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A Comparison of Morphoea and Lichen Sclerosus et Atrophicus In vitro: The Effects of Para-aminobenzoate on Skin Fibroblasts

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To study the effects of para-aminobenzoate on the dermis, fibroblast cell lines derived from lesions of lichen sclerosus et atrophicus, from morphoea and from normal skin were incubated with Potaba in vitro. Monolayer cultures containing Potaba showed a dose-dependent inhibition of proliferation beginning at 1,000 μg/ml with total inhibition at 10,000 μg/ml. Mean ID_{50} values for the three groups were not significantly different. There was a similar dose dependent inhibition of glycosaminoglycan secretion in all 3 groups, except at 10,000 μg/ml where secretion by lichen sclerosus et atrophicus and morphoea fibroblasts was significantly more inhibited than normal lines. Inhibition of the glycosaminoglycan secretion at 10–1,000 μg/ml was a direct effect of the drug rather than an indirect effect of changes in cell density, and lichen sclerosus et atrophicus fibroblasts produced about 40% more GAG than the morphoea or normal lines growing at similar densities. Collagen synthesis was increased in both lichen sclerosus et atrophicus and morphoea cell lines, with increased non-collagenous protein in morphoea lines. These results confirm that there are differences between lichen sclerosus et atrophicus and morphoea, and suggest glycosaminoglycan secretion as a possible target for the therapeutic action of Potaba. ***Keywords: Glycosaminoglycans; Collagen; Fibrosis.***

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Potassium para-aminobenzoate (Potaba) has been used to treat several fibroses, and successful results have been reported in scleroderma (1), dermatomyositis (2). Peyronie's disease (3), rheumatoid arthritis (4), morphoea (4) and most recently in lichen sclerosus et atrophicus (LSA) (5), although other trials have been less favourable. The mode of action of Potaba is unknown, although it has been suggested that monoamine oxidase activity is involved (6). It is likely that therapeutic effects of Potaba will involve alterations in the metabolism of several connective tissue components. The aim of this work was to isolate fibroblasts from normal skin and from lesions of morphoea or LSA, and to compare their production of GAG and collagen. We also examined the effects of Potaba on fibroblast metabolism to determine some therapeutic actions of the drug.

MATERIALS AND METHODS

Sources of cells

Normal skin fibroblasts were obtained from punch biopsies taken from the forearm of five adult volunteers and from two foreskins obtained at adult circumcision. Seven morphoea fibroblast lines from back (n = 3), arm (n = 2), thigh (n = 1) and abdomen (n = 1); and eight LSA fibroblast lines from the arm (n = 2), neck (n = 3) and genital area (n = 3), were grown from portions of skin biopsies taken for histological diagnosis. The mean age of the patients was: LSA 57 ± 3.3 (± s.e.m); morphoea 37.4 ± 6.1; normal 40.2 ± 7.4. Tissue fragments were grown under glass coverslips in Dulbecco-Eagle medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 4 μM glutamine and 20% foetal calf serum (FCS) until first passage and thereafter with 10% FCS (all from Gibco BRL, Paisley, Scotland). The cells were grown in Falcon plastic flasks flushed with 95% air/ 5% CO_2 at 37°C and medium was changed three times weekly. Cells were harvested with trypsin-versene for counting in an electronic cell counter (Coulter model DN).

Proliferation tests

On Day 0,28 flasks (each 25 cm^2 growth area) were seeded with aliquots of cells (1×10^5). All cells were used between passages 4 and 8. On Day 3 four flasks were withdrawn for cell counts while the rest were grouped in fours for treatment with Potaba at 6 concentrations (0, 10, 100, 1,000, 5,000 and 10,000 μg/ml). Media were renewed on Day 4 and on Day 6 the cells were harvested and counted in all flasks. The increase in the numbers of cells in each treated flask was expressed as a percentage of the mean increase in control flasks between Days 3 and 6. Viability was measured by trypan blue exclusion.

