Short Communication

Association between O^6-Alkylguanine-DNA-alkyltransferase Activity in Peripheral Blood Lymphocytes and Bronchial Epithelial Cells[1]

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Abstract

The activity of the DNA repair enzyme O^6-alkylguanine-DNA-alkyltransferase (ATase) may be a risk factor in the pathogenesis of lung cancer. ATase activity has previously been measured in peripheral blood lymphocytes (PBLs), cell extracts from bronchoalveolar lavage fluid, and cell homogenates from resected lung tissue. However, it is not clear whether ATase activity in these samples correlates well with the activity found in bronchial epithelial cells, the progenitor cells for the main types of lung cancer. In this study, cell extracts were prepared from PBLs, bronchial lavage (BL) fluid, and bronchial brushings from normal lung in 20 patients attending for routine bronchoscopy. Bronchial brushing sampled a significantly greater proportion of bronchial epithelial cells than did BL [88 ± 9% (mean ± SD) *versus* 39 ± 19%; $P < 0.0001$]. ATase activity was determined in each of the cell extracts and was found to be higher in PBLs than in bronchial brushings ($P = 0.005$) and higher in bronchial brushings than in BL ($P = 0.005$). No correlation in ATase levels was observed between any of the three samples. We conclude that bronchial brushing is a more specific and reliable way of sampling bronchial epithelial cells than BL and that it samples enough cells for ATase activity to be determined. In addition, in terms of the activity of this potentially critical DNA repair enzyme, PBLs, and cell extracts obtained from BL may not provide good surrogate tissue for bronchial epithelial cells, the critical targets for carcinogenesis.

Introduction

Cigarette smoking accounts for at least 85% of all lung cancer deaths, but fewer than 10% of smokers actually develop the disease (1). In addition to other environmental agents, such as diet (2), genetic factors have been implicated in conferring an increased risk of developing lung cancer (3–5). DNA repair capacity is one such factor that has been associated with a predisposition to malignant change. Reduced DNA repair has been observed in patients with breast and colon cancer (6–12). Evidence for reduced DNA repair in lung cancer is less compelling but has been shown in fibroblast cultures from lung cancer patients (13).

ATase[3] is a DNA repair enzyme that is responsible for the elimination of the potentially mutagenic DNA adducts O^6-methylguanine (14, 15) and O^6-(pyridyloxobutyl)guanine (16, 17), which, in turn, can result from alkylation by tobacco-specific nitrosamines, potent carcinogens that are present in cigarette smoke (18–20). ATase activity has previously been measured in cell extracts from BAL (21), homogenates from resected lung tissue samples (22), and PBLs (21, 23). ATase activity in these samples appears to be higher in smokers than nonsmokers, but no significant difference between lung cancer and noncancer patients has been observed. Statistically significant differences may be difficult to reach due to the large (6–44-fold) interindividual variations in ATase activity (21–23), the possible induction of ATase activity by carcinogens in tobacco smoke (24, 25), and variations in cell composition between samples, as ATase activity shows considerable cellular heterogeneity (26, 27).

The vast majority of lung tumors arise within the bronchial epithelium (28, 29), and variations in the activity of DNA repair enzymes such as ATase within this specific cell population may be critical in terms of lung cancer risk. Sampling substantial numbers of specifically bronchial epithelial cells, however, is not easy.

The epithelium is comprised of pseudostratified columnar cells, which do slough away intermittently into the bronchial lumen and can be sampled in fluid that has been washed into the bronchi and aspirated using a fibreoptic bronchoscope. Such techniques are termed BAL and BL. However, other cell populations (namely, lymphocytes, neutrophils, and macrophages) also exist within the bronchial lumen and alveolar space. The aim of BAL is to sample the contents of alveoli and the distal bronchioles and, hence, the major cellular constituents (up to 85%) of BAL are macrophages, with epithelial cells comprising <5% of the sample under optimal conditions (30). BL generally samples more proximal bronchi and is normally used to enhance diagnostic sensitivity in possible malignant lesions. BL should recover a greater percentage of epithelial cells than BAL (31, 32), but it would seem difficult to control the proportion with this technique. Because we are primarily concerned with the activity of ATase in bronchial epithelial cells and not in the other cell populations, it would be useful to use a more specific and reliable method of sampling the epithelium.

