Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 1771–1778
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Immunohistochemical detection of melphalan–DNA adducts in colon cancer cells in vitro and human colorectal liver tumours in vivo

Joost Rothbarth[a,b], Cindy Koevoets[a], Rob A.E.M. Tollenaar[a], Mike J. Tilby[c], Cornelis J.H. van de Velde[a], Gerard J. Mulder[b], Peter J.K. Kuppen[a,*]

[a]*Department of Surgery, Leiden University Medical Center, Albinusdreef 2, Leiden 2333, The Netherlands*
[b]*Division of Toxicology, Leiden/Amsterdam Center for Drug Research, The Netherlands*
[c]*Department of Biochemistry and Genetics, The Medical School, The University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, UK*

Received 17 October 2003; accepted 17 December 2003

Abstract

Melphalan is a chemotherapeutic drug that exerts its cytotoxic effect mainly through the formation of DNA adducts. We report the specific immunohistochemical detection and visualisation of melphalan–DNA adducts using the monoclonal antibody MP5/73 in cultured tumour cells and solid tumour tissue from colorectal liver metastases from patients treated with melphalan. The human colon cancer cell lines HT29, SW480 and SW1116, and the rat colon cancer cell line CC531 were exposed to different concentrations of melphalan. In addition, tumour samples from 17 patients with colorectal liver metastases treated by isolated hepatic perfusion with high dose melphalan (200 mg) were collected. Cell lines and tumour samples were stained with the MP5/73 antibody against melphalan–DNA adducts and cell viability was determined by an MTT assay. Melphalan–DNA adducts could be visualised by immunohistochemistry in both cultured cells and solid tumour tissue. A correlation between melphalan exposure concentration, the subsequent melphalan–DNA adduct staining intensity, and melphalan cytotoxicity existed for each individual cell line, but the level of both parameters independently differed between cell lines. Specific staining for melphalan–DNA adducts also was feasible in the human solid tumour tissue. There was considerable variation in melphalan–DNA adduct staining, staining intensity, and distribution in the tumour stroma and the tumour epithelium among the different patients. Melphalan–DNA adducts appeared to be more intense in tumour cells at the border of the tumour nodules than in tumour cells in the centre. Thus, visualisation of melphalan–DNA adducts by immunohistochemistry allows the study of distribution of melphalan–DNA adducts in solid tumours.

Keywords: Melphalan; Melphalan–DNA adducts; Colorectal cancer; Cultured cells; Liver metastases; Immunohistochemistry

1. Introduction

The bifunctional alkylating agent melphalan is widely used in cancer treatment [1]. It is used systemically in the treatment of leukaemia, lymphoma and neuroblastoma [2–4], and employed locally in isolated perfusion of a limb (sarcoma and in-transit melanoma metastasis of the upper or lower limb) [5,6] or the liver (metastases confined to the liver), the so called isolated hepatic perfusion [7,8]. The latter involves a method of complete vascular isolation of the liver to enable local treatment of liver metastases with high drug dosages that would cause fatal complications if delivered systemically. Recent clinical studies showed that isolated hepatic perfusion with melphalan results in high tumour response and survival rates [7–9].

Like other alkylating anticancer drugs, melphalan exerts its cytotoxic effect mainly through the formation of DNA adducts [10]. For a few DNA-damaging drugs immunological assays have been developed to enable the quantification of these DNA adducts [11]. Until now the immunological detection and quantification of melphalan–DNA adducts has been performed by an ELISA technique [12] and an immunofluorescence staining method [13,14] using the monoclonal antibodies MP5/73 and Amp4/42. The immunofluorescence technique was only suitable for detection and quantification of melphalan–DNA adducts in suspensions of

Abbreviations: PBS, phosphate-buffered saline.
* Corresponding author: Tel.: +31-71-5262309; fax: +31-71-5266750.
E-mail address: P.J.K.Kuppen@lumc.nl (P.J.K. Kuppen).

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.12.038

individual cells, such as haematological cells and cell lines [15]. Visualisation and localisation of melphalan–DNA adducts in the target tumour tissue by immunohistochemistry have not been previously described. This would allow the study of melphalan–DNA adducts in solid tissue while maintaining detailed histoanatomical information, which is not possible with the current ELISA [12] and immunofluorescence staining techniques [13,14]. Melphalan is representative for a number of related DNA-damaging drugs for none of which the distribution of DNA adducts in different regions of solid tumours are known. Consequently, visualisation of melphalan–DNA adducts in solid tumour tissue would give insight in the distribution of these types of drugs.

In the present study, we report the immunohistochemical detection and visualisation of melphalan–DNA adducts in cultured colon cancer cells in vitro and human colorectal liver tumours in vivo. Initially, we developed a method that enabled us to visualize and semi-quantitatively analyse melphalan–DNA adducts in cultured colon cancer cells using the MP5/73 antibody. This method was then applied to visualize melphalan–DNA adduct formation in colorectal liver metastases of patients treated by isolated hepatic perfusion with high dose melphalan. By double staining of the tumour tissue sections with several tissue markers, including laminin, CD34 (an endothelial cell marker), CD45 (a pan-leukocyte marker), CEA and 3.2.3A3 (tumour markers), the presence and intensity of melphalan–DNA adducts in different types of cells within the tumour were studied.

2. Materials and methods

2.1. Cell lines

The human colon cancer cell lines HT29, SW480 and SW1116, and the rat colon cancer cell line CC531 were cultured in HEPES-buffered RPMI-1640 culture medium supplemented with glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml) and 10% (v/v) fetal calf serum (all Gibco/BRL, Paisley, UK).

