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Alterations induced by cyclosporine A in myocardial fibers and extracellular matrix in rat

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Summary. Cyclosporine A (CsA) is the first choice immunosuppressant universally used in allo-transplantation. However, it has been demonstrated that this drug produces unwanted side effects in several organs and in particular in the kidney and in the heart. While the cardiac toxicity, due to alteration of myocardial prostanoid has been reported, no data are available about the effects of CsA on myocardial cytoarchitecture. We studied the CsA induced alterations of the myocardial structure and of the extracellular matrix components (ECM). To test the ECM enzymatic changes we studied a family of enzymes (matrix metalloproteinase-MMP), responsible for the degradation of extracellular matrix components. In particular we investigated MMP1, MMP2 and MMP9.

The study was carried out on two groups of Wistar rats. The group I animals served as a control and were injected subcutaneously daily with castor oil for 21 days. Group II: animals were subcutaneously injected daily with CsA (dose: 15 mg/Kg in castor oil) for 21 days. The group I animals (control) had normal heart architecture and low levels of MMP1, MMP2 and MMP9. The group II animals showed degenerative changes with myocardial fibrosis, low levels of MMP1 and MMP9 but a clear increase in MMP2.

We suggest that the myocardial fibrosis was a consequence of the cardiotoxic effect of CsA determining the alteration of the balance between synthesis and degradation of ECM. The increase in MMP2 suggests that this enzyme could play a protective role during myocardial damage and represent a compensatory mechanism for the excessive accumulation of collagen.

Key words: Cyclosporine A, Heart, Rat, Matrix-metalloproteinases

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Introduction

The immunosuppressant cyclosporine A (CsA) is widely used to prevent acute rejection after solid organ transplantation (Kahan, 1989). However, the immunosuppressive treatment induces a series of adverse side-effects. The most frequent include the development of severe hypertension and renal failure in most patients treated (Curtis, 1992; Braun-Dullaeus et al., 1998). Systemic or renal vasoconstriction has been suggested to be the principal pathophysiological mechanism responsible for these adverse effects. Several studies have demonstrated that CsA is able to increase the tonus of vessels both in peripheral (Xue et al., 1987; Tronc et al., 1991) and in renal arteries (Carrier et al., 1991; Lanese and Conger, 1993). It has been suggested that CsA exerts its negative effect on vessel tonus through different mechanisms involving an alteration of the renin angiotensin system (Mason et al., 1991; Auch-Schwelk et al., 1994; Kupferman et al., 1994), the nitric oxide-dependent impairment of endothelial relaxation (Gallego et al., 1994), the stimulation of endothelin release (Lanese and Conger, 1993), and the calcium-dependent mechanism (Braun-Dullaeus et al.,1998). However, the most favoured hypothesis of renal failure suggests that CsA alters the balance of the prostacyclin and its vasoconstrictor antagonist tromboxane A_2 in renal cortical tissue (Carrier et al., 1993; Grieve et al., 1993). These prostanoids are also produced within the heart, but their release is low under normal conditions (De Deckere et al., 1977). However under pathophysiological conditions, such as ischemia and reperfusion, elevated levels were found in isolated perfused hearts, thereby contributing to the alteration of cardiac function (Giannessi et al., 1992). Moreover these prostanoids have also been demonstrated to be altered in the heart of patients treated with CsA and to be responsible for cardiac toxicity (Laczkovics et al., 1987). The involvement of CsA in cardiac toxicity, with alteration of myocardial prostanoid balance towards the vasoconstrictive tromboxane A_2, has been reported.

Thus, the aim of this study was to evaluate, in experimental models, the alterations of the myocardial

structure and of the extracellular matrix (ECM) components induced by CsA treatment. The structural changes were evaluated by morphological analysis, and the enzymatic changes by immunohistochemical and immunoblotting analysis. To test the latter, we considered a family of endopeptidase enzymes (matrix metalloproteinases-MMP), that have been classified according to their substrate specificity into gelatinases, stromelysin and collagenases (Basbaum and Werb, 1996). These enzymes are responsible for the degradation of extracellular matrix components including collagen, gelatin, laminin and proteoglycans. In particular we investigated the collagenase (MMP1) and the gelatinase (gelatinase B - MMP2 and gelatinase A - MMP9) enzymes. MMP1 (57 KDa) degrades type I and III native collagen while MMP2 (72 KDa) and MMP9 (92 KDa) act on elastin and type IV collagen. Although MMP2 and MMP9 are similar in their structure, two gelatinase enzymes arise from separate mRNA transcripts on separate genes (Senior et al., 1991).