Bronchial brushing, which samples cells by scraping the epithelial layer with a miniature cylindrical brush, may be just

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[3] The abbreviations used are: ATase, O^6-alkylguanine-DNA-alkyltransferase; BAL, broncheoalveolar lavage; PBL, peripheral blood lymphocyte; BL, bronchial lavage; PCA, perchloric acid.

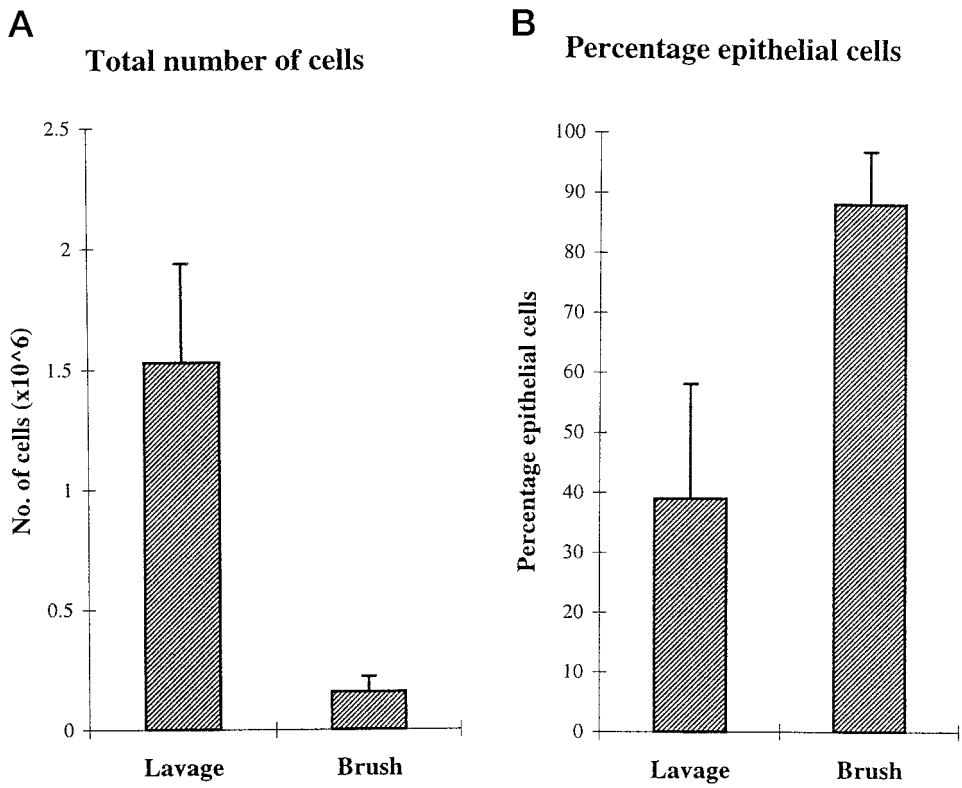

Fig. 1. The total number of cells (*A*) and the percentage epithelial cells (*B*) collected in BL and bronchial brush samples. *Columns*, means from 20 subjects; *bars*, SD.