Measurement of GAG secretion

GAG present in culture medium was measured as its uronic acid moiety. Medium (7 ml) collected on Day 6 of the proliferation tests, and 2 ml of phosphate buffered saline used to rinse the cell layers, was dialysed against 0.1 M sodium acetate buffer. pH 4.9 for three days and concentrated to approximately 4 ml with Lyphogel (Gelman-Sciences Ltd). Each sample was digested with 2 mg testicular hyaluronidase (Sigma, type I) overnight at 37°C before precipitation of protein in cold 5% trichloroacetic acid (TCA). After centrifugation at 18,000 g for 30 min, the supernatant was removed and its uronic acid content was assayed using metahydroxydiphenyl reagent with a glucuronolactone standard (7). Uronic acid output per culture was calculated by subtracting the uronic acid value of the unused medium and values were finally expressed as μg uronic acid/10^7 cells/48 h. Values for cultures containing Potaba were expressed as percentages of those from untreated controls (same cell line) in the same experiment.

Protein synthesis

Total protein synthesis was measured as the incorporation of ^{3}H-proline into TCA-insoluble protein and collagen synthesis as incorporation of ^{3}H-proline into collagenase-sensitive protein. Six-well culture dishes (Linbro, Flow Laboratories, Paisley) were seeded with fibroblasts at a density of 10^5 cells/well. Two wells per dish were allocated to each cell line, and each experiment used one cell line from each of the three groups. Cell lines were selected to match donor ages, sex, site and passage number. A total of seven dishes were used in each experiment. After two days the dishes received medium with 2% FCS supplemented with 50 μg/ml ascorbic acid. Forty-eight hours later, 370 kBq/ml 5-^{3}H proline (Amersham, England) in 1 ml antibiotic-free medium with 2% FCS was added to each well in five dishes. The remaining two dishes received the same treatment without the radioactivity. All seven dishes were then incubated for 24 h. Protein was precipitated and the ^{3}H -collagen was distinguished from the other labelled protein by the use of collagenase (Advance Biofactures, Lynbrook, USA). Labelled protein was measured as previously described (8).

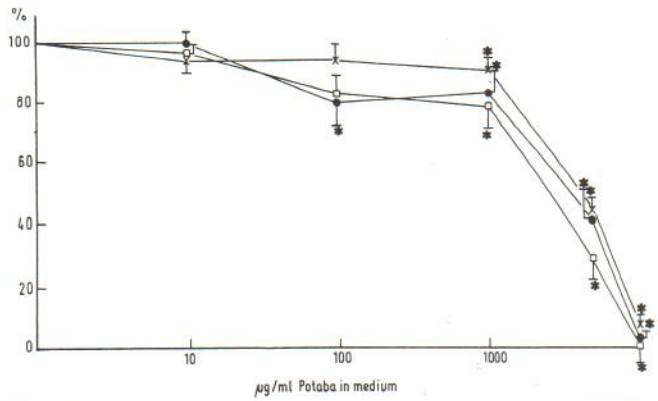

Fig. 1. Effects of Potaba on fibroblast proliferation rates. **x**: normal: □: LSA: ●: morphoea. $^*p < 0.05$ Values shown are the mean of 4 flasks ± SE at each drug concentration. Proliferation rate of untreated cells = 100%.

Urinary GAG excretion

GAG and total uronic acid were measured in 24-h urine samples collected from 11 female patients with LSA (mean age 46 years) and from 8 normal women of similar age (mean age 42 years). The GAG was precipitated with cetyltrimethyl ammonium bromide and measured with m-hydroxydiphenyl (9).

RESULTS

Potaba produced a dose-dependent inhibition of fibroblast proliferation, beginning at 1000 µg/ml for normal and LSA fibroblasts, and at 100 µg/ml for morphoea fibroblasts. Proliferation was completely inhibited at 10.000 µg/ml (Fig. 1). There was no significant difference between the mean ID_{50} levels of the three groups, ($p > 0.05$ Student's *t*-test: normal 4411 ± 574: morphoea 3331 ± 952: LSA 3605 ± 551: mean ± SEM, n = 7). All groups displayed a 90% cell viability at every drug concentration and all cells had normal morphology when examined microscopically, indicating a cytostatic rather than a cytotoxic effect.

