD153 protein, i.e. before cisplatin exposure, was 4.5-fold lower in stromal cells than in tumor cells and this ratio basically remained unchanged after cisplatin treatment. For the cisplatin-DNA adducts, the relationship between the two cell types was reversed, with a sixfold higher adduct level in stromal cells than in tumor cells at 24 h. By combining these results we estimated that approximately 23-fold more GADD153 per adduct was induced in tumor cells than in stromal cells. Since neither of the two assays is truly quantitative, the numerical difference must be interpreted with caution. Nevertheless, a 25-fold difference along with clear differences at visual evaluation of the slides, strongly suggest that different cell types may respond differently to DNA damage caused by cisplatin.

The degree of upregulation of *GADD153 mRNA* in tumor samples from patients treated with cisplatin has previously been shown to correlate with tumor response [1]. When analyzing biopsy material from a tumor one is always faced with the uncertainty of how great the proportion of tumor cells is, compared with other intratumoral cell components. Our study showed clearly higher GADD153 expression in tumor cells than in normal stromal cells. Assuming the same difference in human tumor samples, our results indicate that the proportion of stromal cells in a sample may be of importance for the evaluation of the results.

We used the tumor cell line AB [20], which originated from a patient with cancer of the nasal cavity, xenografted to nude mice. Thus, the tumor cells were of human origin, whereas it can be assumed that most of the stromal cells were of mouse origin [18, 19]. The GADD153 (R-20) antibody was raised against a peptide of 20 amino acids mapping at the carboxy terminus of GADD153 protein of human origin. One may then argue that our results could be explained by different affinity of the antibody to human and mouse tissue. However, the epitope recognized by the antibody differs by only two amino acids (Ala for Thr at 16 amino acids from the carboxy terminus and Asp for Ser at 5 amino acids from the carboxy terminus) between mouse and human tissue (Swiss-Prot, internet address http://expasy.hcuge.ch/sprot), and even though no direct side-by-side comparison of human GADD153 protein next to mouse GADD153 protein has been made, using the antibody under the same conditions and loading the same amounts of protein per lane gave the same results (same intensity bands) on different gels (Catherine Chappell-Ybarra, Technical Service Department, Santa Cruz Biotechnology, Santa Cruz, Calif.; personal communication). This suggests that there should not be any significant difference in affinity between the two species. Besides tumor samples, we have also performed a few preliminary GADD153 immunostainings of mouse kidneys and we have observed a similar staining intensity in kidney cells as in tumor cells. Furthermore, in samples from patients treated with cisplatin we have observed lower levels of *GADD153 mRNA* induction in normal tongue tissue and in white blood cells than in head/neck cancer cells (unpublished observations). These factors suggest that the differences in GADD153 expression in the two cell types observed in the present study were "real".

Another species-related difference between humans and nude mice is the structure of the stroma. It consists of several different cell components: fibroblasts, endothelial cells and inflammatory cells. Nude mice are characterized by a lack of T lymphocytes. It can then be assumed that the proportion of inflammatory cells was lower in our mouse model than in humans, since the T lymphocytes play an important role in the immune response against the tumor. The purpose of the present study was to compare malignant and nonmalignant cells, without trying to analyze the different stromal cell components separately. However, visual evaluation of the slides revealed no obvious difference in staining levels (either for GADD153 or for cisplatin-DNA adducts) between the different stromal cell components. Thus, it is our belief that the conclusions of the present investigation on nude mice also should be valid in humans.

Many previous studies have shown a good correlation between the adduct level and the tumoricidal effect [2, 12, 13, 16], while others have failed to establish such a correlation [8, 14]. The reason for this could be that some cells develop a tolerance to cisplatin-DNA adducts and thereby escape being killed. It may be that this tolerance [14] is due to a decrease in the CIR, but the mechanisms are still unclear. Further studies on the fundamental issue of why some tissues are damaged by chemotherapy, while others escape, are needed. The dual immunohistochemical approach presented here may be a powerful tool to investigate these aspects after cisplatin treatment.

Acknowledgements Many thanks are due to Dr C. Lynch at Nichols Institute in San Juan Capistrano, Calif., for letting us use the CAS 200 and to Mrs Kerstin Björkdahl for staining assistance. This work was supported with Grant # 3681-B95-01XAA from the Swedish Cancer Foundation.