Table 1 Cancer status, smoking status, and ATase activity in BL, brushings, and lymphocytes from 13 subjects[a]

Patient no.	Cancer status	Smoking status	ATase activity (fmol/μg DNA)		
			BL	Brushings	Lymphocytes
5	Normal	Ex-smoker	0.13	3.30	3.09
7	Squamous cell	Nonsmoker	0.80	5.70	4.16
10	Normal	Ex-smoker	0.31	0.57	0.77
11	Normal	Smoker	0.36	1.53	2.38
12	Cancer, type unknown	Nonsmoker	0.70	1.06	9.68
13	Normal	Smoker	0.36	1.09	7.63
15	Normal	Smoker	0.59	0.70	7.60
16	Normal	Smoker	0.17	0.54	6.94
17	Adenocarcinoma	Ex-smoker	0.64	2.84	4.90
18	Small cell	Smoker	1.15	0.69	4.12
19	Normal	Ex-smoker	0.30	1.77	2.22
20	Normal	Ex-smoker	0.79	3.56	4.32
21	Normal	Ex-smoker	1.00	0.97	2.50

[a] The ATase activities from all three sample types show ~10-fold interindividual variability.

such a technique. It is normally used to collect material for cytologic examination but has been shown to sample anywhere from 10^4 to 10^6 epithelial cells (33, 34). It is not known whether this is sufficient cellular material for the assay of DNA repair enzymes in the bronchial epithelium. In the first part of our study, we aimed to compare the total cell number and proportion of bronchial epithelial cells sampled in bronchial brush and BL samples. We will go on to measure ATase activity in these cell extracts.

Bronchial epithelial cells can generally only be sampled by bronchoscopic techniques (as above) that are invasive and sometimes uncomfortable. It is not clear whether more easily obtainable samples, such as PBLs, can act as a surrogate tissue for the bronchial epithelium in terms of work on DNA repair. In the second part of our study, we investigated the comparison and correlation in ATase activity between cell extracts from BL, bronchial brushings, and PBLs from the same individuals.

Materials and Methods

Materials. Substrate for ATase activity was ^{3}H-methylated calf thymus DNA. It was prepared by reacting calf thymus DNA with [^{3}H]methyl-*N*-nitrosurea (Amersham International, Buckinghamshire, United Kingdom; 20 Ci/mmol) for 5 h at room temperature in 2-amino-2-methyl-1,3-propanediol (0.02 M, pH 8; Ref. 35). We used bronchial brushes with 3-mm bristles (Telemed, Marlborough, MA) to collect our bronchial brushing samples.

