Original Article

Altered Expression of Alpha$_2$beta$_1$ Integrin in Kidney Fibroblasts: A Potential Mechanism for CsA-induced Nephrotoxicity

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Abstract

Background: Nephrotoxicity is considered a significant cause of patient morbidity following chronic Cyclosporine A (CsA) treatment. The exact mechanism of CsA-induced nephrotoxicity remains to be fully clarified. Tubulointerstitial fibrosis is widely regarded as a major pathway of CsA toxicity; therefore, the role of integrins as regulators of collagen in the extra-cellular matrix can be deemed pivotal. The objective of the present study was to observe the expression levels of alpha2beta1 integrin following CsA treatment +/- antioxidants.

Methods: Adhesion assay, immunofluorescent and flow cytometric analyses were performed on kidney fibroblasts obtained from rats after administration of CsA (25 mg/kg/day) +/- Vitamin E (vit. E) and Quercetin (Q) for 4 weeks. Total RNA was collected from the aforementioned fibroblasts for semi-quantitative reverse transcriptase-polymerase chain reaction analysis of α_2 and β_1 integrins.

Results: We found that α_2 and β_1 integrins were both markedly reduced following treatment with CsA, i.e., 25% and 13%, respectively, but were normal following subsequent consumption of the antioxidants vit. E and Q. Attachment and spreading of the CsA-treated fibroblasts declined from 82% to 50%; however, this effect was partially reversed to 70% following antioxidant treatment. Similar results were observed in the spreading assay in which the level of spreading decreased from 73% to 21% and was subsequently restored to 46%.

Conclusion: We conclude that cell adhesion, mediated by binding of integrin to collagen, which is a prerequisite of normal cell viability and collagen regulation, may be a novel pathway further explaining the nephrotoxic effects of CsA.

Key words: Alpha$_2$beta$_1$ integrin, cyclosporine A, nephrotoxicity, Quercetin, Vitamin E

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Introduction

Cyclosporine A (CsA) is a potent immunosuppressive drug widely used to prevent graft rejection and treat autoimmune disorders.[1] The introduction of CsA into maintenance therapy for kidney-transplant recipients has played an important role in the improvement of graft survival. CsA is potentially toxic to various tissues including kidney, liver, endocrine pancreas, and the nervous system, and its use is associated with hypertension. Among the reported side effects, malignant tumors have been shown to develop in 15%–20% of patients taking cyclosporine A, 10 years after transplantation.[2] Some of these malignancies occur due to recurrence of a pre-existing condition, either in the recipient or the donor, while others have been attributed to the consumption of immunosuppressive medication.[3] Nevertheless, the capacity of CsA treatment to increase graft survival has been determined for a long time. Optimal doses of CsA blood concentrations have been evolved so as to cause the least kidney toxicity. The magnitude of these effects may also vary with the dose of CsA. Thus, one way for reducing side effects is potential for improvement in dose prescriptions.[4]

The exact mechanism of CsA nephrotoxicity remains unclear; however, clinical and experimental studies have revealed that several factors may be involved.[5,6] Oxidative stress has been proposed as a major causative factor in some diseases[7,8] and in CsA-induced nephrotoxicity.[9,10]

Andres and Cascales showed that the imbalance produced between the activities of superoxide dismutase and catalase (CAT)/glutathione peroxidase (GPx) by CsA is the main mechanism responsible for peroxide accumulation and cell death in cultured hepatocytes.[11] In our recent study, we showed the toxic effects of CsA on rat kidney and illustrated that a combination of two antioxidants, i.e., vitamin E (vit. E) and quercetin (Q) significantly reduces blood urea nitrogen, serum creatinine (Cr) and Thiobarbituric Acid Reactive Substances (TBARS) demonstrating an improvement in the function of the kidneys.[12]

Several studies on gingival tissue samples have explained a relationship between CsA, collagen accumulation and fibrosis. Kataoka, et al., demonstrated a significant decrease in collagen phagocytosis in fibroblasts of rats with cyclosporine-induced overgrown gingiva.[13,14] Other researchers also demonstrated a reduction of fibroblasts isolated from the overgrown gingiva compared to the control.[15] Histopathological studies on the renal tissue indicated that fibrosis induced by CsA could be treated by administration of vit. E and Q.[16]

Lee, et al., reported that the initial binding step in collagen phagocytosis depends on the interaction between fibroblasts and