The inverse relationship between cell density (cells per cm^2) and GAG secretion in human skin fibroblasts (10) was confirmed in the present study using 19 observations from 4 normal cell lines. The relationship was linear and can be described by the equation $y = -0.03x + 2.35$ ($r = -0.85$: df = 17: $p < 0.0005$) where y is log GAG/10^7 cells and x is cell density at 10^5 cells per 25 cm^2 growth area. This equation was used to calculate the GAG values to be expected at a given cell density, and the predicted values for each cell line were compared to the observed values (Table I, Fig. 2). In this way, it is possible to

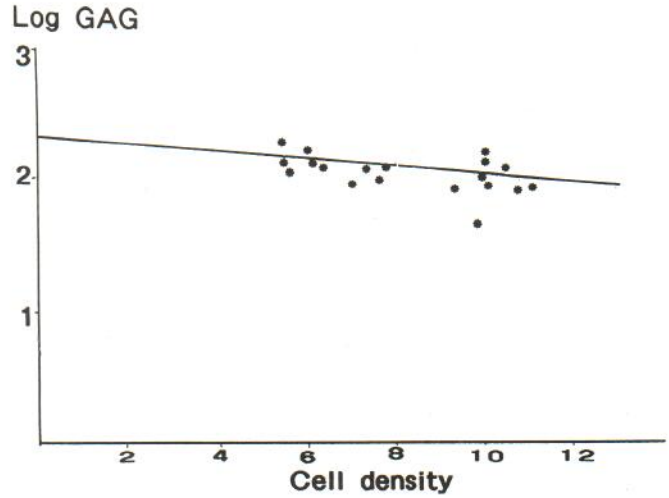

Fig. 2. Inverse relationship between cell density and GAG secretion per cell. GAG secretion is expressed as µg uronic acid per 10^7 cells and cell density as cells × 10^5 per 25 cm^2 flask. In this figure $r = -0.85$: *d.f.* = 17 and $p < 0.0005$.

separate the effects of cell density from the direct effects of Potaba. Each of the LSA fibroblast lines secreted significantly greater amounts of GAG than was predicted ($p < 0.05$: paired *t*-test) but this difference was not found in morphoea cell lines. In cultures containing Potaba there was a significant decrease in GAG at 10.000 µg/ml for LSA and morphoea lines and at 100, 1000 and 5000 µg/ml for normal cells (Fig. 3). The net drug response was expressed as the predicted GAG values subtracted from the observed value (Fig. 4). Using this technique, Potaba caused a significant decrease in GAG secretion at all drug concentrations for normal cells, and at 10 µg/ml and 100 µg/ml for LSA and morphoea lines, respectively. In all cases, the response was dose-dependent. The urinary excretion of GAG was also measured. There were no significant differences between patients and age-matched controls for total GAG, uronic acid or creatinine values: GAG/creatinine ratios were 2.68 ± 0.19 mg/gm for controls and 2.78 ± 0.26 for patients with LSA.

Collagen and other protein synthesis was measured in three cell lines from each group (Fig. 5). The amount of collagen producted by the LSA and morphoea cells was 40% and 138% higher than in normal skin fibroblasts ($p < 0.02$). The amount of other protein produced by morphoea fibroblasts was 97% higher than in controls ($p < 0.005$). Approximately 60% of the ^3H collagen and 48% of the other ^3H protein was secreted into the medium in all three groups.

Table I. *Comparison of GAG secretion in three fibroblast groups*

	HSF	LSA	M
Number of cell lines	4	4	5
Mean cell density			
10^5 cells/25 cm^2 flask	8.74± 1.1	6.73± 2.4	5.05± 0.7
GAG µg/10^7 cells/48 hrs	118.2 ±12.6	201.3 ±22.6	161.6 ±11.9
Predicted GAG from Fig. 2 µg/10^7 cells/48 hrs	125	141	158
Observed/Expected ratios	0.95	1.41	1.03

Values given as Mean ± SEM, LSA, lichen sclerosus et atrophicus; M, morphoea; HSF, normal skin fibroblasts.