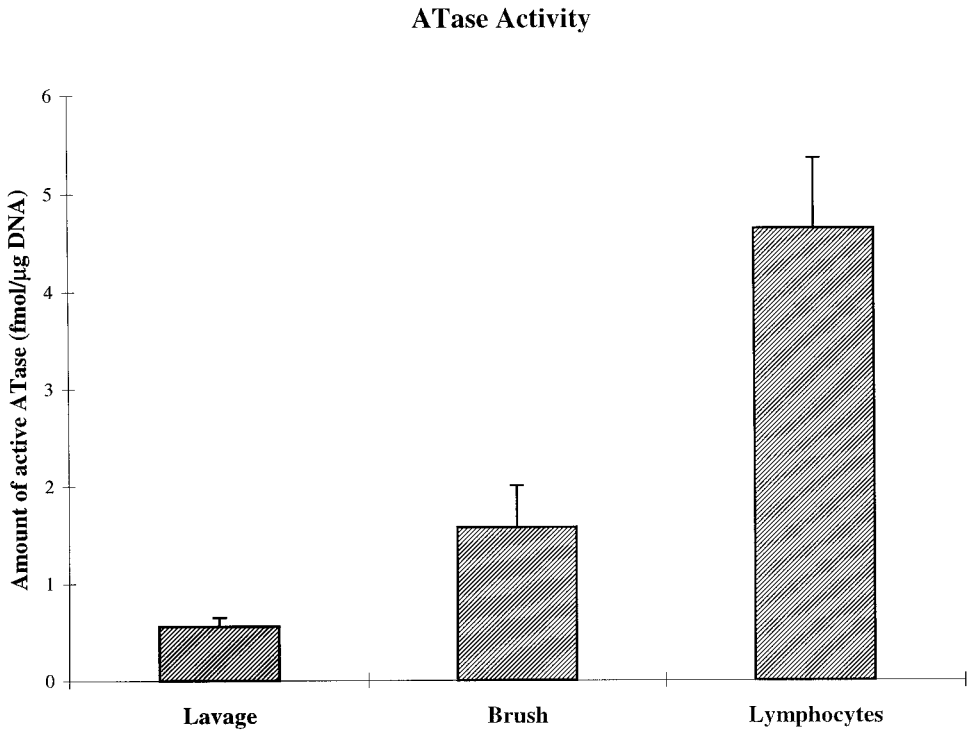

Fig. 2. ATase activity in BL, brush, and PBL samples. *Columns*, means from 13 subjects; *bars*, SD.

Study Subjects. Twenty-one patients attending for fibreoptic bronchoscopy for diagnostic purposes at the North West Lung Centre, Wythenshawe Hospital, were recruited. One patient was excluded due to poor toleration of the bronchoscopy itself. Eight patients were current smokers, 12 were ex-smokers (had abstained for at least 6 months), and 2 had never smoked. Four patients had histologically proven lung cancer (two squamous cell, one small cell, and one adenocarcinoma). One patient was awaiting surgery for presumed lung cancer without proven histology at time of submission.

Tissue Samples. PBLs were extracted from venous blood samples by standard Ficoll-Paque technique. Briefly 10 ml of venous blood were layered onto 10 ml of Ficol medium and centrifuged (600 × *g*) at 20°C, and the lymphocyte layer was then aspirated. The aspirate was washed twice in PBS at 4°C and stored at −80°C.

BL was performed on each subject by gently instilling 30–50 ml normal saline into the right upper lobe bronchus in two aliquots. The fluid was aspirated carefully until a total of 25–30 ml of fluid were recovered. A 10-ml aliquot was used for differential cell counting. The remainder was kept on ice and then centrifuged (1400 × *g*), and the cell pellet was washed twice in PBS at 4°C and stored at −80°C.

Bronchial brushing was performed on the right middle lobe subsegmental bronchi. Ten gentle brush strokes were performed at three different positions on two subsegmental bronchi, to give a total of 60 brushes per subject. This technique appeared to provide optimal coating of the brush without provoking significant visible hemorrhage. The brush tip along with 2–3 cm of wire was then clipped off into a 30-ml universal container containing 20 ml of PBS at 4°C. The tip wire was subsequently held by forceps with the brush end in the PBS, and the brush contents were shaken off by holding the base of the forceps on a vortex for 30 s. The resulting cell suspension was divided in half. One half was sent for differential cell counts, and the other half was centrifuged (1400 × *g*) at 4°C. The cell pellet was then washed twice in PBS at 4°C and stored at −80°C.

All bronchoscopic specimens were sampled by the same investigator (P. N. S. O.) to eliminate interobserver error, and all bronchi sampled were bronchoscopically normal.

Total and Differential Cell Counts. These were performed at the North West Lung Centre, Wythenshawe Hospital, using an improved Neubauer counting chamber and cytospin preparations (36).

Preparation of Cell Extracts. Cell pellets were resuspended in 500 μl of buffer I [50 mM Tris-HCl, 3 mM DTT, and 2 mM EDTA (pH 8.3)] containing leupeptin and sonicated (216 μm peak-to-peak amplitude, 12 s), and then phenylmethylsulfonyl fluoride (5 μl) was added. The sonicate was centrifuged (15,000 × *g*) for 10 min at 4°C. The supernatant was removed into a fresh Eppendorf tube.