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collagen. Therefore it appears that the expression of α_2 integrin, known as a major receptor for collagen in fibroblasts, may play a critical role in regulation of collagen internalization.[17,18] In cell culture, the linkage between $\alpha_2\beta_1$ integrin and collagen occurs at focal adhesions consisting of clusters of integrins that are firmly bound to the extracellular matrix (ECM) interface.[19] Binding of integrins to ECM reorganizes actin filaments and stimulates cell adhesion. It has been shown that cellular adhesion to ECM in the kidney is key to regulating cell proliferation and survival.[20] The renal fibrosis induced by CsA is characterized by an accumulation of collagenous components in the renal connective tissue, with various degrees of inflammatory infiltration.[21]

Collectively, the findings mentioned above suggest that potential alteration in the expression of $\alpha_2\beta_1$ integrin caused by CsA treatment can bring about nephrotoxicity and affect kidney function. Therefore, in order to elucidate a new mechanism for CsA-induced nephrotoxicity, the aim of the present study was to investigate the expression pattern of $\alpha_2\beta_1$ integrin in kidney fibroblasts of CsA-treated rats and observe the effectiveness of antioxidant treatment, namely vit. E and Q, in reversing any changes observed in this pattern. Also, attachment and spreading assays were performed to correlate the expression of $\alpha_2\beta_1$ integrin with changes observed at a microscopic level in the treatment groups.

Materials and Methods

Materials

Quercetin, 2-[N-morpholino] ethanesulfonic acid (MES), bovine serum albumin (BSA), HEPES, type I collagen, Triton X-100, 50% glutaraldehyde, Vectasheild, Fibronectin, SDS and di-methyl sulfoxide (DMSO) were purchased from Sigma Chemical Co (Poole, Dorset, UK); vitamin E, Tris, glycine and acetic acid were obtained from Fluka Chemical Co (Buchs, Switzerland). Sodium azide, Sodium acetate, chloroform, MOPS (3-[N-Morpholino] propanesulfonic acid), sodium chloride, magnesium chloride, formaldehyde (37%), formamide, glucose and EDTA were obtained from Merck (Darmstadt, Germany). Trypsin and ethanol were obtained from BDH-England. Potassium chloride, potassium dihydrogen orthophosphate and isopropanol were purchased from Fluka-England, and Magnesium Chloride and crystal violet from Ridel-Germany. DMEM, MEM (Minimal essential medium), FBS (fetal bovine serum) were purchased from Gibco-BRL (Paisley, UK). PCR mineral oil, DEPC, loading buffer and ethidium bromide were obtained from Cinagen-Iran. Random Hexamer, dNTP, Ribonuclease Inhibitor, 5× RT-reaction buffer and reverse transcriptase primers, Amplification buffer, Taq DNA polymerase and DNA marker (1 Kb DNA ladder) were all obtained from Fermentase-Lithuania and CsA (Neoral) was provided by Novartis Pharma (Basel, Switzerland).

Animal experiments

All animal experiments were approved by the Ethics Committee of Shiraz University of Medical Sciences. Adult male Sprague-Dawley rats (270 ± 15 g) bred and raised at the university animal quarters were housed in a total number of 5 per cage and were given a rat chow diet (Pars Dam Co, Tehran, Iran) and water *ad libitum.* According to the results of our previous studies[12,16] that were done on six groups including a control, a vehicle control, a CsA group, a CsA+vit. E group, a CsA+ Q group and a CsA+ Vit. E+ Q group, we decided to have three groups receiving by gavage the following treatments: Control (CTRL) received daily 1 mL olive oil + 0.5 mL 25% ethanol as vehicle (n = 7); CsA (CsA) received CsA at a concentration of 25 mg / Kg body mass (b.m.)[22] in 1 mL olive oil + 0.5 mL 25% ethanol (n=9); and CsA+ vit. E+ Q (CsA + AO) received CsA (25 mg / Kg b.m.) in 0.5 mL olive oil, vit. E (100 mg / kg b.m., [22] in another 0.5 mL olive oil and Q (15 mg / Kg b.m, [23] in 0.5 ml 25% ethanol per day (n=10). On the final day of treatment (4 weeks), the animals were sacrificed by decapitation and the kidneys were removed and washed with sterile normal saline to remove blood.