2.2. Melphalan treatment of cell lines

Melphalan was purchased from Sigma-Aldrich (St. Louis, MO, USA). A melphalan stock solution (16.4 mM) was prepared by dissolving 1 mg melphalan in 200 μl 0.09% (w/v) hydrochloric acid. The melphalan stock solution was diluted with foetal calf serum-free culture medium to achieve the final melphalan concentrations.

To generate melphalan–DNA adducts for detection by immunohistochemistry the cells were plated in chamber slides with eight chambers per slide (Nunc, Roskilde, Denmark) and cultured for 72 h at 37 °C, 5% CO_2; this resulted in monolayers of cells covering approximately 70% of the chamber's surface. The HT29 and SW480 cells were cultured on poly-L-lysine-coated chamber slides, the SW1116 and CC531 cells on collagen-coated chamber slides (both Sigma-Aldrich). Cells were exposed to 0, 10, 25, 50, 100, 250 or 500 μM melphalan for 1 h at 37 °C, 5% CO_2. Culture medium was removed, cells were washed twice with fresh culture medium and incubated with fresh culture medium for another hour. After removing this culture medium, the slides with the cells were washed twice with phosphate-buffered saline (PBS), fixed in acetone supplemented with 0.3% H_2O_2 (v/v) to block endogenous peroxidase for 10 min at room temperature, air-dried for 10 min at room temperature, and stored at −20 °C until melphalan–DNA adduct detection by immunohistochemistry.

Melphalan cytotoxicity was determined by an MTT assay, an assay designed for the spectrophotometric quantification of cell growth and cell viability [16]. The cells were seeded at 1000 cells per well in a 96-well microtitre plate (Greiner, Alphen a/d Rijn, The Netherlands) in 100 μl culture medium to obtain approximately 4000 cells per well after 2 days at 37 °C, 5% CO_2. Then, the cells were washed and exposed to 0, 10, 25, 50, 100, 250 or 500 μM melphalan for 1 h at 37 °C, 5% CO_2. To control for cytotoxicity from hydrochloric acid, the solvent for melphalan, cells were also exposed to hydrochloric acid at a concentration adjusted to the highest concentration of melphalan (500 μM). After treatment the cells were washed twice with culture medium and 100 μl fresh culture medium was added. The day after exposure to melphalan another 100 μl fresh culture medium was added. After an additional 2 days the culture medium was removed and 200 μl fresh culture medium was added. At day 4 after treatment the culture medium was removed and cells were incubated for 4 h with 100 μl fresh culture medium and 10 μl MTT-labelling agent (Sigma-Aldrich). Subsequently, 100 μl solubilisation solution (BioRad, Hemel Hempstead, UK) (10% (v/v) in 0.01 M HCl) was added. After incubation overnight the absorbance at 590 nm was measured by a microtitre-plate reader (BioRad Laboratories B.V., Veenendaal, The Netherlands).

2.3. Tumour sample collection

Tumour and liver tissue samples were taken from 17 patients with metastases confined to the liver treated with isolated hepatic perfusion [9]. All patients had liver metastases from a histologically confirmed colorectal carcinoma. All patients were treated by isolated hepatic perfusion with 200 mg melphalan dissolved in 40 ml of Wellcome Diluent (Wellcome Pharmaceuticals B.V., Utrecht, The Netherlands) for 1 h [8,9]. Tumour response, i.e. short-term treatment outcome, was evaluated by abdominal CT scans obtained at 3-month intervals [17]. Local medical ethical committee approval was obtained.

Tumour biopsies from liver metastases were taken before and directly after treatment with melphalan. Tissue

samples were frozen by immersion in isopentane at −80 °C and stored at −80 °C. Cryostat sections (5 μm) were cut from frozen tissue and mounted on slides. The sections were dried overnight at 60 °C, fixed in acetone with 0.3% H_2O_2 (v/v) to block endogenous peroxidase for 10 min at room temperature, air-dried for 10 min at room temperature, and stored at −20 °C.

2.4. Immunoreagents

Melphalan–DNA adducts were detected using monoclonal antibody MP5/73 (hybridoma culture supernatant, dilution 1:100). This was raised against DNA that had been alkylated with melphalan [12]. It specifically recognises N7–guanine adducts [18] formed by melphalan in DNA and RNA [12]. It also recognises, with equal sensitivity, DNA adducts formed by the monofunctional derivative of melphalan, monohydroxymelphalan [19]. MP5/73 does not recognise melphalan adducts on protein [12] or adducts formed by related alkylating agents, such as chlorambucil [20]. Exposure of melphalan–DNA adducts to alkali treatment induces a ring-opening reaction of the imidazole moiety of the N7-alkylated guanines to yield *N*-formamidopyrimidine derivatives. These are not recognised by MP5/73 [14]. However, MP5/73 recognises adducts present in G-rich DNA sequences which appear to be relatively resistant to the ring-opening reaction [20]. For double stainings polyclonal rabbit anti-laminin (Sigma-Aldrich), polyclonal rabbit anti-human carcinoembryonic antigen (DAKO, Glostrup, Denmark), monoclonal mouse 323/A3 (Ep-CAM-marker) (obtained from the Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands), monoclonal mouse anti-CD45, or monoclonal mouse anti-CD34 (DAKO) antibodies were used as a second marker. As secondary and tertiary antibodies peroxidase-labelled rabbit anti-rat immunoglobulins (Ig), peroxidase-labelled swine anti-rabbit Ig and peroxidase-labelled rabbit anti-mouse Ig (all DAKO) were used. All primary antibodies were applied at optimal dilutions in PBS containing 1% (w/v) bovine serum albumin. All secondary and tertiary antibodies were applied at optimal dilutions in PBS containing 1% (w/v) bovine serum albumin and 10% (v/v) normal human serum.

