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Original Paper

Immunohistochemical Examination of the Expression of O^6-methylguanine–DNA Methyltransferase in Human Melanoma Metastases

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In tumour cell lines, an inverse relationship has been shown between susceptibility to the cytotoxic effects of the O^6-alkylating agents and the expression of the DNA repair protein O^6-methylguanine–DNA methyltransferase (MGMT). One of the most effective single agents in chemotherapy of metastatic melanoma is the O^6-alkylating drug, dacarbazine. We therefore examined the distribution of MGMT in 37 skin and lymph node melanoma metastases using rabbit antihuman MGMT antiserum. MGMT expression was undetectable in tumours from 2 out of 34 patients and low in 4 further patients. When present, staining was mainly nuclear and showed a marked variation both among tumour cells within the same metastases, between separate metastases in the same patient and between tumours in different patients. MGMT expression determined by immunohistochemistry showed a relation to MGMT activity measurements, but was not related to the number of proliferating cells, as identified by staining with MIB-1 antibody. Tumour cells with moderate to strong immunostaining with MGMT antiserum were significantly more abundant in metastases excised after dacarbazine-based chemotherapy ($n = 8$) than in those excised before treatment ($n = 29$).

Key words: melanoma, DNA repair, O^6-methylguanine–DNA methyltransferase, immunohistochemistry

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INTRODUCTION

THE MOST frequently used drug in the treatment of metastatic melanoma is the monofunctional alkylating agent dacarbazine (5-3′,3′-dimethyl-1-triazenoimidazole-4-carboxamide (DTIC)). With single agent therapy, an objective response rate of 15–20% is achieved [1]. Unfortunately, the majority of patients only obtain partial responses of short duration [1–3].

DTIC undergoes metabolic activation to 5-3′-methyl-1-triazenoimidazole-4-carboxamide (MTIC) which methylates DNA, producing among other lesions, O^6-methylguanine, which is considered to be the principal cytotoxic adduct [4]. Methyl groups bound to the O^6-atom of guanine can be removed by the repair protein O^6-methylguanine–DNA methyltransferase (MGMT) [5] and transferred to a cysteine acceptor residue within the MGMT protein, which is thereby irreversibly inactivated. *De novo* synthesis of the protein is thus required for repair to continue.

A correlation has been found *in vitro* between the expression of MGMT and resistance to MTIC [6–8]. It is, therefore, possible that MGMT expression could have an influence on clinical response to DTIC treatment.

We have previously analysed MGMT activity in melanoma tumours using a biochemical assay [9, 10]. This technique has the disadvantage of only showing the average MGMT activity of all cells in the biopsy, i.e. both tumour and normal cells. Thus, if the biopsy material contains a substantial proportion of the latter, the result may not accurately reflect the MGMT activity in the tumour cells.

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Another possible pitfall is due to the phenomenon of tumour cell heterogeneity: a tumour with low overall MGMT activity could contain a small fraction of tumour cells expressing high levels of MGMT. These problems can be addressed using immunohistochemistry in which the MGMT levels of individual cells can be determined.

The aim of the present study was to examine MGMT expression in human melanoma metastases using rabbit polyclonal MGMT antibodies [11, 12] and to compare the results of immunohistochemistry with MGMT activity measurements using a biochemical assay. We also investigated whether the expression of MGMT is related to the proliferative activity of the tumour cells.

PATIENTS AND METHODS

Patients and melanoma biopsies

Thirty-seven melanoma metastases, comprising 22 lymph node and 15 skin tumours, were excised from 34 patients at the Department of Oncology, Radiumhemmet, Karolinska Hospital. There were 18 male and 16 female patients with a mean age of 52.4 years (range 20–84 years). Twenty-nine metastases were excised from patients who had not received chemotherapy, while eight were excised from 5 patients who were non-responders after DTIC treatment. Normal tissue surrounding the tumour was removed and the tumours were divided into small pieces, which were frozen in liquid nitrogen and stored at −70°C until assayed. Local ethical approval was obtained for the study.

