Defective repair of *O*⁶-methylguanine-DNA in primary Sjögren's syndrome patients predisposed to lymphoma

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Abstract

Objective—To investigate a role for mutation in the aetogenesis of autoimmune disease by examining levels of repairing enzyme for the promutagenic DNA base lesion, *O*⁶-methylguanine, in lymphocyte extracts from patients with autoimmune diseases. We included primary Sjögren's syndrome (PSS) patients because of the additional relevance of their being at increased risk (>40-fold) of developing lymphoma.

Methods—Lymphocytes were prepared from patients with PSS (n = 22) (12 with parotid gland enlargement, an indicator of extensive lymphoproliferation), rheumatoid arthritis (n = 12), primary biliary cirrhosis (n = 11), osteoarthritis (n = 12), and healthy individuals (n = 11). MGMT amounts were determined in lymphocyte extracts by direct enzyme assay and expressed in relation to total extract DNA, protein, or cell number.

Results—We found no defect in the repairing methyltransferase enzyme between any of the groups, except in PSS patients at increased risk of developing lymphoma (those with enlarged parotid glands): *p* < 0.0001 and *p* = 0.0056, compared with healthy controls and PSS patients without parotid gland swelling, respectively.

Conclusions—Our findings implicate persistence of *O*⁶-methylguanine-DNA in the aetiology of lymphoma associated with PSS, and raise the possibility that an alternative repair process for *O*⁶-methylguanine-DNA, nucleotide excision repair, might be defective in autoimmune disease.

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Much evidence from animal model and human studies indicates that, in common with carcinogenesis, autoimmune diseases are multifactorial in origin, with both genetic and acquired factors involved. Exact aetiological mechanisms in either type of disorder, however, remain unknown. DNA repair deficiency and hypersensitivity to genotoxic agents have been proposed to predispose individuals to the development of autoimmune diseases through somatic mutation in the immune system.^{1 2} Somatic mutation and

DNA repair deficiency are also strongly implicated in carcinogenesis. That an aetiological factor(s) might be common to both conditions is supported by the finding of several associations of malignancy with autoimmune disease. One of the most significant of these is primary Sjögren's syndrome (PSS). These patients show an approximately 40-fold increased relative risk of developing lymphoma, those most at risk being individuals displaying parotid gland enlargement and other indicators of extensive lymphoproliferation.³ Lymphomas associated with PSS are largely confined to the B cell lineage, although an increased number of T cell malignancies have been reported recently.⁴ Another well documented association is that between lymphoid malignancy and autoimmune haemolytic anaemia.⁴

One explanation for these disease associations may relate to the finding in a mouse model of carcinogenesis that a single, small dose of a methylating agent can induce both thymic lymphoma and autoimmune haemolytic anaemia in susceptible strains.⁵ These agents, which occur ubiquitously in the environment, react with many sites on DNA. Of all these sites, formation and persistence of promutagenic *O*⁶-methylguanine appears to be the most important with regard to carcinogenesis and induction of autoimmune disease in animal models.

*O*⁶-methylguanine mispairs with thymine, then DNA replication leads to propagation of a G-to-A transition mutation, a known mechanism of human oncogene activation and tumour suppressor gene inactivation. *O*⁶-methylguanine is repaired by *O*⁶-methylguanine-DNA methyltransferase (MGMT), which transfers the methyl group to one of its cysteine residues. Automethylation leads to inactivation of MGMT which, unusually for an enzyme, is not regenerated.⁶ Thus, in the absence of new enzyme synthesis, a cell contains a finite capacity for removal of *O*⁶-methylguanine by MGMT.

Removal of *O*⁶-methylguanine from the DNA of intact lymphocytes, presumably by an enzymic repair process(es), was previously shown to be defective in patients with a variety of autoimmune diseases.¹ Further studies with larger numbers of patients have confirmed this observation in RA. In an attempt to explain this finding, we commenced a preliminary study of MGMT levels in lymphocyte extracts from patients with PSS, included for the first

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time because of the additional relevance of its association with lymphoma, and other autoimmune diseases.

Patients and methods

Patients were recruited from a cohort of subjects with PSS.⁷ All 22 were female and fulfilled Fox's criteria for definite or probable Sjögren's syndrome. None was a smoker or taking drugs considered likely to interfere with DNA repair proficiency assay (see below). Ten patients had existing parotid gland swelling, as detected by the examining physician. Two further patients had either previous excision of the parotid gland or radiotherapy to the parotid. In neither case was there histological evidence of malignancy. In total, therefore, 12 patients were classified as having or having had clinically significant salivary gland enlargement. Ten 'control' PSS patients without salivary gland enlargement were recruited. Anti-Ro antigen antibodies were present in approximately 50% of the subjects in each group. Median age (~57 years) and disease duration (~7.5 years) did not differ significantly between the groups. Other autoimmune diseases investigated were patients with primary biliary cirrhosis (PBC) (n = 11), and rheumatoid arthritis (RA) (n = 12). Controls comprised 12 patients with osteoarthritis (OA) who served as autoimmune connective tissue disease controls, and 11 healthy individuals (women aged 45–65 years—age range and gender chosen to reflect those of most of the patients under investigation).