Kidney fibroblast primary culture

One half of each kidney was used to establish cell culture. Kidney tissues were washed twice with phosphate buffered saline containing no Ca^{+2} and Mg^{+2} (PBS^- buffer) and then cut into small pieces. A falcon tube containing 15 mL 0.25% trypsin in PBS and the kidney pieces were agitated in a shaker incubator at 37°C for 10 minutes. This process was repeated three times and after every digestion period (10 minutes), the cell suspensions were removed and trypsin was inactivated with cold Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum (FBS). The whole suspension was then filtered and centrifuged at 1000 rpm for 10 minutes. The cell pellet was later re-suspended in 5mL DMEM supplemented with 20% FBS, 100 U/mL penicillin and 100µg/mL streptomycin. Aliquots of this stock cell suspension were added to either 25 or 75 cm^2 flasks containing DMEM with 10% FBS and were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The medium was changed on the following day and then every 3–4 days.

Cell Attachment Assay

Cell attachment assay was performed using flat-bottomed 96-well microtitre tissue culture plates (Nunc-Denmark). Microtitre wells were coated with 100 µL aliquots of COLI (serial concentrations: 0.7, 1.25, 2.5, 5, 10 µg/mL) diluted with phosphate buffered saline containing Ca^{2+} and Mg^{2+} (PBS^+) for a minimum of one hour at room temperature as described previously.[24]

Cell Spreading Assay

Cell spreading assay was performed using flat-bottomed 96-well microtitre tissue culture plates. Wells were coated with 5, 10, or 25 µg/mL COLI and cell spreading assays were performed as described previously.[25]

Inhibition Assay

Cell inhibition assay was performed in the same manner as attachment assay. In order to determine the effects of antibodies on cell attachment, 50 µL aliquots of CD49b (integrin α2 antibody) and CD29 (integrin β1 antibody) (BD Biosciences, Mississauga, Canada) were added at 37°C for 1 hour prior to the addition of 50 µL 4×10^5 cells / mL. In this assay, the COLI concentration was 10µg/mL and the rest of the procedure was performed in the same way as the cell attachment assay.[24]

Immunofluorescence assay for determination of focal adhesion formation

Immunofluorescence analyses were performed essentially as described previously.[26]

Flow Cytometry

Kidney fibroblast cells were grown to confluency in 25 cm^2 culture flasks. Cells were detached with a mild dissociation buffer (10 mM HEPES and 2 mg/mL EDTA in PBS^-) and then prepared for flow cytometry as described previously.[25]

RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from fibroblasts with RNX (RNA extraction kit from Cinagen, Tehran, Iran), and cDNA was synthesized from 1 μg of total RNA. After an initial denaturation for 2 min at 95°C, the samples were amplified for 30 cycles, consisting of annealing at 55°C for 1 minute, elongation at 72°C for 2 minute, and denaturation at 95°C for 1 minute. Duration of the final elongation reaction was increased for 10 minutes at 72°C to permit completion of reaction products. The PCR products were separated on a 2% (w/v) agarose gel and visualized by ethidium bromide staining. The sequences of each 5'- and 3'-primers were as follows: 5'-cta gca ctc caa cgg aga gg-3' was used as α2 integrin forward primer and 5'-cac tgc acc tag cat cag ga-3' was used as α2 integrin reverse primer. A 200 bp amplicon was expected upon performing PCR using the above α2 integrin primers.

Beta1 integrin forward primer was 5'-gac ctg cct tgg tgt ctg tgc-3' and its reverse primer was 5'-agc aac cac acc agc tag aat-3'. A 313 bp amplicon was expected performing PCR using the above β1 integrin primers. PCR primers for the analysis of rat glyceraldehyde-3-phosphate dehydrogenase mRNA level as an internal control consisted of: 5'-cct ctc tct tgc tct cag tat-3' as forward primer, and 5'-gta tcc gtt gtg gat ctg aca-3' as the reverse primer. Such primers will result in a 344 bp amplicon.

Statistical analysis

Data were statistically analyzed by one-way Anova and Kruskal-Wallis using SPSS software (version 16; SPSS, Chicago, IL, USA).

Results

Primary fibroblast culture establishment

Fibroblasts were successfully established from all kidney explants. Sample explants were from half of both kidneys. Primary fibroblasts were prepared from rat kidneys of the following three groups: a vehicle control group (CTRL), a CsA group (CsA) and a CsA+ vit. E+ Q group (CsA + AO). The monolayer cells (single layer of growing cells) grew after three days and had spindle-shaped morphology (normal appearance of fibroblasts). The three groups of cells had normal morphology after ten days.