MGMT immunohistochemistry

Tumour material was thawed before fixation in 95% ethanol overnight and embedded in paraffin. Sections (4 μm) were prepared, mounted on slides, dewaxed and rehydrated. Endogenous peroxidase activity was blocked with 0.5% H_2O_2 in methanol for 10 min at room temperature and the slides were washed in water for 5 min and in phosphate-buffered saline (PBS) (pH 7.4) for 10 min. The sections were blocked with 1.5% normal goat serum for 20 min at room temperature and were then exposed to rabbit polyclonal antihuman MGMT antiserum (1:2500) [11, 12] or normal rabbit serum overnight at 4°C. Human MGMT-transgenic mouse-liver tissue was used as a positive control. The slides were then washed three times in PBS and incubated with a biotinylated goat antirabbit antibody (1:100) for 1 h at room temperature using the Vectastain Elite avidin–biotin complex (ABC) kit (Vector Laboratories, Inc., Burlingame, California, U.S.A.) [13]. They were then washed three times in PBS and incubated with ABC for 1 h at room temperature after which they were again washed three times in PBS. For chromogen development, they were incubated for 5 min in 0.1 M Tris–HCl (pH 7.2) containing 0.5 mg/ml 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma Company, St Louis, Missouri, U.S.A.) and 0.01% H_2O_2. The slides were washed in water, counterstained with haematoxylin, dehydrated and mounted with coverslips.

For each biopsy, 100–400 tumour cells were counted and the intensity of staining of each cell was determined visually and classified according to the following score: negative = no staining; 1 + = weak staining; 2 + = medium staining; 3 + = strong staining. The fraction of negative, 1 +, 2+ and 3+ cells was calculated for each tumour slide. The material was reviewed independently by two persons who were in agreement with each other.

The fraction of cells was divided into two groups, one with medium to strong staining (2 + to 3+) and one with no or weak staining (negative to 1+). The fraction of cells with medium to strong (2 + to 3+) staining of MGMT in the area with the highest staining intensity in skin and lymph node metastases was calculated.

MIB-1 antibodies

Proliferation was estimated using immunohistochemistry with a Ki-67 clone, MIB-1 antibody, which detects all cells in G1, S and G2 + M phases of the cell cycle [14]. Sections (4 μm) were prepared as described above. The slides were placed in heat-resistant boxes filled with 10 mM citrate buffer (pH 6.0) and processed twice in a microwave oven (700 W) for 5 min each. The slides were allowed to cool in the boxes for approximately 20 min and were then rinsed in Tris-buffered saline (TBS) (pH 7.6) for 5 min. Endogenous peroxidase activity was blocked with 0.5% H_2O_2 in water for 10 min and the slides were washed three times with water. The slides were exposed overnight at 4°C to the monoclonal mouse anti-Ki-67 clone MIB-1 antibody (2 μg/ml) (Immunotech S.A., Marseille, France) or normal mouse serum. After washing in PBS, the slides were covered with biotinylated goat antimouse antibody (1:200) for 30 min at room temperature followed by three washes in PBS. The slides were incubated with ABC-complex for 30 min at room temperature and after washing in PBS, exposed to DAB (as above) for 7 min, followed by another three washes in PBS. They were then treated as above. The fraction of positive and negative tumour cells were determined by counting more than 100 tumour cells/sample.

Assay of MGMT activity

An approximately 0.5 cm^3 piece of the tumour biopsy was homogenised in a microdismembrator II (B. Braun, Melsungen AG, Germany) for 30 sec. The dry powder was suspended in equal volumes of lysis buffer (300 mM potassium chloride (KCl), 50 mM Tris–HCl (pH 7.5), 10 mM dithiothreitol, 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride) and left on ice for 30 min. Debris was removed by centrifugation for 30 min at 13000 rpm at 4°C [15]. The MGMT activities of cell extracts were measured by removal of [^{3}H]methyl groups from the O^6-position of guanine in [^{3}H]MNU-methylated *M. luteus* DNA as previously described [16]. The protein concentrations of all extracts were determined by the Bradford method (Bio-Rad) using bovine serum albumin (BSA) as standard [17].

Statistical analysis

Where indicated, statistical significance was tested using Student's *t*-test; a P value <0.05 was considered significant.

RESULTS

In the present study, biopsies of 37 melanoma metastases from 34 patients were analysed with MGMT antiserum. The MGMT protein was undetectable in two of the metastases, and four metastases with low expression (>90% of cells negative to 1+) were also observed. These six tumours were all obtained from different patients, thus 17.6% of the patients had tumours with low or undetectable MGMT

expression. When present, staining was predominantly nuclear in most cases, although some samples also showed cytoplasmic staining. Figure 1 shows examples of antibody staining of metastases from 3 patients. The tumour from Patient 1 exhibited a uniform nuclear staining of medium intensity while Patient 2's tumour showed weak, nuclear staining of most tumour cells. The majority of the cells in Patient 3's tumour sample had strong nuclear staining, but some cells lacked nuclear staining.