2.5. Immunohistochemical assay

Frozen chamber slides and tissue slides were allowed to rise to room temperature followed by washing twice in PBS for 5 min. The chamber slides were treated with 0.1 M NaOH for 5 min and the tissue slides for 3 min to denature the DNA and were then washed three times with PBS for 5 min. Sections were then incubated overnight with MP5/73. The next day the sections were washed three times for 5 min with PBS and incubated with peroxidase-conjugated rabbit anti-rat Ig for 30 min. After washing three times with PBS for 5 min, the sections were incubated with peroxidase-conjugated swine anti-rabbit for 30 min. After washing another two times with PBS for 5 min and washing 5 min in a buffered 0.05 M Tris–HCl (pH 7.6) solution, visualisation of binding antibodies was achieved by a 10-min incubation with diaminocarbazole substrate in a buffered 0.05 M Tris–HCl (pH 7.6) solution containing 0.0018% (v/v) H_2O_2.

For single stainings, the sections were counter stained using haematoxylin and washed twice for 5 min in PBS, rinsed with water, air-dried and mounted using Aquatex (Merck, Darmstadt, Germany). For double stainings, the sections were washed twice in PBS and subsequently incubated with a second primary antibody for 1 h at room temperature. After incubation, the slides were washed three times for 5 min with PBS. In case of mouse second primary antibodies, the sections were incubated for 30 min with peroxidase-conjugated rabbit anti-mouse Ig. In case of rabbit second primary antibodies, the sections were incubated for 30 min with peroxidase-conjugated swine anti-rabbit Ig. In case of anti-CD34, amplification was done by another incubation with peroxidase-conjugated swine-anti-rabbit Ig for 30 min. After washing three times with PBS for 5 min, the immune complexes were visualised by a 12-min incubation in a buffered 0.05 M Tris–HCl (pH 7.6) solution containing per 100 ml: (1) 40 mg 4-chloro-1-naphthol dissolved in 200 μl dimethylformamide and 300 μl ethanol; and (2) 200 μl of a 30% (v/v) H_2O_2 solution. The sections were washed two times for 5 min in PBS and rinsed with water, air-dried and mounted with Aquatex.

2.6. Analysis

Melphalan–DNA adduct staining intensities in the cell lines were semi-quantitatively scored by defining different groups of staining intensity as described in Section 3: 0 = none, 1 = low, 2 = moderate, 3 = high and 4 = very high staining intensity. A general assessment was made of the whole sample. In the human colorectal liver metastasis tissue we assessed the melphalan–DNA adduct staining (negative or positive) and the melphalan–DNA adduct distribution in both the tumour epithelial and tumour stromal cells.

3. Results

Specific staining for melphalan–DNA adducts proved to be feasible in all cultured colon cancer cell lines tested: the tumour cells exposed to melphalan and stained with the MP3/75 antibody showed specific nuclear staining for melphalan–DNA adducts (brown staining), whereas the unexposed controls did not. Fig. 1 shows this staining for the SW480 cell line. In most cell lines melphalan–DNA adducts could already be visualised after exposure of the cells to a concentration of 10 μM.

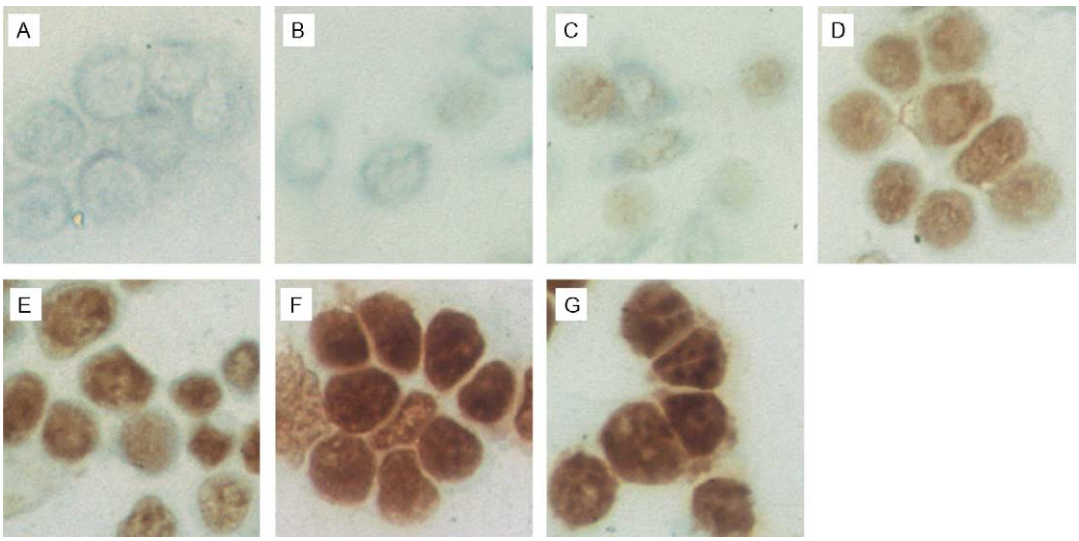

Fig. 1. Representative fields of view of immunohistochemical staining for melphalan–DNA adducts (brown staining) of SW480 cells exposed to different concentrations of melphalan. Melphalan–DNA adduct intensity increased with melphalan concentration: panels A = 0 μM, B = 10 μM, C = 25 μM, D = 50 μM, E = 100 μM, F = 250 μM, G = 500 μM melphalan. Melphalan–DNA adduct staining intensities in the cell lines were semi-quantatively scored by defining different groups of staining intensity: 0 = none, 1 = low, 2 = moderate, 3 = high and 4 = very high staining intensity. In this figure melphalan–DNA adduct staining intensity was considered 'none' in panels A and B, 'low' in panel C, 'moderate' in panel D, 'high' in panel E and 'very high' in panels F and G. Original magnification 700×.

