Genomic Instability in Scleroderma

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Scleroderma (systemic sclerosis) is an uncommon systemic rheumatic disorder of unknown etiology. Further, most genetic and environmental studies have provided little insight into the etiopathogenesis of scleroderma and for the majority of patients there is no good explanation as to why they have the disorder.¹ In our own recent epidemiological study of a population based cohort of 548 patients with scleroderma in South Australia we were unable to define any predisposing genetic or environmental factors to account for the disease.² However, an analysis of the age-specific incidence suggested that scleroderma could be considered as a stochastic illness resulting from a number of random events occurring in a predisposed population. We hypothesized that these random events might involve acquired mutations in pivotal genes on a predisposing genetic background.³

There is preliminary evidence of acquired genetic damage in patients with scleroderma. This evidence includes increased chromosomal breakage rate, deletions and acentric fragments,⁴ decreased telomere length,⁵ the detection of clastogenic activity in sera⁶ and the finding of increased somatic mosaicism/mutations in variable number tandem repeats in patients and family members.⁷ Furthermore, we have recently observed from our population-based cohort study that the standardized incidence ratio (SIR) for all cancers in patients with scleroderma was significantly increased (SIR = 2.0, 95% CI = 1.46-2.65).⁸ Patients with diffuse scleroderma had a SIR for all cancers of 2.73 (95% CI = 1.31-

SUMMARY Scleroderma is an enigmatic rheumatic disorder of uncertain etiopathogenesis. Cancer has an approximately two-fold higher incidence in scleroderma patients than in the general population. There are preliminary data of acquired genetic damage in scleroderma but the significance of these observations are uncertain. To determine somatic mutation frequency at the glycoptorin-A (GPA) locus in patients with limited and diffuse cutaneous scleroderma. The GPA assay measures the total somatic mutation frequency (Vf), composed of gene inactivating mutations (NO) and mutations arising from mitotic recombination (NN) in individuals heterozygous for the GPA MN blood group. Mutation frequency was determined using a validated GPA flow cytometric assay using fluorescent labeled monoclonal antibodies specific for the GPA blood groups M and N. This assay detects and enumerates progeny of red blood cell (rbc) precursor cells which have acquired genetic damage resulting in a loss of expression of one of the GPA alleles. It was found that patients with scleroderma (n = 23) had significantly elevated Vf as compared with young healthy controls (p < 0.001) and elderly controls (p = 0.03). Patients with diffuse scleroderma had higher mean Vf as compared with limited scleroderma (p = 0.055). In comparison with controls, patients with scleroderma exhibit a higher proportion of mitotic recombiant mutations than inactivating mutations (p < 0.002). There was no correlation between Vf and disease duration, age at onset or autoantibody status. We have documented evidence of acquired genetic damage at the GPA locus in scleroderma. Evidence of acquired genetic damage in this disorder may be important in explaining both the etiopathogenesis of scleroderma and the association of scleroderma with cancer.
5.02) as compared with 1.85 (95% CI = 1.23-2.68) for patients with limited scleroderma. A possible explanation for this enhanced cancer risk in scleroderma may relate to acquired somatic genetic damage. Accordingly we examined the somatic mutation frequency at the glycoporphin-A (MN) blood group locus in patients with scleroderma and controls. We chose this particular locus as, a priori, we would not anticipate it being directly involved in the disease process of scleroderma.

**PATIENTS AND METHODS**

**Patients and controls**

**Patients**

Scleroderma patients were recruited from the South Australian Scleroderma Register. Patients were subdivided into limited cutaneous and diffuse cutaneous variants according to the diagnostic criteria of LeRoy and colleagues. Those with the blood group MN were identified for further analysis. They consisted of 13 female patients with limited scleroderma (mean age 63.9 years, mean disease duration of 18.5 years and eight with anti-centromere antibody) and 10 female patients with diffuse scleroderma (mean age 58.8 years, mean disease duration 10.6 years and two with ScI-70 antibody). Four patients with diffuse scleroderma and one with limited scleroderma had received the antime-tabolite azathioprine prior to testing.

**Controls**

The informative controls consisted of 86 young healthy females (43 twin pairs obtained from the Australian Twin Registry and of mean age 17.9 years) and 11 elderly females with degenerative arthritis (obtained from a rheumatology Consulting Clinic and of mean age of 70.5 years).

**Glycoporphin-A (GPA) somatic mutation assay**

Somatic mutation frequency in heterozygous (blood group MN) individuals was determined by a validated flow cytometric technique which detects and enumerates progeny of red blood cell (rbc) precursor cells which have acquired prior genetic damage resulting in loss of expression of one of the GPA alleles. The method was as described by Jensen and Bigbee and uses fluorescent labeled specific monoclonal antibodies to detect the GPA-M and GPA-N blood groups on rbc. Among MN typed subjects, rbc with an NO phenotype reflect the presence of erythrocyte precursors predominantly bearing intragenic mutations.