Primary rat kidney fibroblast cell attachment to collagen type I (COLI) and fibronectin (FN)

The primary fibroblasts were tested for attachment to COLI and FN and compared with their attachment to bovine serum albumin (BSA) which does not promote any attachment (Figure 1). A dose-dependent attachment of the cells from the three groups to COLI was observed, with a maximum attachment at a coating concentration of 10 mg/mL. The extent of attachment, however, varied among the three different groups. After 1 hour of plating, more than 82% of the CTRL cells had attached to COLI. Groups CsA and CsA + AO showed a maximum of attachment to COLI of approximately 50% and 70%, respectively. The protein concentration of approximately 2–5 μg/mL was required for half maximal attachment (Figure 1A).

Primary fibroblasts were tested in the same assay for attachment to FN. Cells from the three groups showed a dose dependent attachment to FN with a maximum value at a coating concentration of 10 μg/mL, the same as that of COLI. The pattern of cell attachment to FN (Figure 1B) for the three groups (CTRL, CsA and CsA + AO) was 47%, 37%, and 40%, respectively, lower than those for COLI. These results indicate that the level of cell attachment is higher on COLI than on what we observed for FN. Also, CsA can decrease the attachment of fibroblasts to COLI but not to FN.

The effect of anti-integrin antibodies on cell attachment

In order to determine the relative involvement of $\alpha_2\beta_1$ integrin in the attachment of the cells to COLI (10 μg/mL), attachment in the presence of 0,10, 25, and 50 μg/mL of anti-α_2 integrin antibody (Figure 2A) and anti-β_1 integrin antibody (Figure 2B) was investigated. As shown in Figure 2A, the attachment of CTRL cells to COLI was approximately 79%, 50.5%, 27.7%, and 22.5%, respectively, while the attachment of group CsA cells was 50%, 31.7%, 15.7% and 13.7% and that of CsA + AO cells was 73.5%, 40.8%, 22.3%, and 17.7%. Cell attachment to COLI in CTRL cells, as shown in Figure 2B, was approximately 79%, 50.3%, 32.7% and 25.8%. However, the attachment of CsA cells was 50%, 31.3%, 22.5% and 20.7% and that of CsA + AO cells was 73.2%, 40.7%, 31.3% and 22.8%, respectively. The results suggest that anti-α_2 integrin antibody inhibits the attachment of fibroblasts to COLI-coated substrata. The inhibitory effect of anti-β_1 integrin was less than that of anti-α_2 integrin, although it was also dose-dependent as the anti-α_2 integrin yielded maximum inhibition at a concentration of 50 μg/mL. CTRL cells were inhibited more than the other two groups by either antibody.

Primary fibroblasts spreading on COLI

COLI was then tested for its ability to support fibroblast cell spreading in a dose-dependent manner. CsA cells spread much more slowly on COLI than the other two groups. CTRL cells spreading on COLI was maximal at a level of 73% while it was only 21% for CsA cells and 46% for CsA + AO cells as shown in Figure 3. COLI was found to support cell spreading in a dose-dependent manner with an optimal coating concentration of approximately 5 μg/mL for half-maximal spreading of the cells in the three groups (Figure 3).

Expression of α_2 and β_1 integrin mRNA in kidney primary fibroblasts with RT-PCR

As shown in Figure 4, the RT-PCR products corresponding to α_2 and β_1 subunits of integrin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were detected as a single band of the predicted size, and the level of GAPDH mRNA was relatively constant during the experiments. The level of α_2 subunit of integrin mRNA in the CsA cells was apparently lower than that of the CTRL cells. On the other hand, the RT-PCR products for α_2 integrin subunit mRNA in fibroblasts from CsA + AO showed an α_2 integrin expression higher than that of fibroblasts from group B and only slightly lower than the control cells.