In all cell lines staining intensity increased with melphalan concentration exposure (see, e.g. Fig. 1). By semi-quantitative analysis of the melphalan–DNA adduct staining intensity, we were able to compare the DNA adduct intensity in the different cell lines exposed to different melphalan concentrations (Fig. 2A). Both melphalan–DNA adduct staining intensity (Fig. 2A) and melphalan cytotoxicity (Fig. 2B) in each cell line increased with melphalan concentration. This implicates that melphalan–DNA adduct staining intensity and melphalan cytotoxicity are correlated, which is indeed the case as is shown in Fig. 2C. Between different cell lines a difference in melphalan–DNA adduct staining intensity at equal melphalan concentrations existed (Fig. 2A). We analysed whether these differences reflected differences in melphalan cytotoxicity, but this was not the case: equal melphalan–DNA adduct staining intensity did not result in equal melphalan cytotoxicity (Fig. 2C). For instance, at moderate staining intensity, scored as '2', the number of cells as a percentage of the control was 83% in the CC531, 72% in the SW1116, 49% in the HT29 and 22% in the SW480 colon cancer cells. No difference in cytotoxicity occurred between control cells exposed to the highest concentration hydrochloric acid and the control cells exposed to culture medium.

Specific staining for melphalan–DNA adducts also proved to be feasible in human colorectal liver metastasis tissue from patients treated with high dose melphalan by isolated hepatic perfusion. The presence of melphalan–DNA adducts (brown staining) was evident in tumour tissue exposed to melphalan (sample taken after melphalan treatment) (Fig. 3B), but not in the unexposed tumour tissue (sample taken before melphalan treatment) (Fig. 3A). Specific staining of melphalan–DNA adducts was achieved in 15 out of the 17 patients. Double staining enabled us to distinguish tumour cells from other cells, such as fibroblasts, lymphocytes, and endothelial cells. This showed that melphalan–DNA adducts were also present in fibroblasts located in the tumour stroma, lymphocytes and in endothelial cells (Fig. 3B). We rarely observed melphalan–DNA adducts in hepatocytes: only in hepatocytes in the tumour margin.

In colorectal tumours, the blood vessels are exclusively situated in the tumour stroma surrounding the tumour epithelial nodules (see Fig. 3B). Analysis of the localisation of melphalan–DNA adducts in the tumour tissue indicated that the intensity of staining in the tumour epithelial cells was related to their proximity to blood vessels. Melphalan–DNA adducts appeared to be more intense in tumour epithelial cells at the border of the tumour nodules than in the centre (Fig. 3C).

There was considerable variation in staining intensity and distribution in the tumour stroma and the tumour epithelium. In all 15 tumour samples, in which melphalan–DNA adducts were present, the tumour stromal cells stained positive for melphalan–DNA adducts, and in all except for one sample, the tumour epithelial cells stained positive for melphalan–DNA adducts.

4. Discussion

Melphalan alkylates DNA, RNA and protein with similar efficiencies [21]. On DNA, it induces predominantly monofunctional adducts, the majority of which are formed at N7–guanine. Guanine–guanine and guanine–adenine