In 2 patients more than one metastasis was analysed, and in both cases MGMT expression differed between the metastases from the same patient. Figure 2 shows analyses of three metastases from patient 4: a considerable variation in staining intensity between the metastases was observed.

The fraction of cells with 2+ to 3+ staining of MGMT in skin and lymph node metastases showed wide variation (Figure 3). The average fraction of cells with 2+ to 3+ staining of MGMT in skin ($42.7 \pm 28.9\%$ mean $\pm$ S.D.) and lymph node metastases ($39.5 \pm 21.0\%$) were similar.

The levels of MGMT activity determined using the biochemical assay were compared with the expression of MGMT analysed by immunohistochemistry in 16 melanoma metastases. Only an approximate comparison could be made since the ABC method is not as quantitative as the biochemical assay (i.e. a doubling of immunostaining does not necessarily correspond to a doubling of the amount of protein) [13]. We found that metastases with MGMT activities less than 0.2 pmol/mg protein showed a mean 2+ to 3+ staining of $23.8 \pm 20.7\%$ ($n = 6$), while metastases with MGMT activities higher than 0.2 pmol/protein showed a 2+ to 3+ staining of $54.9 \pm 18.1\%$ ($n = 10$) ($P < 0.01$).

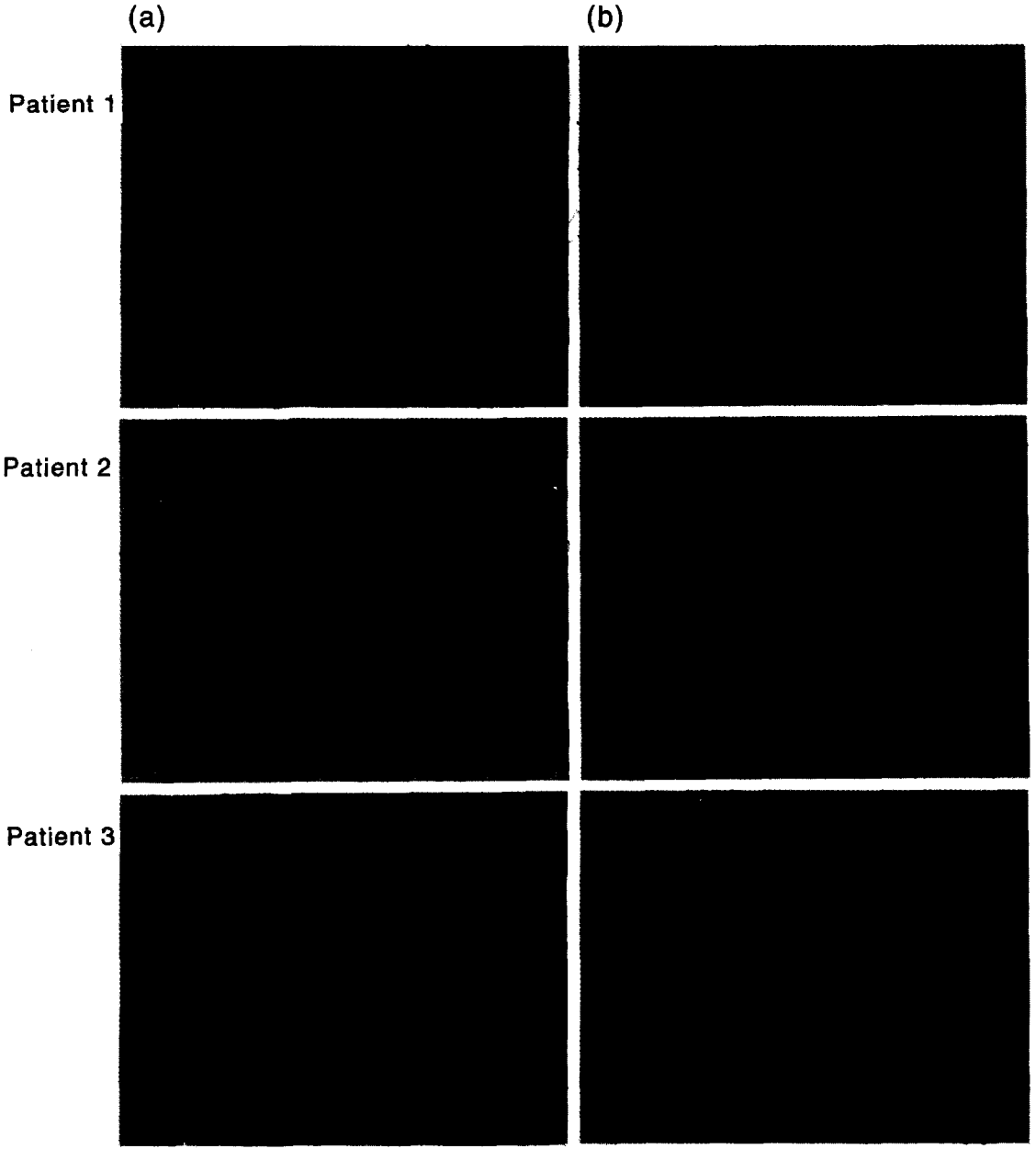

Figure 1. Immunostaining of melanoma metastases from three patients with (a) anti-human MGMT antiserum and (b) normal rabbit serum. Magnification ×570.

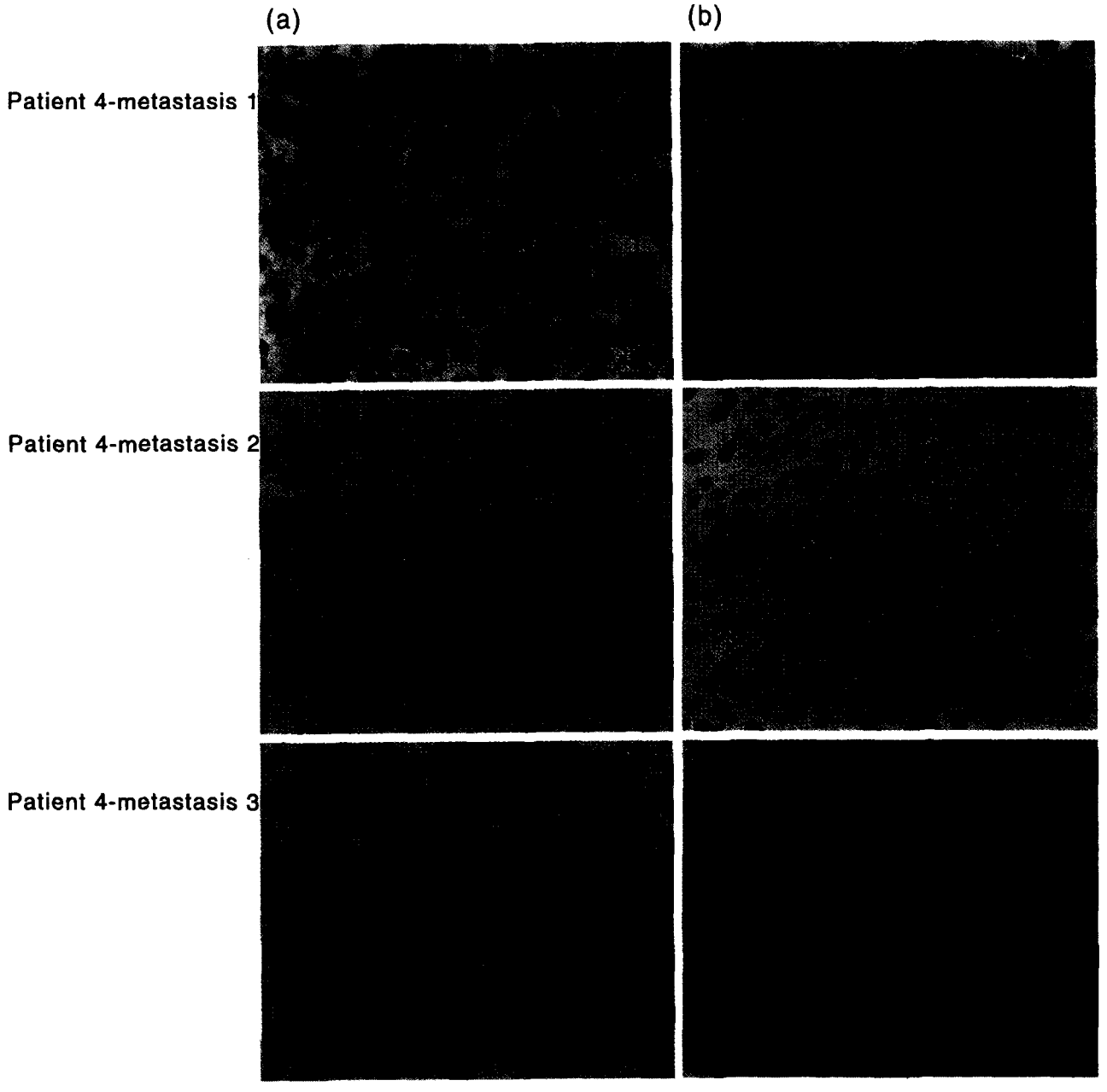

Figure 2. Immunostaining of three melanoma metastases from the same patient with (a) anti-human MGMT antiserum and (b) normal rabbit serum. Magnification ×570.