Assay of Cell Extract ATase Activity. Increasing volumes of cell extract were added to scintillation tubes containing 100 μl of ^{3}H-methylated calf thymus DNA (1.3 μg, 0.6 nCi). The mixture was incubated for 30 min at 37°C. BSA (100 μl) was added followed by 4 M PCA (100 μl) and 2 M PCA (2 ml). The mixture was incubated at 75°C for 50 min to ensure complete hydrolysis of the DNA. The protein precipitate was collected by centrifugation (2100 × *g*) for 10 min at 20°C, washed by resuspending in 1 M PCA (4 ml), and then suspended in 10 mM NaOH (300 μl). Ecoscint (3 ml) was then added, and the radioactivity of the solution was determined in a scintillation counter. A number of BL samples were quite mucinous. This would have increased the protein content of the sample, meaning that ATase activity estimation of the cellular content would have been falsely lowered if expressed per mg of protein. Therefore, levels of active ATase were expressed in fmol per μg DNA (microfluorimetric DNA determination using Hoescht 33258; Ref. 37).

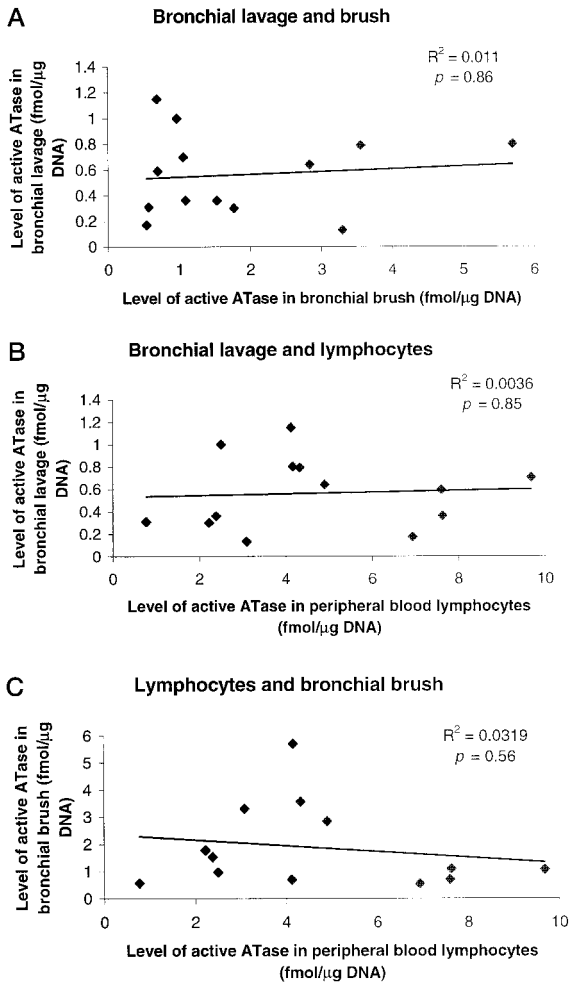

Fig. 3. Linear regression analyses of the ATase activities in BL (*A*), bronchial brush (*B*), and PBL (*C*) samples. No correlation is demonstrated in any of the three possible pairings. Values were obtained from 13 subjects.

Statistical Evaluations. Student's paired *t* test was used for comparison of the paired data on total cell counts and differential cellular proportions in bronchial lavage and bronchial brush samples. Linear regression analysis was used for investigation of the correlation of ATase activity between the different sample groups.

Results

No deleterious side effects were reported by any of the 20 patients who were subjected to both bronchial brushing and BL. Bronchoscopically, brushing was seen to provoke a small amount of self-limiting contact bleeding in a minority of patients. This never gave rise to hemoptysis and was essentially well tolerated.

The total and differential cell counts of BL and bronchial brushing samples are shown in Fig. 1. Clearly, significantly more cells are collected in the lavage samples ($P = 0.002$). Brushing appears to sample $\sim 10^5$ cells, which is consistent with amounts (10^4–10^6 cells) recovered in previous studies (33, 34). Bronchial brushing, as expected, samples a significantly greater percentage of epithelial cells than BL [88 ± 9% (mean ± SD) *versus* 39 ± 19%, $P < 0.0001$]. It would also appear that this method is more reproducible (note SDs), in terms of the proportion of epithelial cells collected, than BL.