References

1. Alas M de las, Christen RD, Kirmani S, D'Agostino HR, Plaxe S, Darrah D, McClay EF, Howell SB, Los G (1996) Correlation between tumor response and change in GADD 153 mRNA level in human biopsies after paclitaxel treatment. Proc Proc Am Soc Clin Oncol 37: 571
2. Eastman A (1990) Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells 2: 275

3. Gately DP, Jones JA, Christen R, Barton RM, Los G, Howell SB (1994) Induction of the growth arrest and DNA damage-inducible gene GADD153 by cisplatin in vitro and in vivo. Br J Cancer 70: 1102
4. Howell SB, Gately D, Christen RD, Los G (1996) The cisplatin-induced cellular response. In: Schornagel JH, Pinedo HM (eds) Platinum and other metal coordination compounds in cancer chemotherapy. Plenum Press, New York, p 269
5. Johnsson A, Cavallin-Ståhl E (1995) A topographic study on the distribution of cisplatin in xenografted tumors on nude mice. Anticancer Drugs 7: 70
6. Johnsson A, Olsson C, Andersson H, Cavallin-Ståhl E (1994) Evaluation of a method for quantitative immunohistochemical analysis of cisplatin-DNA adducts in tissues from nude mice. Cytometry 17: 142
7. Johnsson A, Olsson C, Nygren O, Nilsson M, Seiving B, Cavallin-Ståhl E (1995) Pharmacokinetics and tissue distribution in nude mice: platinum levels and cisplatin-DNA adducts. Cancer Chemother Pharmacol 37: 23
8. Los G, Blommaert FA, Barton R, Engelse L den, Hanchett C, Vicario D, Weisman R, Robbins KT, Howell SB (1995) Selective intra-arterial infusion of high-dose cisplatin in patients with advanced head and neck cancer results in high tumor platinum concentrations and cisplatin-DNA adduct formation. Cancer Chemother Pharmacol 37: 150
9. Los G, Weisman R, Weiner D, Benbatoul K, Veelen L van, Barton R, Alas M de las, Christen R, Kirmani S, Robbins KT, Howell SB (1996) Identification of markers reflecting the extent of injury induced in human tumors following treatment with chemotherapeutic agents. Proc Am Assoc Cancer Res 37: 135
10. Los G, Benbatoul K, Gately D, Barton R, Christen R, Robbins KT, Vicario D, Orloff LA, Kirmani S, Weisman RL, Howell SB (1998) Quantitation of gadd153 nRNA levels in human malignancies: a molecular marker for the tumor injury response. Clin Cancer Res (in press)
11. Luethy JD, Holbrook NJ (1992) Activation of the gadd 153 promoter by genotoxic agents: a rapid and specific response to DNA damage. Cancer Res 52: 5
12. Plooy ACM, Dijk M van, Berends F, Lohman PHM (1985) Formation and repair of DNA interstrand cross-links in relation to cytotoxicity and unscheduled DNA synthesis induced in control and mutant human cells treated with cis-diamminedichloroplatinum(II). Cancer Res 45: 4178
13. Reedijk J, Lohman PHM (1985) Cisplatin: synthesis, antitumour activity and mechanism of action. Pharm Weekbl (Sci) 7: 173
14. Schmidt W, Chaney SG (1993) Role of carrier ligand in platinum resistance of human carcinoma cell lines. Cancer Res 53: 799
15. Sherman SE, Lippard SJ (1987) Structural aspects of platinum anticancer drug interactions with DNA. Chem Rev 87: 1153
16. Terheggen PMAB (1989) Interaction of cisplatin and carboplatin with cellular DNA (Doctorial thesis). The Netherlands Cancer Institute, Amsterdam
17. Terheggen PMAB, Floot BGJ, Scherer E, Begg AC, Fichtinger-Schepman AMJ, Engelse Lden (1987) Immunocytochemical detection of interaction products of cis-diamminedichloroplatinum(II) and cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II) with DNA in rodent tissues. Cancer Res 47: 6719
18. Warenius HM (1980) Identification and separation of mouse and human components of heterotransplanted human tumors. In: Sparrow S (ed) Immunodeficient animals for cancer research. Macmillan Press, London, p 207
19. Wennerberg J, Alm P, Lögdberg L, Tropé C (1984) Beta 2-microglobulin in squamous cell carcinoma of the head and neck and in tumours heterotransplanted into nude mice. Acta Otolaryngol 98: 335
20. Wennerberg J, Biörklund A, Tropé C (1988) The effect of cisplatin and fluorouracil on xenografted human squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 114: 162