Thus, an increased expression of MGMT protein as detected by immunohistochemistry was related to an increased MGMT activity.

Proliferative activity was analysed in the melanoma metastases with MIB-1 monoclonal antibodies and compared to the MGMT expression. Since the sections require processing in a microwave oven to make the epitopes available to the MIB-1 antibody, it was not possible to assay the same slide with both antibodies. In order to relate proliferative activity to MGMT expression, the two analyses were carried out on adjacent sections, and the tumour cells in the same area of the two slides were analysed. The proliferative activity was analysed in 16 of the tumour samples and the mean fraction of proliferating tumour cells was $34.8 \pm 25.6\%$. We observed that the number of non-proliferating cells was not related to a lack of expression of MGMT ($r = 0.016$) and we also found no correlation between the fraction of proliferating cells and the fraction of cells with 2+ to 3+ MGMT staining ($r = 0.063$).

Eight metastases were excised after chemotherapy. A comparison of the MGMT expression was made between the unpaired tumours excised before and after chemotherapy (Figure 4). The biopsies obtained after chemotherapy had a mean fraction of cells with 2+ to 3+ staining of $55.6 \pm 25.6\%$ whereas the corresponding fraction in tumours excised before treatment was $36.7 \pm 22.5\%$ ($P < 0.05$). Thus, although the number of tumour biopsies obtained from patients who received chemotherapy is small compared to the tumours excised before treatment, a significantly higher MGMT intensity was observed in metastases removed after treatment.

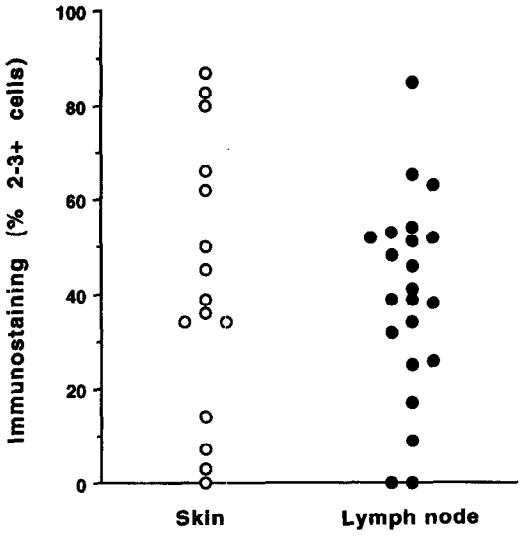

Figure 3. Intensity of staining of tumour cells from melanoma metastases with MGMT antiserum. In the area with the highest intensity of staining more than 100 tumour cells/ tumour sample were examined. The figure shows the fraction of cells with 2+ to 3+ staining of MGMT in 15 skin (○) and 22 lymph node (●) metastases.

DISCUSSION

The results presented in this paper are consistent with those of other reports [11, 12, 18] showing that staining with MGMT antiserum is mainly nuclear. In contrast, Ishibashi and associates [19] reported that 90% of MGMT immunostaining was located in the cytoplasm of tumour cell lines, and not until the DNA was damaged was the protein observed in the nucleus. The question, therefore, arises whether localisation of immunostaining varies in different cell types, or if different antibodies prefer to bind to the protein either in the nucleus or in the cytoplasm. Lee and associates [12] compared the distribution of MGMT in two different malignancies, Hodgkin's disease and ovarian carcinoma. Staining was very intense and essentially confined to the nucleus in the ovarian tumours whereas in Hodgkin's disease the staining was much weaker and found both in the nuclei and in the cytoplasm. These findings strengthen the hypothesis that there may be differences in the localisation of MGMT protein in different cell types.