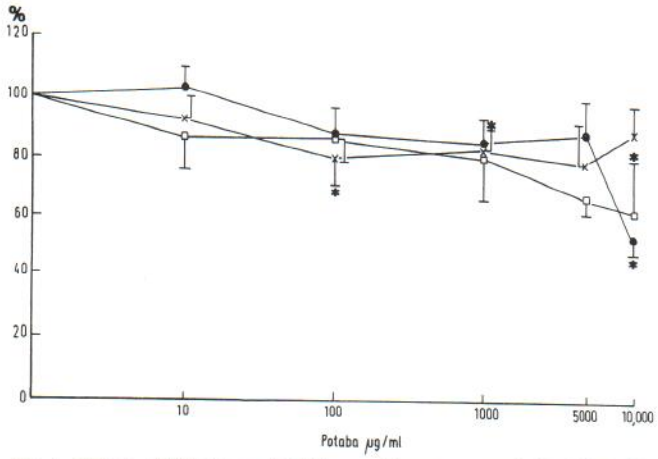

Fig. 3. Effect of Potaba on GAG secretion. **x**: normal: □: LSA: ●: morphoea. $^*p < 0.025$ Values shown are the mean ± SE of four flasks at each drug concentration. GAG secretion by untreated cells = 100%.

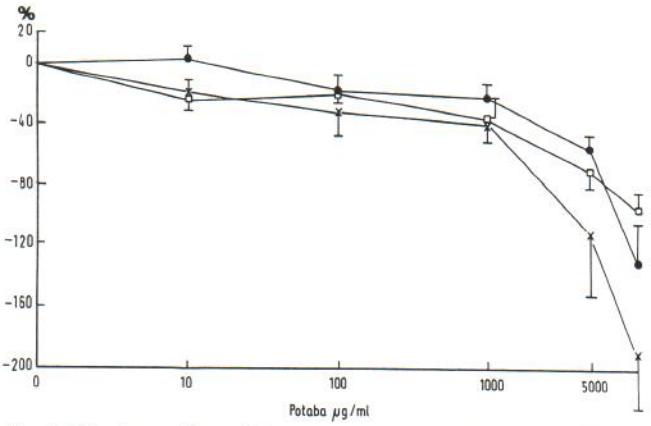

Fig. 4. Net drug effect of Potaba on GAG secretion **x**: normal: □: LSA: ●: morphoea. The net drug effect was the GAG value predicted from Fig. 2 subtracted from the observed value and was expressed as the % change from untreated controls. GAG secretion was significantly reduced ($p < 0.05$) in all cases except 10µg/ml Potaba in morphoea cell lines. Values shown are the mean ± SE of four flasks. Net drug effect on untreated cells = 0%.

DISCUSSION

A previous study (6) examined the effects of Potaba on the excessive collegen synthesis of skin fibroblasts from scleroderma lesions and the abnormal GAG secretion of rheumatoid synoviocytes. We have now examined LSA and morphoea fibroblasts to see if similar alterations were detectable, and if these were affected by treatment with Potaba. The LSA cell lines displayed an increase in GAG secretion over normal fibroblasts which was not found with morphoea cell lines. Juhlin et al. (11) reported greatly increased levels of hyaluronic acid in suction blisters raised in the skin of two LSA patients, which agrees with the increase in total GAG in the cultures, since fibroblast GAG consists predominantly of hyaluronic acid (8).

Once the levels of GAG secretion had been established in the normal cell lines, it was possible to measure the effect of treatment with Potaba. The net drug response showed significant decreases in GAG secretion at low drug levels (10–100 µg/ml) obtainable during treatment of patients with Potaba. Peak blood levels of Potaba measured by Priestley et al. (12) were in the range of 29–138 µg/ml. It is important to note that GAG secretion was depressed at concentrations of Potaba which did not affect cell proliferation.

There were differences in protein synthesis between the three groups of cell lines. Both the LSA and morphoea cell lines synthesised more collagen than the normal fibroblasts, with the morphoea lines giving the highest values. This is compatible with the fibrotic nature of the skin lesions observed in these conditions. The morphoea lines also synthesised more non-collagenous protein. Vuorio et al. (13) described an increase in fibronectin production by morphoea fibroblasts. It has been suggested that this connective tissue glycoprotein may act as a collagenase inhibitor. It seems possible that fibronectin may account for some of the increase in non-collagenous protein seen in this study.

For many years there has been disagreement on whether LSA and morphoea are variants of one disease. Patterson & Ackerman (14), however, defined pathological differences between the two conditions and declared them to be separate entities. Our experiments have shown different alterations in metabolism in fibroblasts from LSA and morphoea patients. In LSA fibroblasts, GAG metabolism was primarily affected and in morphoea fibroblasts it was collagen metabolism. If treatment with Potaba can reduce excess GAG secretion in vivo, this might be a useful therapy for LSA.