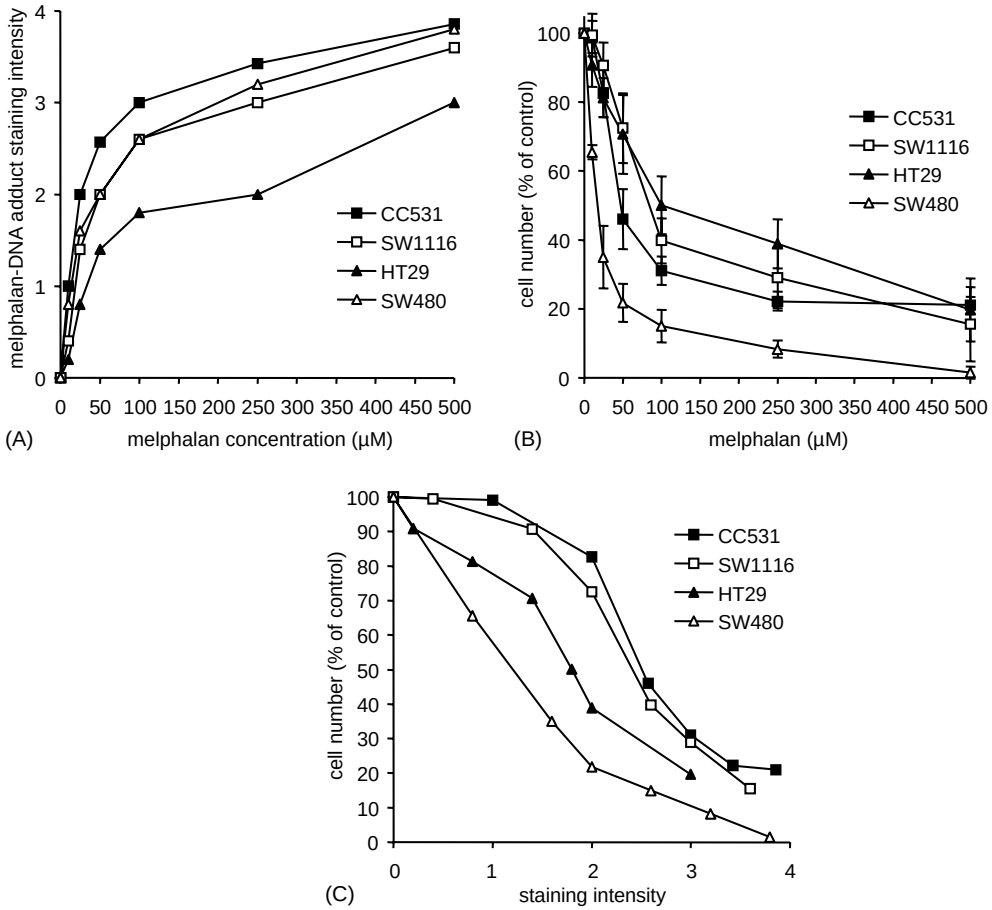

Fig. 2. Melphalan–DNA adduct intensity and cell number after melphalan treatment in the different colorectal cancer cell lines. Melphalan–DNA adduct staining intensity was scored semi-quantatively as explained in Fig. 1. (A) Melphalan–DNA adduct intensity after exposure to different melphalan concentrations; (B) cell number after exposure to different melphalan concentrations; (C) the relation between the melphalan–DNA adduct staining intensity and cell number. All experiments were performed at least four times.

cross-links are also formed [18,21]. The monoclonal antibody used in this study to detect DNA adducts recognises adducts on guanine [18] and appears to recognise mono- and bifunctional adducts with comparable sensitivity [19]. Alkali treatment of samples is necessary to denature the DNA so as to maximise immunorecognition of the melphalan adducts, which are very poorly recognised by MP5/73 on native DNA [12]. However, it also causes degradation of histological structure, and thus needs to be minimised. Furthermore, alkali treatment causes ring-opening of N7–guanine adducts. The ring-opened derivatives are more stable to subsequent depurination but are not recognised by MP5/73 [20]. The alkali treatments used in the present procedures (0.1 M NaOH for 3 or 5 min) were found previously to achieve optimal denaturation but to cause no significant loss of immunoreactivity due to ring opening [13,20]. Melphalan preferentially alkylates DNA at sequences of several contiguous guanines [19]. It was previously concluded, from immunological evidence, that the alkali-induced ring-opening reaction proceeds more slowly at such sites. Both effects were attributed to the electronegative properties of these sequences [20,22]. These features favour the development of a useful staining technique. The alkaline treatment also induces hydrolysis of RNA, which minimises potential cytoplasmic staining due to RNA adducts. Another antibody was developed to recognise the stable ring-opened guanine derivatives [14]. However, it was found that to achieve full ring-opening requires such extensive alkali treatment [14,20] that histological structure would be lost.

We report a new immunohistochemical staining method for the detection and visualisation of melphalan–DNA adducts in both cultured tumour cells and human solid tumour tissue. The experiments on cultured cells showed that melphalan–DNA adducts can be detected and visualised by this method. Two methods have been published previously for detection of melphalan DNA adducts using antibody MP5/73. An ELISA method [12] was applicable to analysis of extracted DNA and, although reliably quantitative, did not permit assessment of inter-cell or histological distribution of the adducts. An immunofluorescence method [13] was developed for quantification of adducts in individual cells, but since this entailed embedding suspended cells in agarose it was not applicable to histological sections. The present method complements the above methods by permitting, a semi-quantitative assessment

