Amino Acids (1993) 5: 91–97

Amino
Acids

Cyclosporin A reduces skin collagen content in renal graft recipients

B. Lubec[1], **X. Stockenhuber**[2], **M. Weninger**[1], **J. Zezula**[1], and **G. Lubec**[1]

[1] Department of Pediatrics, and [2] Division of Nephrology, 3rd Department of Medicine, University of Vienna, Austria

Accepted September 3, 1992

Summary. Stimulated by the observation of a direct cytopathic effect of cyclosporin A on dermal fibroblasts we have examined total skin collagen content and collagenase activity in three groups of patients. Group 1 (controls) consisted of 16 patients without internal diseases, group 2 of 12 patients with renal transplantation on cyclosporin A therapy and group 3 of six patients with renal transplantation on corticosteroid/azathioprine therapy.

Total skin collagen was measured by hydroxyproline/protein determination, collagenase activity according to the principle of Wünsch. SDS page was employed in order to show collagen split products.

Mean skin collagen content (expressed by hydroxyproline/protein) was significantly lower in patients on cyclosporin A treatment (42.4 ± 12.2 μg/mg) compared to controls (78.6 ± 14.2 μg/mg) and patients on corticosteroid/azathioprine therapy (73.7 ± 11.2 μg/mg) . Mean collagenase activity was significantly higher in patients on cyclosporin A treatment (0.59 ± 0.16 IU) compared to controls (0.21 ± 0.09 IU) and patients on corticosteroid/azathioprine treatment (0.25 ± 0.11 IU). Total skin collagen content and collagenase activity were significantly inversely correlated in patients on cyclosporin A treatment ($r = -0.82$, $p < 0.01$, $y = -0.011x + 1.053$). Patients on cyclosporin A treatment showed remarkable reduction of alpha 1 and alpha 2 collagen chains and significantly prominent split products.

The results of our study could be explained either by the activation of collagenase or as a consequence of cyclophilin (peptidyl-prolyl cis-trans-isomerase) inhibition.

Keywords: Amino acids – Collagenase – Collagen split products – Connective tissue – Cyclosporin A side effect – Kidney transplantation – Peptides

Introduction

Steinmann and coworkers showed earlier this year the effect of cyclosporin A (CsA) on procollagen folding in chicken embryos (Steinmann et al., 1991). These authors have found that folding of procollagen I is slowed by CsA: the time needed for 50% of the molecules to reach a completely helical conformation is 8.5 minutes in the absence and 13.5 minutes in the presence of 5 μmol CsA. In cultured human fibroblasts CsA caused intracellular degradation and hence decreased production of collagen. The proposed mechanism of action is that CsA is binding to an intracellular enzyme, peptidyl-prolyl cis-trans-isomerase (Drugge and Handschuhmacher, 1988). This enzyme (synonym: cyclophilin) controls isomerization steps of the in vitro folding several proteins (Lang et al., 1987; Davis et al., 1989). Its inhibition should delay the triple helical conformation; the consequences of delayed helical conformation are that nonhelical collageneous material i.e. procollagen chains are readily degraded by proteolytic enzymes (Uitto et al., 1984) resulting into net negative collagen synthesis.

Stimulated by a paper on the direct cytopathic effect of CsA on dermal fibroblasts in culture (Nickoloff et al., 1988) we investigated skin collagen and collagenase in patients with renal transplantation on CsA in 1989/90. In the light of Steinmann's observations we are herewith presenting the findings of this study.

Methods

Patients

Skin of 16 patients (controls) without internal diseases, of 12 patients with renal transplantation on CsA therapy and of six patients with renal transplantation on corticosteroid/ azathioprine medication was examined. No patient was on medications known to influence collagen metabolism, had fibrotic processes or acute or chronic infection or inflammation of the skin. Patients with renal transplantation had no concomitant chronic internal diseases. Mean age was 52 years for the CsA group, 58 years for the controls and 45 years for the corticosteroid/azathioprine treated group. Skin punch biopsies were taken after written consent and information of the patients.