LYMPHOCYTE MGMT LEVELS

Lymphocytes were isolated from approximately 20 ml of venous blood by centrifugation through Lymphoprep according to the manufacturer's (Nyegard) instructions. Lymphocyte extracts were prepared by microhomogenisation as described previously,⁸ and used immediately for determination of MGMT by direct enzyme assay.⁹ Briefly, this assay measures MGMT by monitoring the transfer of radiolabelled methyl groups to methyltransferase protein, following repair of a high molecular weight DNA substrate produced to contain O⁶-[Me-³H]methylguanine. Auto-methylation inactivates the enzyme, which is not regenerated. The stoichiometric reaction between MGMT and its substrate permits calculation of molar enzyme amount from knowledge of the specific radioactivity of the tritiated methylating agent used to make the methylated DNA substrate. MGMT amounts determined in this manner were expressed in relation to total extract DNA and protein content, measured by the methods of Cesarone *et al*¹⁰ and Bradford,¹¹ and cell number used to make the extracts. In the course of this work, we found that a modification of the DNA assay method, namely prior treatment of the sample with the anionic detergent sodium dodecyl sulphate¹⁰ (SDS) (1%), produced an increased yield of extract DNA, the same treatment being without effect on the DNA assay

calibration standards (purified calf thymus DNA). For this reason we considered that the modified procedure provided a more accurate reflection of cellularity than the previously used assay and, therefore, we continued to use the modified assay to generate all DNA data reported here. For comparison purposes, it should be noted that, as the modified assay generated a greater yield of extract DNA, MGMT amounts expressed in relation to total extract DNA reported here are smaller than those we reported previously.⁸ Additionally, and as before,⁸ we omitted the sonication step used by most other laboratories for extract preparation. This was because of our finding in preliminary studies, performed with the aim of optimising enzyme extraction efficiency, that sonication inactivated about 90% of MGMT in cultured Epstein-Barr virus immortalised human lymphocytes (LCL cells) (data not shown).

STATISTICAL METHODS

Analysis of variance (*F* test) was used to compare differences in MGMT levels between groups of patients, whose mean enzyme levels were compared by *t* test.

Results

We found highly significant differences in MGMT concentrations between the two PSS patient groups and healthy controls, the mean activity being less in PSS patients showing parotid gland enlargement (table 1). We found no significant difference in amounts of MGMT between RA and PBC patients compared with either of the control groups (healthy individuals or subjects with osteoarthritis). These findings applied irrespective of whether activities were expressed in relation to total extract DNA or lymphocyte number used to make the extract. However, MGMT amounts expressed in relation to total extract protein were not significantly different between any of the groups (table 1; data not shown for PBC, RA, and OA groups). This contradictory 'protein' result, showing no significant enzyme deficiency in PSS patients with parotid gland enlargement, seemed to arise because lymphocytes from these patients contained significantly less extractable protein, thereby producing an apparent increase in MGMT expressed in relation to total cellular

Table 1 Amounts of O⁶-methylguanine-DNA methyltransferase (MGMT) in lymphocyte extracts prepared from patients with primary Sjögren's syndrome and healthy controls

MGMT	Healthy controls	Primary Sjögren's syndrome	
		No parotid enlargement	Parotid enlargement
fmol/μg DNA†	249 (26)	234 (41)	109 (15)
fmol/10 ⁶ cells†	69 (6.8)	62 (7.6)	44 (5.7)
fmol/mg protein	910 (80)	887 (123)	810 (79)

Mean values (SEM). †Significant differences within three groups (*F* = 8.1, *p* = 0.0015 for 'DNA'; *F* = 4.0, *p* = 0.0282 for 'cells') and between parotid enlargement and both no parotid enlargement (*p* = 0.0056, 'DNA') and healthy controls (*p* < 0.0001, 'DNA'; *p* = 0.0096, 'cells').

Table 2 Amounts of total protein and DNA in lymphocyte extracts prepared from patients with primary Sjögren's syndrome and healthy controls

Extract	Healthy controls	Primary Sjögren's syndrome	
		No parotid enlargement	Parotid enlargement
Protein (µg)	1480 (159)	1796 (382)	667 (99)
DNA (µg)	5.31 (0.34)	6.52 (0.82)	4.97 (0.67)
Protein/DNA	279	275	134

Mean values (SEM).

protein; total extract DNA did not appear to vary between the groups (table 2). Why lymphocytes from PSS patients with enlarged parotid glands might show less extractable protein is not known, and requires clarification.