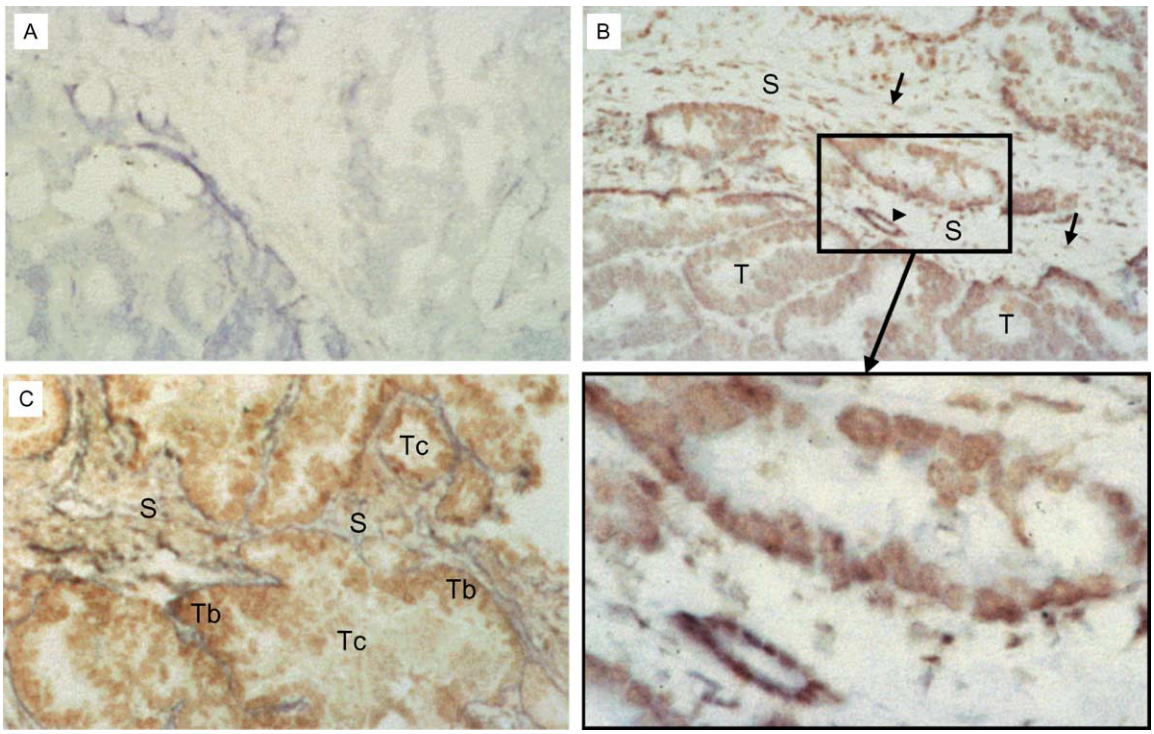

Fig. 3. Immunohistochemical staining for melphalan–DNA adducts (brown staining) of human liver tumour tissue taken from a patient treated with high dose melphalan by isolated hepatic perfusion. Sample taken before melphalan treatment stained with MP5/73 antibody (A); sample taken after melphalan treatment stained with MP5/73 (B); sample taken after melphalan treatment double stained with MP5/73 and anti-laminin (C). Melphalan–DNA adducts were present in tumour tissue exposed to melphalan (B), but not in the unexposed tumour tissue (A). Melphalan–DNA adducts were also present in fibroblasts (arrow) and endothelial cells (arrowhead) situated in the tumour stroma. Panel C shows that the tumour nodules were surrounded by a basal membrane-like structure of laminin, which is represented by the blue lining around the tumour nodules. Melphalan–DNA adducts were more intense in the tumour epithelial cells at the border of the tumour nodules (Tb) compared to tumour cells in the centre of the tumour nodules (Tc). Tumour epithelial cells (T); tumour stroma (S). Original magnifications: 150× (A); 150× and 450× (B); 300× (C).

of adduct distribution across histological sections for the first time for any alkylating agent in clinical tissue.

We found a correlation between the ease of formation of melphalan–DNA adducts (i.e. staining intensity in proportion to drug concentration) and melphalan sensitivity of the various cell lines. Staining intensity for melphalan–DNA adducts seemed to be predictive for the antitumour efficacy in each individual cell line. These data are consistent with the results of previous studies on DNA adducts. Tilby et al. already reported a linear correlation between the melphalan dose and the level of melphalan–DNA adducts in a human lymphoblastoid cell line as measured by competitive ELISA [15]. Several studies on DNA adducts induced by cisplatin, another DNA-reactive drug, report a correlation between cisplatin–DNA adduct levels and cytotoxicity [23–25]. Moreover, a correlation has been observed between cisplatin–DNA adduct levels in buccal cells and leukocytes of cancer patients treated with cisplatin and treatment response [26–31]. In these studies the cellular DNA adduct level proved to be a good biomarker for cytotoxicity and tumour response after cisplatin treatment.

Among cell lines these correlations for melphalan were different: treatment with the same melphalan concentration led to different melphalan–DNA adduct staining intensity, and the same melphalan–DNA adduct staining intensity led to different melphalan cytotoxicity among the four cell lines that we used. A possible explanation may be that differences exist in actual cellular melphalan uptake, and thus melphalan–DNA adduct formation, among different cell lines. Differences in active transport systems between cell lines can play a role in cellular melphalan uptake [32–34]. For instance, Harada et al. [33] reported that down-regulation of CD98 results in decreased melphalan uptake, and Larrivee and Averill [34] showed that P-glycoprotein can mediate active melphalan efflux.

As equal staining intensity after melphalan exposure in the various cell lines was not associated with equal cell melphalan cytotoxicity, apparently differences exist in the lethality of melphalan–DNA adducts between cell lines. This may be caused by variations in DNA repair mechanisms among different cell types, which is assumed to play a major role in cellular resistance to alkylating agents [35,36].

In all but two human colorectal liver metastasis samples melphalan–DNA adducts were observed in both the tumour stroma (fibroblasts, lymphocytes and endothelial cells) and the tumour epithelial cells, indicating that the melphalan treatment had been effective in terms of melphalan exposure and DNA damage. We can only speculate why no melphalan–DNA adducts were detected in two of

the patients. It is possible that the tumour sample was taken from a tumour area that was poorly vascularised and, as a result, not exposed to melphalan. The lack of adducts could also be due to the tumour being drug resistant through reduced access of drug to DNA.

We did not observe melphalan–DNA adducts in hepatocytes; this occurred only rarely in the tumour margin. In a pilot study, however, we detected melphalan–DNA adducts in liver tissue as measured by ELISA (unpublished data). The fact that we could not visualize melphalan–DNA adducts in hepatocytes may be due to a technical artefact, e.g. the pre-treatment with NaOH which is essential for exposing the melphalan–DNA adducts in tumour cells to the MP5/73 antibody. We found that the duration and concentration of NaOH treatment was rather critical for different cell types. Possibly, the 3-min pre-treatment in tissue sections is not sufficient to denature the DNA of hepatocytes and expose the melphalan–DNA adducts to the MP5/73 antibody.

Much variation was seen in the melphalan–DNA adduct staining pattern and staining intensity of the tissue sections of the different patients, although all patients received the same dose of melphalan, treatment duration was equal and inter-patient variation in pharmacokinetics would have been minimal because the drug was administered by isolated perfusion. This seems consistent with our finding that melphalan–DNA adduct staining intensity at equal melphalan concentrations can differ among different cell lines. Because much variation exists in colorectal cancer characteristics of patients in general and antitumour efficacy of chemotherapy [37], the colorectal liver metastasis characteristics in our series of patients are also expected to be heterogeneous, and thus may result in variable melphalan–DNA adduct staining patterns. Furthermore, tumour structure is variable among patients in terms of tumour stroma/tumour epithelium ratio [38]; moreover, the tumour microenvironment is characterised by a mixture of well-vascularised regions and less vascularised regions, inevitably resulting in variable exposure to melphalan. This is supported by our findings that the presence and intensity of melphalan–DNA adducts in the tumour epithelium was related to the proximity of a blood vessel. In colorectal tumours the blood vessels are not situated in the actual tumour epithelium, but in the tumour stroma surrounding the tumour epithelial nodules [38,39]. To reach these tumour nodules the melphalan, entering the tumour through the blood vessels in the tumour stroma, has to pass the tumour stroma before reaching the tumour epithelium. As a result, the tumour epithelial cells at the border of a tumour nodule, adjacent to the tumour stroma, are exposed to a higher concentration of melphalan than tumour epithelial cells in the centre of a tumour nodule. The melphalan–DNA adduct staining pattern we found corresponded with this route of melphalan in a tumour. All together, the variable staining intensity of melphalan–DNA adducts in our group of patients was not surprising. Because of this variation, staining intensity of melphalan–DNA adducts in one biopsy sample of all patients' colorectal metastases in the liver may not be predictive for treatment outcome.