Skin biopsy

Skin biopsies were taken after disinfection and local anaesthesia (Xylocain 0.5%) from the proximal part of the upper arm using biopsy punches (Stiefel S.A.®, Belgium) with a diameter of 6 mm. Skin was used for determination of total collagen content, collagenase activity and characterization of collagen on SDS-Page.

Total collagen content

A standard protocol (Sykes et al., 1977) was used and the results expressed in μg hydroxyproline/ mg protein. Protein determination was carried out according to the method of Bradford (1976). Hydroxyproline was determined according to the principle of Lindblad and Diegelmann (1984).

Collagenase activity

Collagenase activity in the skin tissue homogenate was carried out according to the method by Wünsch and Heidrich (1963). The principle uses the substrate 4 phenyl-azo-benzyloxy-

carbamoyl-prolyl-leucyl-glycyl-prolyl-D-arginine dihydrate (Fluka 27667) and as test substance 4 phenyl-azo-benzoyl- carbamoyl-L-prolyl-L-leucin (Fluka 27668). The enzyme collagenase E.C.3.4.24.3. (Fluka 27665) served as calibration substance.

One unit of the enzyme activity is defined by the enzyme amount which catalyses 1 μM of PZ-Pro-Leu-Gly-Pro-Leu-Gly-Pro-Arg/min at pH 7.1 at 25 centigrades (PZ = 4 phenylazobenzyl-oxycarbonyl).

Units of collagenase activity were related to the protein content of the homogenate.

SDS-Polyacrylamide gel electrophoresis

The extraction of collagen was performed according to the principle of Dixit (1979). 80 μg of the extract were applied onto each well of the 8% SDS-Page which was carried out according to the principle of Lämmli (1970).

Statistical analysis

Comparison of groups were done by the Kruskall-Wallis and Wilcoxon test. Correlations were calculated by linear regression analysis.

Results

Skin collagen content

Mean collagen content expressed by hydroxyproline/protein was 78.6 $\pm$ 14.2 μg/mg in controls, 73.7 $\pm$ 11.2 in patients on corticosteroid/azathioprine

Table 1. Total skin collagen content (expressed as μg hydroxyproline/mg protein) of patients with renal transplantation on cyclosporin A (CsA) therapy, in patients with renal transplantation on corticosteroid/ azathioprine therapy and in controls

Nr	Controls	Corticosteroid/ azathioprine therapy	CsA therapy
1	73	77	42
2	84	84	21
3	62	72	36
4	59	63	45
5	79	87	51
6	104	59	23
7	83		39
8	59		47
9	69		65
10	89		52
11	94		47
12	58		41
13	79		
14	82		
15	87		
16	96		
mean	78.6	73.7	42.4*

* $p < 0.01$

treatment and 42.4 ± 12.2 in patients on CsA treatment, respectively. Mean collagen content was significantly lower in skin of CsA treated patients ($p < 0.01$). Details are given in Table 1.

Collagenase activity

Collagenase activity was 0.21 ± 0.09 IU in controls, 0.25 ± 0.11 IU in patients on corticosteroid/azathioprine treatment and 0.59 ± 0.16 IU in patients on CsA treatment. Mean collagenase activity was significantly higher in skin of CsA treated patients ($p < 0.01$). Details are given in Table 2.

Table 2. Collagenase activity (IU) in skin of patients with renal transplantation on cyclosporin A (CsA) therapy, in patients with renal transplantation on corticosteroid/azathioprine therapy and in controls

Nr	Controls	Corticosteroid/ azathioprine therapy	CsA therapy
1	0.17	0.29	0.49
2	0.42	0.32	0.72
3	0.31	0.41	0.87
4	0.22	0.22	0.53
5	0.30	0.11	0.57
6	0.11	0.16	0.82
7	0.12		0.72
8	0.17		0.57
9	0.27		0.32
10	0.23		0.47
11	0.11		0.46
12	0.16		0.54
13	0.21		
14	0.33		
15	0.13		
16	0.17		
mean	0.21	0.25	0.59*

* $p < 0.01$

Skin collagen content and collagenase activity were significantly negatively correlated in patients on CsA treatment ($r = -0.82$, $p < 0.01$) (Fig. 1). In controls and in patients on corticosteroid/azathioprine treatment no correlation between skin collagen content and collagenase activity was found.