As shown in Figure 4, although β_1 integrin expression is diminished in CsA cells compared to the CTRL group, the effect of CsA on inhibition of β_1 integrin expression is much less than its

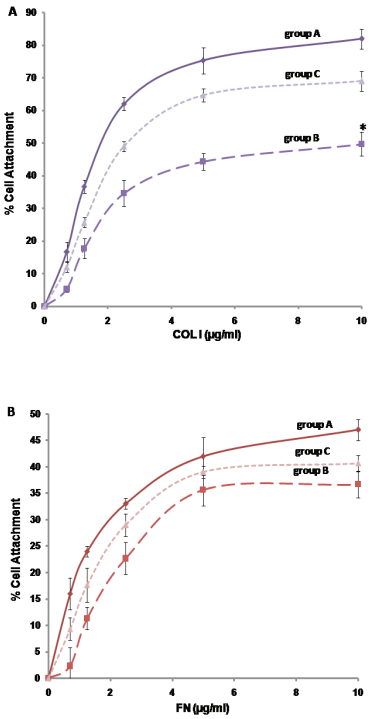

Figure 1. Attachment of primary kidney fibroblasts to COLI and FN coated wells at various concentrations of the proteins. The level of non-specific binding, determined from fibroblast cell attachment to wells coated with BSA alone, was subtracted. Values shown are mean ± standard deviation of percent attachment in triplicate wells. Results are representative of repeated experiments. Statistical analyses were performed using Kruskal-Wallis test. **A)** CsA cells show significant differences ($P < 0.05$) in %cell attachment compared CTRL and CsA + AO on COLI. **B)** CsA cells do not show significant differences in %cell attachment on FN.

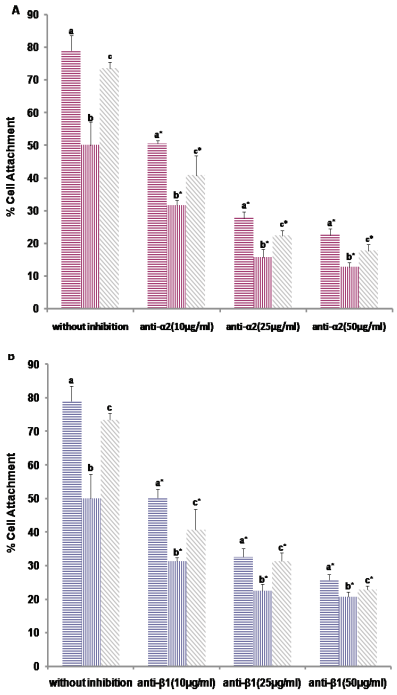

Figure 2. Comparison of the **(A)** anti-α_2 integrin antibody and **(B)** anti-β_1 integrin antibody on percent attachment of primary kidney fibroblast cells on COLI (10µg/mL). In three groups: CTRL, CsA and CsA + AO, cells were incubated with either anti-α_2 or anti-β_1 integrin antibodies at a concentration of 10, 25 and 50 µg/mL. The level of non-specific binding, determined from fibroblast cell attachment to wells coated with BSA alone, was subtracted. Values are mean ± standard deviation of percent cell attachment in triplicate wells. Results shown are representative of repeated experiments. Statistical analysis for studying the effect of each level of antibody on individual groups (a, b or c) and their comparison with the control (0 µg/mL antibody) were done by Friedman test. Each group with the significant differences ($P < 0.05$) compared to its respective control group is shown by an asterisk (*).

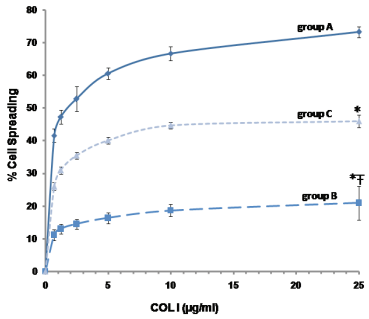

Figure 3. Spreading of primary kidney fibroblasts on COLI. The level of non-specific binding, determined from fibroblast cell spreading on wells coated with BSA alone, was subtracted. Values are mean ± standard deviation of percent cell spreading in triplicate wells. Results shown are representative of repeated experiments. Statistical analysis was performed using Kruskal-Wallis test. Group CsA shows significant differences ($P < 0.05$) compared to CTRL and group CsA + AO.