This method allowed for the first time the visualisation of melphalan–DNA adducts in morphologically intact tumour tissue. As a consequence, it was possible to investigate how melphalan–DNA adducts are distributed within a tumour. This not only gives insight in the melphalan-induced DNA damage in different tumour areas, but also may lead to clues for improving the melphalan antitumour efficacy. For instance, our finding that tumour epithelial cells in the centre of a tumour nodule are less intensely stained for melphalan–DNA adducts than tumour epithelial cells at the border of a tumour nodule indicates that we should explore new ways of improving the melphalan transport and uptake in these tumour epithelial cells.

Acknowledgments

This study was supported by Grant 2000–2198 from the Dutch Cancer Society.

References

[1] Rothbarth J, Vahrmeijer AL, Mulder GJ. Modulation of cytostatic efficacy of melphalan by glutathione: mechanisms and efficacy. Chem Biol Interact 2002;140:93–107.
[2] Kyle RA. Update on the treatment of multiple myeloma. Oncologist 2001;6:119–24.
[3] Kulkarni S, Powles R, Treleaven J, Singhal S, Horton C, Sirohi B, et al. Melphalan/TBI is not more carcinogeneic than cyclophosphamide/TBI for transplant conditioning: follow-up of 725 patients from a single centre over a period of 26 years. Bone Marrow Transplant 2000;25:365–70.
[4] Kaufmann H, Urbauer E, Ackermann J, Huber H, Drach J. Advances in the biology and therapeutic management of multiple myeloma. Ann Hematol 2001;80:445–51.
[5] Eggermont AM, Schraffordt KH, Lienard D, Kroon BB, Van Geel AN, Hoekstra HJ, et al. Isolated limb perfusion with high-dose tumor necrosis factor-alpha in combination with interferon-gamma and melphalan for nonresectable extremity soft tissue sarcomas: a multicenter trial. J Clin Oncol 1996;14:2653–65.
[6] Fraker DL, Alexander HR, Andrich M, Rosenberg SA. Treatment of patients with melanoma of the extremity using hyperthermic isolated limb perfusion with melphalan, tumor necrosis factor, and interferon gamma: results of a tumor necrosis factor, dose–escalation study. J Clin Oncol 1996;14:479–89.
[7] Alexander Jr HR, Bartlett DL, Libutti SK, Fraker DL, Moser T, Rosenberg SA. Isolated hepatic perfusion with tumor necrosis factor and melphalan for unresectable cancers confined to the liver. J Clin Oncol 1998;16:1479–89.
[8] Vahrmeijer AL, Van Dierendonck JH, Keizer HJ, Beijnen JH, Tollenaar RA, Pijl ME, et al. increased local cytostatic drug exposure by isolated hepatic perfusion: a phase I clinical and pharmacologic evaluation of treatment with high dose melphalan in patients with colorectal cancer confined to the liver. Br J Cancer 2000;82:1539–46.
[9] Rothbarth J, Pijl ME, Vahrmeijer AL, Hartgrink HH, Tijl FG, Kuppen PJ, et al. Isolated hepatic perfusion with high dose melphalan for the treatment of metastatic colorectal cancer confined to the liver. Br J Surg 2003;90:1391–7.