Polyacrylamide gel electrophoresis

As given in Fig. 2 bands of collagen eluted from skin of patients on corticosteroid/azathioprine treatment and controls showed a comparable pattern. Collagen eluted from skin of patients treated with CsA showed remarkable

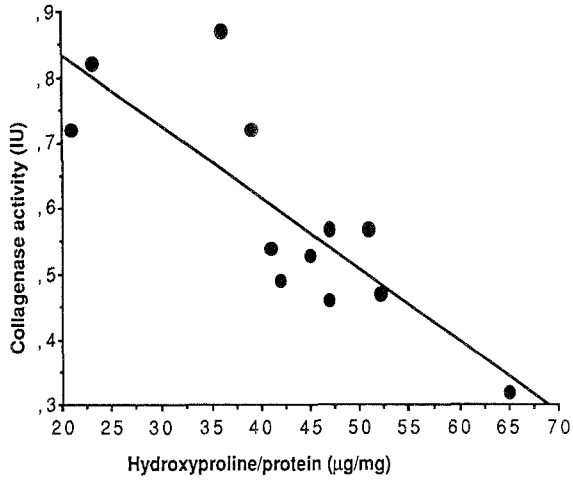

Fig. 1. Correlation between skin collagen content (expressed by hydroxyproline/protein) and skin collagenase activity in patients with renal transplantation on CsA treatment ($r = -0.82$, $p < 0.01$, $y = -0.011x + 1.053$)

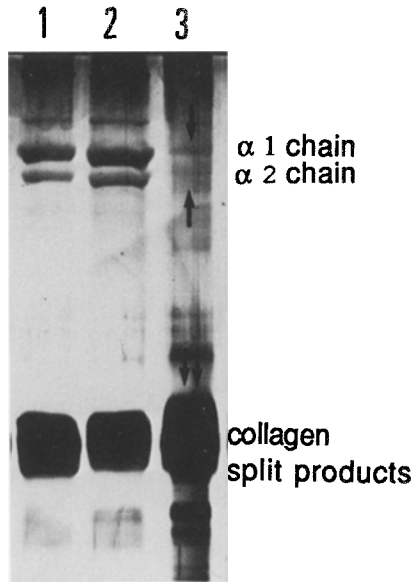

Fig. 2. Presentation of eluted skin collagen of controls (*1*), patients on corticosteroid/azathioprine treatment (*2*) and patients on CsA treatment (*3*). Note reduced intensity of alpha 1 and alpha 2 collagen bands (arrow) as well as intense split products (double arrow) in lane 3

reduction of alpha 1 and alpha 2 collagen chains and significantly prominent split products (Fig. 2).

Discussion

Our study clearly demonstrates that the skin collagen content in CsA treated patients was significantly lower and the collagenase activity was significantly

higher than in patients treated with corticosteroid/azathioprine or in controls. The decreased collagen content in the CsA group could be caused by increased collagenase activity as demonstrated by the negative correlation found in our study. The increased collagenolytic activity could be due to a) CsA mediated effect on macrophages (Matsushima and Baba, 1990), b) cytopathic effect of CsA on dermal fibroblasts and keratinocytes (Nickoloff et al., 1988), which might lead to a release of collagenolytic enzymes.

In consideration of Steinmann's data (1991), intracellular degradation of procollagen chains could be another explanation for reduced collagen content. Procollagen chains should be to a certain degree in a nonhelical conformation in the presence of CsA and therefore readily degradable by intracellular proteases. The fact that nonhelical procollagen is highly susceptible to intracellular proteolytic enzymes is well known from experiments with proline analogues (Uitto et al., 1984).