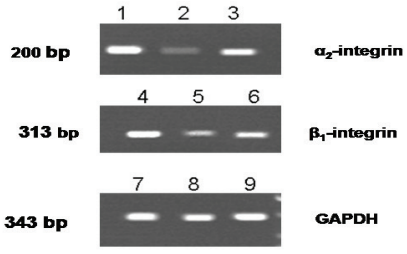

Figure 4. Ethidium bromide stained agarose gel electrophoresis of RT-PCR products of kidney primary fibroblast cells. Five µg of RNA were reverse transcribed and 6 µl of the cDNA were amplified for 30 cycles. Note the low intensity of cDNA from group B cells.**1)** α_2-integrin in control fibroblast cells; **2)** α_2-integrin in fibroblasts from CsA-treated rats; **3)** α_2-integrin in fibroblasts from the CsA+vit. E+Q- treated rats; **4)** β_1-integrin in control fibroblast cells; **5)** β_1-integrin in fibroblast cells from CsA-treated rats; **6)** β_1-integrin in fibroblasts from CsA+vit. E+Q treated rats; **7)** GAPDH in control fibroblast cells; **8)** GAPDH in fibroblasts from CsA-treated rats; **9)** GAPDH in fibroblast cells from CsA+vit. E+Q-treated rats.

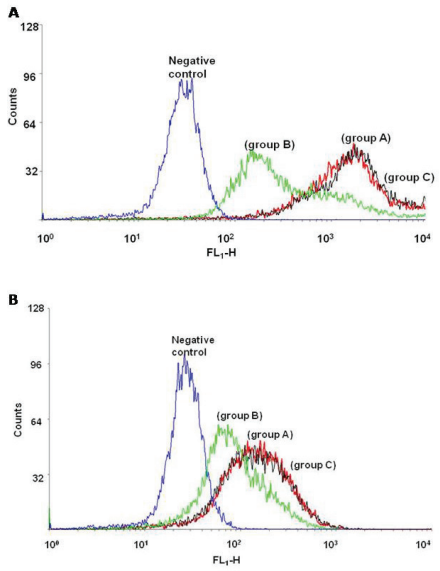

Figure 5. Flow cytograph analysis of **(A)** α_2 integrin and **(B)** β_1 integrin staining in fibroblasts derived from the kidneys of rats fed with a diet of CsA (green), CsA+vit.E+Q (red) and a control group (black). The blue curve represents the binding of anti-keyhole limpet hemocyanine monoclonal antibody, used as a negative control.

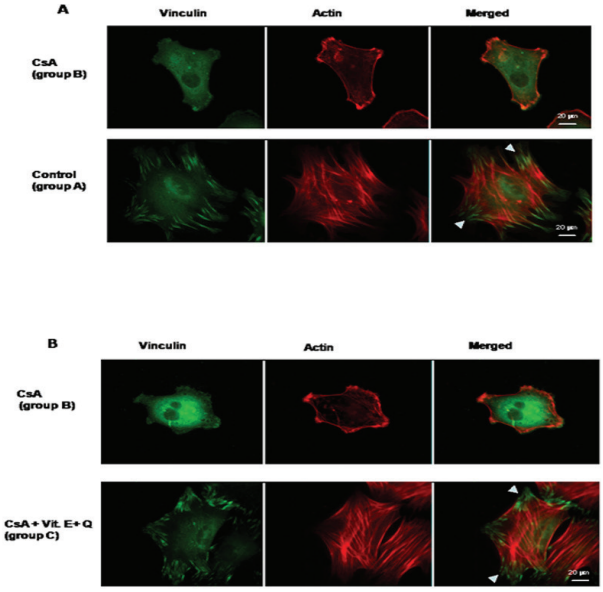

Figure 6. Focal adhesion and actin stress fiber formation in fibroblasts from CsA cells (CsA) vs. **(A)** CTRL cells **(B)** CsA + AO on COLI. Cells were incubated for 2 hours on COLI, fixed and double-stained for vinculin and actin. Arrowheads in the merged images indicate localization of vinculin at the end of actin bundles.

inhibitory effect on α_2 integrin expression. Using a combination of antioxidants could also induce the expression of β_1 integrin. These experiments were repeated with two different cultures in each group, and a similar tendency of mRNA expression was confirmed in both cultures.