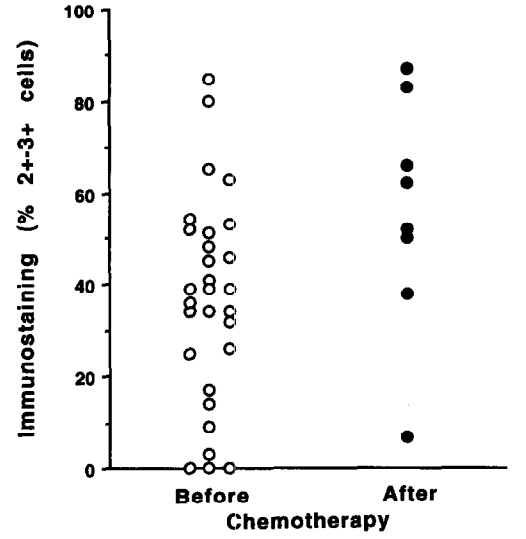

Figure 4. Intensity of staining of tumour cells from melanoma metastases with MGMT antiserum. In the area with the highest intensity of staining more than 100 tumour cells/ tumour sample were examined. The figure shows the fraction of cells with 2+ to 3+ staining of MGMT in 29 metastases excised before (○) and eight metastases excised after (●) chemotherapy.

We have previously described the MGMT activity in human melanoma tumours [9, 10]. A disadvantage with such activity measurements is that they only estimate the average MGMT activity of the tissue, and do not take into account possible differences in MGMT expression among the individual cells of the tumour. In the present study, we examined the MGMT expression of individual tumour cells in human melanoma metastases using immunohistochemistry, which provides the possibility of identifying subpopulations of cells which differ in MGMT expression in the tumours and also to analyse specifically the tumour cells and to disregard normal cells which might otherwise compromise the evaluation of the data. The mean levels of MGMT activity in skin and lymph node metastases were similar, in agreement with our earlier analyses of mean MGMT activity in melanoma metastases [10]. The staining intensity was variable in the tumours. Heterogeneity in MGMT expression between different metastases in the same patient as well as between individual cells within the same metastasis was frequent. If we presume that MGMT has an influence on the clinical response to chemotherapy, the heterogeneity in MGMT expression could explain why many of the melanoma patients only obtain partial responses after treatment with DTIC [1–3].

The reason for the variability in MGMT expression among metastases in the same individual is unclear, but may in part be due to the influence of the surrounding host tissue cells. Kjønniksen and colleagues [20] established tumours in nude rats by injection of LOX cells, a human melanoma cell line which lacks expression of MGMT: these produced metastases in several different locations including subcutaneous tissue, bone and lungs. The rats were then treated with a variety of agents including DTIC, doxorubicin or *cis*-diamminedichloroplatinum(II) (cisplatin). MGMT has not been implicated in resistance to the two latter drugs. Despite the fact that the cells in the metastases originated from the same cell line, they responded differently to treatment with DTIC but not to doxorubicin and cisplatin. These authors did not analyse MGMT activity in the different metastases, but the differences in DTIC sensitivity could be explained by an upregulation of MGMT in melanoma cells in some of the anatomical locations.

Since MGMT activity is variable during the cell cycle [21, 22], being high prior to DNA replication, declining during replication and then increasing again, some of the heterogeneity in MGMT expression between tumours might depend on differences in the levels of expression of MGMT in non-proliferating versus proliferating cells. In the present study, we related MGMT intensity to the fraction of proliferating cells in the metastases, but found no correlation.

Although the number of tumour biopsies obtained after chemotherapy was small ($n = 8$) compared to that from untreated patients ($n = 29$), the average number of tumour cells with 2+ to 3+ MGMT staining was significantly higher in the metastases excised after chemotherapy with DTIC.

Metastases removed after chemotherapy may, therefore, have an increased MGMT activity, as might be expected if drug-resistant cells are selected for by this treatment. In agreement with our findings, Moriwaki and colleagues [23] reported that MGMT activity was higher in melanoma tumours after chemotherapy with DTIC or 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (ACNU) than before treatment.

Prospective studies are required to establish whether the MGMT protein is an important determinant of resistance to chemotherapy with agents such as DTIC. We have, therefore, started to obtain fine needle biopsies in order to analyse the MGMT levels in individual tumour cells with the antibodies used in the present investigation. These analyses will be supplemented by semiquantitative analyses of MGMT mRNA with reverse transcription-polymerase chain reaction. This technique will enable us to monitor the therapeutic response of the individual metastases that were analysed for MGMT, as well as to obtain repeated samples from the same tumours during and after chemotherapy with DTIC. Immunocytochemistry will allow detection of heterogeneity in MGMT levels between subpopulations of tumour cells within the individual metastasis. This may be an important advantage, since a small proportion of cells with high levels of MGMT might determine the clinical outcome of chemotherapy.

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