The NO phenotype also characterizes major deletions but we have shown in studies using another codominantly expressed locus on human lymphocytes that major deletions represent fewer than 5% of single allele null phenotype somatic mutations. By analogy, NN phenotype cells from constitutively MN subjects are likely to represent the progeny of erythrocyte precursor cells which have undergone mitotic recombination proximal to the GPA locus on chromosome 4q. In support, previous studies of somatic mutations at the HLA-A focus demonstrated that reduction to homozygosity was predominantly by mitotic recombination and that gene conversion was rare. The total variant GPA mutation frequency (Vf), consisting of the sum of simple gene inactivating mutations (NO), and mitotic recombination mutations (NN), were determined by counting ~ 2.5 x 10^6 labeled rbc in the FACSscan (Becton-Dickinson). A typical flow cytometric result is shown in Fig. 1 where Vf was determined as the sum of the events counted in the gates assigned to NO and NN mutations divided by total events. Using multiple stored aliquots of spheroplasts obtained from a single subject we determined that the inter-assay coefficient of variance for this technique was 12.4%.

**Statistical analysis**

Differences between groups were analyzed by Wilcoxon's sum of ranks (non-parametric) testing with p value of 0.05 being considered as statistically significant. Association between groups was analyzed by Fisher's exact test.

**RESULTS**

Approximately 50% of both the scleroderma patients and the control subject studied were of the blood group MN (as anticipated) and hence were informative (for determining mutation frequency). The demographics and clinical features of these subjects are documented in the Methods section.

Patients with scleroderma had significantly increased Vf when compared with young healthy females (p < 0.001) and elderly females (p = 0.03) (Fig. 2). Patients with diffuse scleroderma had a higher mean Vf as compared with patients with limited scleroderma...
Fig. 1  FACS analysis of spheroplasts for MN labeled erythrocytes. N-fluorescence in the X-direction. (a): contour plot including 95% of all cells in the major gated population in (b), R4 in the Table. (b): scatter plot of all forward and side scatter events gated in (c), R1 in the Table. Mutant cells (O/N and N/N) gated in (b) have intensity ≤ 10% of the geometric mean M-labeled intensity for the wild-type MN population. NO cells are dynamically gated to be centered on the MN geometric mean N intensity with gate width encompassing 95% of all cells. NN cells are centered around twice the NO mean with gates at the equivalent geometric fluorescence intensity width. 2.5 x 10^6 cells counted.
Patients with scleroderma had a significantly higher mean frequency of mitotic recombinant (NN) mutations than controls \( (p < 0.002) \) (Table 1). No correlation was observed between \( V_f \) and disease duration, age at disease onset and autoantibody status. Four diffuse scleroderma patients and one limited scleroderma patient with prior use of azathioprine had \( V_f \) values of 14.3, 6.8, 5.5, 2.3 and 6.6 \( \times 10^{-5} \), respectively, but these values were not significantly different to those scleroderma patients whom had no prior azathioprine \( (p = 0.1) \).

**DISCUSSION**

The GPA flow cytometric assay is a validated method for determining total variant mutation frequency at the GPA gene locus\(^4\) and by inference gene inactivating mutations (NO) and mitotic recombination mutations (NN). Using this technique we observed a wide inter-individual variation of mutation frequency in healthy subjects. In our concurrent study of monozygotic and dizygotic twins we have demonstrated that \( \sim 50\% \) of the variance in mutation frequency is due to additive genetic factors (Male D, Turner D, unpublished observation). Here we have also confirmed the anticipated increase in mutation frequency with age as our older female controls have a \( 70\% \) higher mean mutation frequency compared with the young female controls which is consistent with the \( \sim 1\% \) per year increment in mutation frequency observed in other studies.\(^7\)

Such studies have also identified that other factors such as gender, cigarette consumption, exposure to irradiation, chemicals and drugs can each influence mutation frequency.\(^7\) With this in mind it is prudent to note

![Graph showing mutation frequency](image-url)
that 5 of our scleroderma patients had prior administration of the antipurine metabolite azathioprine, but whilst the mean Vf value of these patients was higher than those without prior exposure the difference was not significant.