Flow cytometry results for expression of α_2 and β_1 integrin subunits

FACS scan analysis was performed for quantitative assessment of the expression of α_2 and β_1 integrin subunits in fibroblasts. The percentage of cells expressing α_2 and β_1 integrin subunits was calculated on the basis of the percentage of mean fluorescent cells (%MFC), that is:

%MFC = %F1.mAb − %F2.mAb where 1.mAb represents the anti-rat α_2 or β_1 integrin antibodies and 2.mAb shows anti-keyhole hemocyanine monoclonal antibody. When %MFC of fibroblasts from all three groups was represented as the mean ± SD from different experiments, %MFC related to α_2 integrin of CsA fibroblasts was 45.7 ± 5.2 (Figure 5A), whereas those of the groups CsA and CsA + AO cells were 74.5 ± 6.4 and 72.7 ± 7.8, respectively. The differences between the CsA group and the other two group cells were significant ($P < 0.05$) according to independent Student *t*-test. %MFC of β_1 integrin of group CsA cells was 78.5 ± 5.3 (Figure 5B), whereas those of the fibroblasts from groups CTRL and CsA + AO cells were 92.1 ± 8.3 and 88.4 ± 4.9, respectively, which was also statistically significant (Figure 5B, $P < 0.05$). This experiment confirms that CsA inhibits the expression of α_2 integrin subunit by 25% and that of β_1 integrin subunit by 13% in the fibroblasts. Treatment with a combination of the two antioxidants could ameliorate it to some extent.

Organization of actin stress fibers and vinculin localization of fibroblasts on COLI

The ability of COLI to support focal adhesion formation and microfilament polymerization in three groups of cells was assessed by double immunofluorescent microscopy using anti-vinculin antibody and rhodamine-conjugated phaloidin for actin filament staining. The CTRL cells formed well-organized actin stress fibers and focal contacts containing vinculin, while cells from the CsA group did not spread well on COLI and there was actin accumulation at the edge of the cells with no accumulation of vinculin compared to the CTRL cells (Figure 6A). Disruption of actin filament rearrangement was also observed (Figure 6A).

The group CsA + AO cells also formed actin stress fibers and focal contacts to the same degree as the control cells compared with the cells from the CsA group (Figure 6B).

Discussion

The adverse effects of chronic CsA use are not limited to the kidneys, and other organs have been mentioned as possible targets. Heart disease, cancer, pulmonary disorders and hepatotoxicity are just some of the side effects associated with CsA in literature. CsA-induced nephropathy is considered as the main factor hindering extensive prescription of the drug.[27]

Numerous studies have reported the nephrotoxic effects of CsA and many have suggested the possible mechanisms involved; yet much is still unclear on this subject. Formation of ROS, activation of the renin-angiotensin-aldosterone system, renal vasoconstriction and up-regulation of TGF-β as a promoter of fibrosis have been described as possible pathways which can lead to nephropathy. Histopathologic studies have illustrated that tubulointerstitial fibrosis is a final common pathway in chronic CsA nephrotoxicity, stressing the importance of collagen regulation.[28] Through a series of studies on rats, Kataoka, et al., correlated α_2 subunit integrin expression with collagen accumulation in the gingiva leading to gingival overgrowth.[13] We postulated that the same mechanisms

described by Kataoka could be applied to CsA-induced nephrotoxicity; hence, we designed the present study to investigate this hypothesis. In their studies, Kataoka, et al., demonstrated that expression of the α_2 subunit of integrin is significantly reduced following treatment with CsA. This is in agreement with the findings of the present study, in which, using RT-PCR analysis, we found that CsA specifically inhibits mRNA expression of α_2 integrin subunit in kidney fibroblasts. Similarly, expression levels of β_1 integrin subunit were measured and a mild decrease was noted. We complemented the findings obtained from the RT-PCR analysis with FACS, in which 25% and 13% reductions were observed respectively for α_2 and β_1 integrin subunits.

There is increasing evidence in literature suggesting that protein folding and generation of reactive oxygen species (ROS) due to protein oxidation in the ER are linked,[29] although Staron, et al., have demonstrated that the expression of integrins is controlled by gp96 in ER.[30] Finally, Bouvier's findings suggest that endoplasmic reticulum stress initiated by CsA induces endothelial-to-mesenchymal transition and fibrosis generation.[31] Therefore, the reduction in integrin expression could be due to CsA-induced ER stress; however, future studies are required to understand how these stresses affect protein misfolding and studies are also required to elucidate if protein misfolding may cause oxidative stress or CsA-induced ROS production effect on integrin misfolding and expression.

It has been shown that antioxidant treatment can improve kidney function in rats treated with CsA.[32,33] This finding can be explained by the results of the present study, in which $\alpha_2\beta_1$ expression returned to near normal values following the administration of vit E and Q.