For detectable ATase activity to be obtained, experience dictated that the cell pellets, once resuspended in 500 μl buffer I, should provide at least 10 μg of DNA per ml. Six of the brushings and one of the lavage samples did not meet this criteria, and therefore, these seven subjects were excluded from the analysis. In the 13 remaining individuals, there was no correlation between the amount of DNA in the sample and the ATase level (data not shown). The smoking status, cancer status, and ATase activities in these subjects are shown in Table 1. Comparisons of the ATase activity between smokers, ex-smokers, nonsmokers, and patients with or without lung cancer is not advisable in such a small study group, due to the large interindividual variability in ATase activity supported by previous observations (21–23). There were, however, significant differences observed in the ATase activity in the three different sample types: lymphocytes, BL, and bronchial brushings (Fig. 2). ATase activity was greater ($P = 0.005$) in lymphocytes (4.64 ± 2.61 fmol of ATase/μg of DNA) than in brushings (1.87 ± 1.55 fmol of ATase/μg of DNA) and greater ($P = 0.005$) in brushings than in lavage (0.56 ± 0.32 fmol of ATase/μg of DNA).

The linear regression analyses examining the association between all three paired data sets are shown in Fig. 3. No significant correlation was detectable between any of the three sample groups. All three sample types show an interindividual variability in ATase activity of ~10-fold.

Discussion

This study demonstrates that bronchial brushing is a more specific and reproducible way of sampling bronchial epithelial cells than BL. Some work has been performed using this technique to investigate the pathogenesis of asthma and chronic bronchitis (34, 38, 39) but it has not been used to study potential factors impacting lung cancer risk. Because the majority of primary lung tumors arise in the epithelial layer, it could be useful in this respect. In particular, we have shown that bronchial brushing, as performed here, is well tolerated and can sample enough cellular material for the activity of the DNA repair enzyme, ATase, to be measured. In our study, we obtained sufficient material from 13 of 20 individuals, but half of each initial sample had been sent for differential cell counting. In the absence of splitting, we should have obtained sufficient material from everyone. It may be possible, using this method, to evaluate the levels of other DNA repair proteins in bronchoscopically normal epithelium from smokers and nonsmokers and those with and without lung cancer.

A noteworthy advantage of bronchial brushing is that it provides very clean, mucous-free samples, whereas with BL, fluid is often more mucinous and cells are consequently more difficult to collect by centrifugation, despite multiple washes. One disadvantage of bronchial brushing is that the brushes are not reusable and are, therefore, expensive compared to lavage techniques that use washable receptacles. Unfortunately, sampling the bronchial epithelium generally requires bronchoscopy to be performed, and therefore, using surrogate tissue that is easier to obtain is a more attractive option for large studies on predisposition to lung cancer.

Our study focuses on the potential for using PBLs as a surrogate sample for bronchial epithelial cells in work relating to ATase activity. The lack of correlation between activity in brushings and PBLs, albeit in a small sample group, suggests that we should be wary of using them in this respect. Although using PBLs in studies of ATase activity in lymphomas and hematological malignancies is undoubtedly appropriate, it is unclear whether PBL ATase activity correlates well with that observed in other tissues. One study has suggested a correlation between ATase

levels in gastric mucosa and in PBLs (40), but no similar study has previously been reported on the bronchial epithelium.

The substantial interindividual variability in ATase activity that exists requires that quite large studies will have to be performed before any significant differences between study groups can be demonstrated. ATase also exhibits considerable cellular heterogeneity. To avoid this possible confounding factor, it is important to ensure homogeneity of cell samples and suitability of the tissue used in the study of this potentially critical DNA repair enzyme. In future work on DNA repair enzymes in lung cancer, we intend to use bronchial brushing as the optimal method to collect bronchial epithelial cells.

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