Using the GPA assay we have documented that scleroderma patients have a significantly increased mean Vf compared with both young and elderly female controls. Further, patients with diffuse scleroderma had higher Vf than patients with limited scleroderma. Interestingly the proportion of mutations attributable to mitotic recombination was higher in scleroderma patients than in controls (Table 1). Comparing the geometric mean mutation frequencies of patients with elderly controls, nearly all the increase in somatic mutation in patients was attributable to increase in mitotic recombination (data not shown). Finally there was no correlation between Vf and disease duration.

What is the significance of these findings of acquired genetic damage at the GPA locus in scleroderma? We note that other studies have revealed evidence of a number of forms of genetic damage in scleroderma. This damage includes increased chromosomal breaks\(^4\) deletions, and acentric fragments;\(^4,9\) clastogenic activity in sera and cell extracts detected in patients with scleroderma;\(^10\) increased chromosomal breakage rate in first degree relations of patients with scleroderma;\(^7\) and increased variable number random repeat mutations in patients with scleroderma and first degree relatives.\(^11\)

Explanations for this genetic instability in scleroderma may include the effect of prior exposure to exogenous or endogenous mutagens and inherited factors. Case reports and retrospective analysis have linked exposure to aromatic or aliphatic hydrocarbons (e.g. vinyl chloride monomers, trichlorethylene, benzene and other solvents), the duration and cumulative dose varying between cases.\(^18,19\) In addition exposure to trichlorethylene and its metabolites has been shown to induce antibodies to nucleic acids in murine models.\(^20\)

Endogenous factors include those derived from the immune/inflammatory response found in scleroderma. These include the various oxygen derived free radicals and/or cytokines which have the capacity to damage DNA and chromatin. This might be relevant in explaining the known weak association between prior silica exposure and scleroderma (particularly in males),\(^19\) the silica microcrystals being a potent fibrogenic agent causing local release of DNA damaging mediators.

In addition to a possible role for environmental factors in the etiology of scleroderma the observation that mutational endpoints such as instability in VNTRs and increased chromosomal breakage rates are seen in first degree relatives of scleroderma patients\(^21\) suggests that genetic factors are also etiologic. Indeed, family history is the greatest risk factor for scleroderma to date.\(^22\) Such increased genetic instability and increased SIR of cancer in patients\(^12\) and their relatives\(^23\) together with our results described here indicate a possible role for aberrant response to DNA damage and its repair in the etiopathogenesis of scleroderma. There are a number of well described cellular responses to DNA damage that have been strongly evolutionarily conserved and that are essential to maintain genetic integrity.\(^21\) Rare syndromes of inherited failure of DNA repair processes are commonly (but not invariably) associated with increased somatic mutation and lifetime risk of cancer.\(^21\) Some of these disorders, such as ataxia telangiectasia and the progeroid syndromes also exhibit scleroderma-like features.\(^21\) But complete failure of a genetic stability maintenance function may not be necessary for phenotypic effect. Functional polymorphism in one or more of the many genes involved in maintaining genetic stability may result in pathology. Currently there are a number of studies that have demonstrated increased cancer risks associated with common polymorphisms in DNA repair related genes.\(^21\)

### Table 1

| Comparison of ratio of mitotic recombination mutations (NN) to inactivating mutations (NO) in each scleroderma patient and control |
|----------------------------------|---------------|----------------|
| Young controls                  | 31            | 55             |
| Elderly controls                | 1             | 10             |
| Scleroderma                     | 17            | 6              |


The relative risks of predisposing genotypes are generally low, comparable with that seen in scleroderma. Evidence of genetic instability, increased somatic mutation and possible inherited contribution suggests that a subset of genes maintaining genomic stability may be important in the etiology of scleroderma. The study of DNA repair enzyme polymorphism in scleroderma is merited; particularly as in the event of genetic heterogeneity even modest increased relative risks may indicate very large population variation in genetic susceptibility. Thus scleroderma patients may harbour a more restricted range of polymorphisms with high relative risk of scleroderma but modest relative risk of cancer.

In conclusion we observed in our present study that patients with scleroderma had a significant higher glycoprotein-A somatic mutation frequency than healthy young and old female controls (including significant more mitotic recombinant mutations). This observation adds to the body of evidence indicating an increase in acquired genetic damage among patients with scleroderma. It does not distinguish the extent to which such acquired damage is due to exogenous or endogenous agents or the effects of defective repair of genetic damage. Nonetheless increased somatic mutation may be relevant in explaining the enhanced risk of common cancers and possibly be contributory to the etiopathogenesis of the disease itself.

ACKNOWLEDGEMENTS

We thank Mrs. C. Thomas for secretarial assistance and Drs. M. Rischmuller, C. Hill, K. Pile, S. Proudman, L. Cleland, W. Hill, R. Geddes and G. Champion for their support of the South Australian Scleroderma Register.

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