[10] Kohn KW. Molecular mechanisms of crosslinking of alkylating agents and platinum complexes. In: Sartorelli AC, Lazo JS, Bertino JR, editors. Molecular actions and targets for cancer chemotherapeutic agents. New York: Academic Press; 1981. p. 3–16.
[11] van Zeeland AA. Molecular dosimetry of alkylating agents: quantitative comparison of genetic effects on the basis of DNA adduct formation. Mutagenesis 1988;3:179–91.
[12] Tilby MJ, Styles JM, Dean CJ. Immunological detection of DNA damage caused by melphalan using monoclonal antibodies. Cancer Res 1987;47:1542–6.
[13] Frank AJ, Proctor SJ, Tilby MJ. Detection and quantification of melphalan–DNA adducts at the single cell level in hematopoietic tumor cells. Blood 1996;88:977–84.
[14] Tilby MJ, McCartney H, Cordell J, Frank AJ, Dean CJ. A monoclonal antibody that recognizes alkali-stabilized melphalan–DNA adducts and its application in immunofluorescence microscopy. Carcinogenesis 1995;16:1895–901.
[15] Tilby MJ, Newell DR, Viner C, Selby PJ, Dean CJ. Application of a sensitive immunoassay to the study of DNA adducts formed in peripheral blood mononuclear cells of patients undergoing high-dose melphalan therapy. Eur J Cancer 1993;29A:681–6.
[16] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[17] Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. Cancer 1981;47:207–14.
[18] Tilby MJ, Lawley PD, Farmer PB. Alkylation of DNA by melphalan in relation to immunoassay of melphalan–DNA adducts: characterization of mono-alkylated and cross-linked products from reaction of melphalan with dGMP and GMP. Chem Biol Interact 1990;73:183–94.
[19] Tilby MJ, Mccartney H, Gould KA, O'Hare CC, Hartley JA, Hall AG, et al. A monofunctional derivative of melphalan: preparation, DNA alkylation products, and determination of the specificity of monoclonal antibodies that recognize melphalan–DNA adducts. Chem Res Toxicol 1998;11:1162–8.
[20] Mccartney H, Martin AM, Middleton PG, Tilby MJ. Antibody recognition of melphalan adducts characterized using immobilized DNA: enhanced alkylation of G-rich regions in cells compared to in vitro. Chem Res Toxicol 2001;14:71–81.
[21] Osborne MR, Lawley PD, Crofton-Sleigh C, Warren W. Products from alkylation of DNA in cells by melphalan: human soft tissue sarcoma cell line RD and *Escherichia coli* WP2. Chem Biol Interact 1995; 97:287–96.
[22] Mattes WB, Hartley JA, Kohn KW. DNA sequence selectivity of guanine–N7 alkylation by nitrogen mustards. Nucleic Acids Res 1986;14:2971–87.
[23] Hansson J, Fichtinger-Schepman AM, Edgren M, Ringborg U. Comparative study of two human melanoma cell lines with different sensitivities to mustine and cisplatin. Eur J Cancer 1991;27:1039–45.
[24] Terheggen PM, Emondt JY, Floot BG, Dijkman R, Schrier PI, den Engelse L, et al. Correlation between cell killing by *cis*-diamminedichloroplatinum(II) in six mammalian cell lines and binding of a *cis*-diamminedichloroplatinum(II)–DNA antiserum. Cancer Res 1990; 50:3556–61.
[25] Terheggen PM, Floot BG, Scherer E, Begg AC, Fichtinger-Schepman AM, den Engelse L. Immunocytochemical detection of interaction products of *cis*-diamminedichloroplatinum(II) and *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) with DNA in rodent tissue sections. Cancer Res 1987;47:6719–25.
[26] Reed E, Parker RJ, Gill I, Bicher A, Dabholkar M, Vionnet JA, et al. Platinum–DNA adduct in leukocyte DNA of a cohort of 49 patients with 24 different types of malignancies. Cancer Res 1993; 53:3694–9.
[27] Parker RJ, Gill I, Tarone R, Vionnet JA, Grunberg S, Muggia FM, et al. Platinum–DNA damage in leukocyte DNA of patients receiving carboplatin and cisplatin chemotherapy, measured by atomic absorption spectrometry. Carcinogenesis 1991;12:1253–8.
[28] Reed E, Ostchega Y, Steinberg SM, Yuspa SH, Young RC, Ozols RF, et al. Evaluation of platinum–DNA adduct levels relative to known prognostic variables in a cohort of ovarian cancer patients. Cancer Res 1990;50:2256–60.
[29] Reed E, Ozols RF, Tarone R, Yuspa SH, Poirier MC. The measurement of cisplatin–DNA adduct levels in testicular cancer patients. Carcinogenesis 1988;9:1909–11.
[30] Reed E, Ozols RF, Tarone R, Yuspa SH, Poirier MC. Platinum–DNA adducts in leukocyte DNA correlate with disease response in ovarian cancer patients receiving platinum-based chemotherapy. Proc Natl Acad Sci USA 1987;84:5024–8.
[31] Gupta-Burt S, Shamkhani H, Reed E, Tarone RE, Allegra CJ, Pai LH, et al. Relationship between patient response in ovarian and breast cancer and platinum drug–DNA adduct formation. Cancer Epidemiol Biomarkers Prev 1993;2:229–34.
[32] Goldenberg GJ, Lam HY, Begleiter A. Active carrier-mediated transport of melphalan by two separate amino acid transport systems in LPC-1 plasmacytoma cells in vitro. J Biol Chem 1979;254:1057–64.
[33] Harada N, Nagasaki A, Hata H, Matsuzaki H, Matsuno F, Mitsuya H. Down-regulation of CD98 in melphalan-resistant myeloma cells with reduced drug uptake. Acta Haematol 2000;103:144–51.
[34] Larrivee B, Averill DA. Melphalan resistance and photoaffinity labelling of P-glycoprotein in multidrug-resistant Chinese hamster ovary cells: reversal of resistance by cyclosporin A and hyperthermia. Biochem Pharmacol 1999;58:291–302.
[35] Pu Q, Bianchi P, Bezwoda WR. Alkylator resistance in human B lymphoid cell lines: (1). Melphalan accumulation, cytotoxicity, interstrand-DNA-crosslinks, cell cycle analysis, glutathione content in the melphalan-sensitive B-lymphocytic cell line (WIL2) and in the melphalan-resistant B-CLL cell line (WSU-CLL). Anticancer Res 2000;20:2561–8.
[36] Eder Jr JP, Chan VT, Ng SW, Rizvi NA, Zacharoulis S, Teicher BA, et al. DNA topoisomerase II alpha expression is associated with alkylating agent resistance. Cancer Res 1995;55:6109–16.
[37] Adlard JW, Richman SD, Seymour MT, Quirke P. Prediction of the response of colorectal cancer to systemic therapy. Lancet Oncol 2002;3:75–82.
[38] Menon AG, Fleuren GJ, Alphenaar EA, Jonges LE, Janssen van Rhijn CM, Ensink NG, et al. A basal membrane-like structure surrounding tumour nodules may prevent intraepithelial leucocyte infiltration in colorectal cancer. Cancer Immunol Immunother 2003;52:121–6.
[39] Hagenaars M, Ensink NG, Basse PH, Hokland M, Nannmark U, Eggermont AM, et al. The microscopic anatomy of experimental rat CC531 colon tumour metastases: consequences for immunotherapy? Clin Exp Metastasis 2000;18:189–96.