Strong support for our data comes from our experiments on SDS – page: in contrast to the controls and corticosteroid/azathioprine group patients' the CsA-group showed only faible bands of alpha 1 – and alpha 2 – bands but prominent split products. Unfortunately, Steinmann and coworkers presented the upper part of their gels revealing the alpha-chains only, which does not allow to compare findings with respect to the occurrence of split products. A recent observation by Nast et al. (1991) finding elevated procollagen alpha 1 – mRNA levels in renal cortex of CsA treated rats do not contradict our findings of reduced collagen content in CsA treated patients. Poiani et al. (1990) also found an enormous increase of procollagen alpha 1 – mRNA levels in the experimental therapy using cis-4-hydroxyproline for bleomycin induced lung fibrosis reflecting increased procollagen chain synthesis. Those chains, however, undergo rapid intracellular degradation as the chains containing cis-4-hydroxyproline do not form stable helical conformation resulting in reduced collagen content of lungs.

In agreement with our findings of reduced collagen content are the observations of Movsowitz et al. (1988), who reported osteopenia in CsA treated rats. It must be mentioned that CsA has no effect on total protein synthesis (Steinmann et al., 1991).

No dermatological symptoms were observed in all patients investigated but diagnostic assays for mechanoelastic properties which might be altered in CsA patients were not performed.

References

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72: 248–252

Davis JM, Boswell BA, Bächinger HP (1989) Thermal stability and folding of type IV procollagen and effect of peptidyl-prolyl cis-trans-isomerase on the folding of the triple-helix. J Biol Chem 264: 8956–8962

Dixit SN (1979) Isolation and characterization of two alpha chain size collagenous polypeptide chains C and D from glomerular basement membrane. FEBS Lett 106: 379–384

Drugge RJ, Handschuhmacher RE (1988) Cyclosporine – mechanism of action. Transplant Proc 20/2: 301–309

Lämmli UK (1970) A cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–682

Lang K, Schmid FX, Fischer G (1987) Catalysis of protein folding by prolyl isomerase. Nature 329: 268–270

Lindblad WJ, Diegelmann RF (1984) Quantification of hydroxyproline isomers in acid hydrolysates by HPLC. Ann Biochem 138: 390–395

Matsushima Y, Baba T (1990) The in vivo effect of cyclosporin A on macrophages. J Exp Pathol 5/2: 39–48

Movsowitz C, Epstein S, Fallon M, Ismail F, Thomas S (1988) Cyclosporin – A in vivo produces severe osteopenia in rat: effect of dose and duration of administration. Endocrinology 123/III: 2571–2577

Nast CC, Adler SG, Artishevsky A, Kresser CI, Ahmed K, Anderson PS (1991) Cyclosporin induces elevated procollagen alpha 1 (I) mRNA levels in the renal rat cortex. Kidney Int 39/4: 631–638

Nickoloff BJ, Fisher GJ, Mitra RS, Voorhees JJ (1988) Direct cytopathic effects of cyclosporin A on rapidly proliferating cultured keratinocytes and dermal fibroblasts. Transplant Proc 20/3: 85–90

Poiani GJ, Sumka JM, Tozzi CA, Liao WO, Riley DU (1990) Local delivery of liposome encapsulated proline analogue prevents pulmonary hypertension in rats. In: Lubec G, Rosenthal GA (ed) Amino acids. Escom, Leiden, pp 634–642

Steinmann B, Bruckner P, Superti-Furga A (1991) Cyclosporin A slows collagen triple-helix formation in vivo: indirect evidence for a physiologic role of peptidyl-prolyl cis-trans-isomerase. J. Biol Chem 226/2: 1299–1303

Sykes B, Francis MJO, Smith R (1977) Altered relation of two collagen types in osteogenesis imperfecta. N Engl J Med 296: 1200–1203

Uitto J, Ryhänen L, Tan EML, Oikarinen AI, Zaragoza EJ (1984) Pharmacological inhibition of excessive collagen deposition in fibrotic diseases. Fed Proc 43: 2815–2820

Wünsch E, Heidrich HG (1963) Darstellung von Prolinpeptiden, III. Ein neues Substrat zur Bestimmung der Kollagenase. Hoppe-Seylers Z Physiol Chem 332: 300–304

Authors' address: Prof. Dr. G. Lubec, Department of Pediatrics, University of Vienna, Waehringer Guertel 18–20, A-1090 Wien, Austria.

Received October 4, 1991