In conclusion, this study has identified abnormalities in the metabolism of LSA and morphoea fibroblasts in vitro, and has confirmed a difference between the two conditions. A possible therapeutic mechanism, which is effective at the blood levels of Potaba achieved during oral treatment of patients, is suggested by the drug's inhibition of glycosaminoglycan secretion by skin fibroblasts.

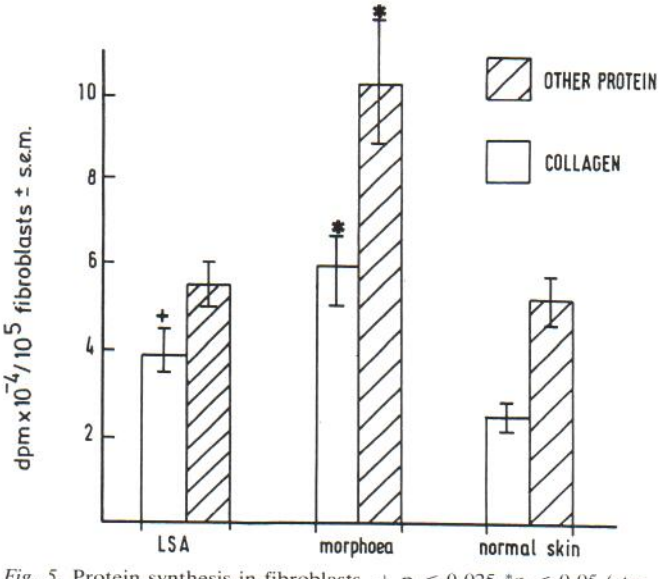

Fig. 5. Protein synthesis in fibroblasts. + $p < 0.025$ $^*p < 0.05$ (students 2-sample *t*-test) This was expressed as the mean ± SE for three cell lines from each group.

REFERENCES

1. Zarafonetis CJD. Treatment of scleroderma. Ann Int Med 1959; 5: 343–365.
2. Zarafonetis CJD, Curtis AC, Gulick AE. Use of para-aminobenzoic acid in dermatomyositis and scleroderma. Arch Int Med 1950; 85: 27–43.
3. Hasche-Klunder R. The treatment of Induratio penis plastica. Urologe 1970; 9: 335–336.
4. Zarafonetis CJD. Clinical use of para-aminobenzoic acid. Texas State J Med 1953; 49: 666.
5. Penneys NS. The treatment of lichen sclerosus with potassium para-aminobenzoate. J Am Acad Dermatol 1984; 10: 1039–1042.
6. Priestley GC, Brown JC. Effects of potassium para-aminobenzoate on growth and macromolecule synthesis in fibroblasts cultured from normal and sclerodermatous human skin and rheumatoid synovial cells. J Invest Dermatol 1979; 72: 161–164.
7. Blumenkrantz N, Asboe-Hansen G. New methods for quantitative determination of uronic acids. Anal Biochem 1973; 54: 484–489.
8. Priestley GC. Hyperactivity of fibroblasts cultured from psoriatic skin II. Synthesis of macromolecules. Br J Dermatol 1983; 109: 119–126.
9. Priestley GC. Urinary excretion of glycosaminoglycans in psoriasis. Arch Dermatol Res 1988; 280: 77–82.
10. Oakley CA. Priestely GC. Density-dependent regulation of skin-fibroblast glycosaminoglycans in vitro: Control by a secreted factor. Arch Dermatol Res 1985; 277: 264–269.
11. Juhlin L. Tengblad A. Ortonne JP. Lacour J-P. Hyaluronate in suction blisters from patients with scleroderma and various skin disorders. Acta Derm Venereol (Stockh) 1986; 66: 409–413.
12. Priestley GC. Heyworth R. Buxton PK. Blood levels of para-aminobenzoic acid during oral therapy. J Dermatol Treatment 1989; 1: 13–14.
13. Vuorio T, Mäkelä JK, Vuorio E. Activation of type I collagen genes in cultured scleroderma fibroblasts. Arch Dermatol Res 1987; 279: 154–160.
14. Patterson J, Ackerman AB. Lichen sclerosus et atrophicus is not related to morphoea. Am J Dermatopath 1984; 6: 323–335.