Discussion

The aetiology of lymphoma seen in association with PSS is unknown. It is often imprecisely ascribed to the emergence of a malignant clone following persistent antigenic stimulation of an immune system that either develops progressive abnormalities or was abnormal at the outset. Genetic translocation (proto-oncogene bcl-2, t(14;18), in PSS) and viruses (for example Epstein-Barr virus in PSS) have been implicated in the aetiology of lymphoma. Rather than being directly causal, however, these agents may act by prolonging the life of a somatic cell, thereby increasing its likelihood of accumulating the genetic lesions required to become malignant. A more crucial determinant of susceptibility may be an individual's ability to repair these lesions.¹² In support of this, the rare inherited disorder ataxia-telangiectasia confers defects related to the repair of DNA damaged by ionising radiation, and is associated with an increased incidence (> 100-fold) of lymphoid tumours in affected individuals.¹³

With regard to autoimmunity, somatic mutation might operate to disorder T lymphocyte regulation or B lymphocyte activity,

leading to cell mediated autoimmunity, or aberrant lymphocyte differentiation and immunoglobulin gene expression (processes normally involving somatic mutation and DNA recombination) causing autoantibody production. Current evidence suggests that several types of repair that normally occur in response to DNA damage caused by alkylating and oxidative agents and ionising radiation, might be generally defective in the blood lymphocytes of patients with autoimmune disease.²

Our finding of deficient repair of O⁶-methylguanine as a result of depressed MGMT levels suggests that lymphoid cells in this subgroup of PSS patients are at risk of transformation to the malignant phenotype because of persistence of O⁶-methylguanine. Further, lymphoid tissue undergoing extensive proliferation and DNA replication in these patients would be expected to make them additionally susceptible. Because of the possible value of lymphocyte MGMT levels being used for the early detection of patients at increased risk of lymphoma, it will be important to determine in future studies if those patients with the smallest MGMT values are indeed more likely to develop lymphoma. In support of a role for defective O⁶-methylguanine repair in the development of lymphoma are the previous observations of decreased amounts of lymphocyte MGMT in lymphoma patients, and in a group of patients with therapy related leukaemia. Before developing leukaemia, these patients were treated with the genotoxic agent procarbazine which, after metabolic activation, can methylate the O⁶-position of guanine.¹⁴ Our finding also raises the questions: do all the peripheral lymphocytes of PSS patients at increased risk of lymphoma show a defect in MGMT, and is the defect inherited or acquired, persistent or transient? Further, our observation of less total extractable protein in lymphocytes from these patients raises the possibility that deficiency in MGMT, which is normally not an abundant protein in cells, occurs as part of a more generalised down-regulation of protein synthesis, possibly in a 'dominant' clone of circulating lymphocytes that are present in greatly expanded number. Attempting to answer these questions will form the basis of future investigation in our laboratory.

An unusually high incidence of two different diseases of multifactorial aetiology (both occurring in the same individual) may, in part, be explained by a shared aetiogenic factor operating in both conditions. Deficient removal of O⁶-methylguanine from DNA in intact lymphocytes, presumably by an enzymic repair process(es), was previously seen in patients with various autoimmune diseases.¹ Our present and previous findings imply a deficiency in MGMT in association with premalignant states in lymphomagenesis and human hepatocarcinogenesis.⁸ Here, we have also shown that any defect in O⁶-methylguanine-DNA removal in association with autoimmune disease does not appear to be

O⁶-Methylguanine-DNA methyltransferase (MGMT) levels in lymphocyte extracts prepared from healthy controls (HC) and patients with primary Sjögren's syndrome (PSS) with (+pge) or without (-pge) parotid gland enlargement, primary biliary cirrhosis (PBC), rheumatoid arthritis (RA), or osteoarthritis (OA). Horizontal bar indicates mean values.

caused by decreased levels of repairing MGMT. However, previous studies reported relatively small *O*⁶-methylguanine removal deficiencies and detected a narrower range of normal values (about twofold less than we obtained). Therefore, direct enzyme assay, as used in our study, might not have permitted the detection of such deficiency purely on statistical grounds. Nevertheless, an alternative possibility suggested by this inconsistency is that there are other mechanisms in cells, besides MGMT, which remove *O*⁶-methylguanine, which may offset observed interindividual variations in levels of MGMT. One such mechanism may be nucleotide excision repair (NER), possibly so-called 'long patch' NER directed by *O*⁶-methylguanine-thymine base pair mismatches in DNA. In *Escherichia coli*, NER performs a large proportion of *O*⁶-methylguanine removal following DNA alkylation.¹⁵ If it operated to remove *O*⁶-methylguanine from DNA in normal human lymphocytes, then it is conceivable that defective NER, against a background of normal MGMT, might explain the reduced *O*⁶-methylguanine removal from DNA previously seen in patients with autoimmune diseases. Such an involvement of NER raises the possibility of two separate pathways for the repair of the same DNA lesion, *O*⁶-methylguanine; a defect in one path would be associated with carcinogenesis/lymphomagenesis, and a defect in the other with autoimmune disease.

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