Alpha2beta1 is widely accepted as the main receptor for COLI on fibroblasts. It has been demonstrated by Arora, et al., that regulation of extracellular matrix by phagocytosis of collagen fibrils is dependent on the presence of $\alpha_2\beta_1$ integrin, a receptor that mediates initial cellular recognition and binding to collagen fibrils of the ECM.[34] This recognition and binding of $\alpha_2\beta_1$ integrin to COLI is key to integrin-mediated attachment and spreading. The mechanism involved has been explained by Sjaasted, et al.[35] They demonstrated that binding of cell surface integrins to ECM ligands leads to a transient increase in intracellular free Ca^{2+} which, through a feedback loop, alters integrin affinity for ECM molecules, enhancing integrin-mediated cell adhesion. We attempted to investigate whether the decrease in $\alpha_2\beta_1$ integrin observed following CsA treatment could affect attachment and spreading of the kidney fibroblasts. If so, this phenomenon could be attributed to a reduction in initial bonding of integrin to COLI.

The results from the adhesion assay indicated that CsA treatment decreased the attachment of fibroblasts to COLI, an effect which was reversed to some extent by administration of vit. E and Q. The same pattern was also observed in the spreading assay; however, the efficiency of antioxidant treatment in reversing the adverse effect of CsA on spreading was slightly less pronounced as it did not proximate the normal values measured in the control group. These results are consistent with other studies in which CsA treatment reduced cell attachment and spreading.[36,37] Herzberg, et al. demonstrated that altered integrin expression has a direct effect on cellular adhesion.[36] Analyzing these findings, we arrived at the conclusion that CsA reduced cell attachment and spreading on COLI via its effect on $\alpha_2\beta_1$ integrin expression. To further investigate this matter, we performed inhibition assay using specific antibodies against α_2 and β_1 subunits of integrin. We observed that when these antibodies were administered, attachment was reduced in the same manner as was noted during CsA treatment, further strengthening the idea that CsA exerts its effect by reducing $\alpha_2\beta_1$ integrin expression.

Focal adhesions are sites where integrin binds to the ECM proteins and molds the intracellular framework, by means of actin filament rearrangement.[38] Using double labeling immunofluoresence assay with antibodies directly against actin filaments and the cytoskeleton protein vinculin, focal contacts were detected by the co-localization of ligand-occupied clustered $\alpha_2\beta_1$ integrin on the surface of group CTRL and CsA + AO cells. As it can be noted from Figure 6, the cells of group CTRL and CsA + AO were of similar shape and neither the number nor the location of focal contacts was distinguishable. No focal contacts were observed in group CsA cells, indicating the adverse effect of CsA on focal contact formation. CsA reduces the expression levels of $\alpha_2\beta_1$ integrin, as was demonstrated in the RT-PCR assay; it also inhibits focal contact formation and disrupts actin filament rearrangement (Figure 6). Therefore, we can hypothesize that these two effects are related and the reduction of $\alpha_2\beta_1$ integrin expression can be detrimental for focal contact formation because the binding of this integrin to its ligand is required for initiating this process.

Focal adhesion kinase[39] is a protein tyrosine kinase which regulates the cytoskeleton and thus mediates many of the downstream responses attributed to focal adhesions[40] The binding of integrins to their ligands can lead to ROS generation which inhibits low molecular weight phosphotyrosine phosphatases by oxidation, thereby preventing the enzyme from dephosphorylating and inactivating FAK.[41] Therefore, lower levels of $\alpha_2\beta_1$ on the cell surface following CsA treatment can be a possible mechanism explaining the reduction of focal contact formations via FAK deactivation.

In conclusion, we demonstrated that CsA treatment reduces expression of $\alpha_2\beta_1$ integrin which in turn leads to attenuation of spreading and attachment of kidney fibroblasts. Due to the importance of integrins in normal cell viability and function, other potential side effects of $\alpha_2\beta_1$ integrin down-regulation can also be revealed pending further investigations. Therefore, the results of this study illustrate a novel mechanism for the nephrotoxic effects of CsA through down-regulation of $\alpha_2\beta_1$ integrin. The promising results obtained from administrating vit. E and Q gives hope that antioxidant treatment can reverse the side effects of CsA to some extent.

Conflict of interest statement

The authors do not have any conflict of interest to disclose.

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