Materials and methods

The study included 28 male Wistar rats with an average weight of 200-250 g. The animals were housed in individual cages with food and water ad libitum and kept in an animal house at a constant temperature of 22 °C with 12 h alternating light-dark cycle. Every effort was made to minimise animal suffering according to the "Guiding Principles in the use of animals in Toxicology" and to the Italian Ministry of Health. The animals were divided into two groups (each of 14 animals). Group I: the animals served as controls and were injected subcutaneously daily with castor oil for 21 days. Group II: the animals were injected subcutaneously daily with CsA (Sandimmun, Sandoz; 15 mg/Kg in castor oil) for 21 days according to Schuurman et al. (1991).

The animals were killed at the end of the 21 day treatment of period. The hearts were removed, washed in phosphate buffer 0.1 M, pH 7.4 and cut transversely into small pieces containing both the atria and the ventricles. The samples were fixed with 10% buffered formalin, processed according to standard procedures, embedded in paraffin and serially sectioned at 5 μm by a microtome. Half of the sections were treated with sirius red to stain collagen. Collagen volume fraction was determined by quantitative morphometry of sirius-stained sections with an automated image analyzer (Imageproplus, Immagini e Computer, Milan, Italy). The collagen volume fraction was calculated as the sum of all connective tissue areas of the entire coronary section divided by the sum of all connective tissue and muscle areas in all fields of the section according to Brilla et al. (1991). Five sections per rat were examined. All data were subjected to T-test to evaluate the differences between the collagen content in controls and in CsA-treated rats.

The other half of the sections was treated for MMP1, MMP2 and MMP9 immunohistochemical and immunoblotting analysis.

Immunohistochemical localization of matrix metalloproteinases (MMP1, MMP2, MMP9)

Briefly, 5 μm-thick sections were first deparaffinized and rehydrated, and then immersed in 3% hydrogen peroxide in methanol for 30 min to block the endogenous peroxidase activity. Sections were then incubated with goat serum (diluted 1:5) for 60 min, then serially treated with MMP1, MMP2 and MMP9 polyclonal antibodies (diluted 1:100) overnight. The sections were washed in TBS buffer and sequentially incubated with: a) biotinylated rabbit anti-goat IgG; and b) avidin-biotin horseradish peroxidase complex according to the manufacturer's instructions (ABC kit; Dakopatts, Milan, Italy). The sections were stained by immersing slides in a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.03% hydrogen peroxide. All slides were counterstained with haematoxylin, dehydrated and mounted. Control reactions were performed in the absence of MMP1, MMP2 and MMP9 antibodies.

Immunoblotting analysis

The tissue, transversely cut, was homogenised in a buffer system according to Laemmli (1970) and centrifuged at 10.000 g for 20 min. Protein concentrations were determined by absorption spectroscopy with bovine serum albumin (BSA) serving as standard protein. Supernatants were analysed by SDS-PAGE using a 10% polyacrylamide gel under reducing conditions followed by silver staining of the gel and by Western blotting. The molecular weights of the proteins were evaluated by using prestained marker proteins with a molecular weight in the range of 15-200 KDa (Life Technologies, Milan, Italy).

Western Blot analysis

The blots were blocked with 5% milk in Tris-buffered saline (TBS, 0.05 M, pH 7.4) for 2 hours. Then, the solution was removed and the blots were incubated with a 1:2500 dilution in TBS-0.5% Tween (TBS-T) of goat antibodies against MMP1, MMP2 and MMP9 at 4 °C overnight, followed by incubation with anti-goat IgG (1:5000) and avidin-biotin horseradish peroxidase complex (1:1000) according to the manufacturer's instructions (ABC kit; Dakopatts, Milan, Italy). The blots were developed with the same solution used for the immunohistochemical method.

Results

The structural and enzymatic data were similar both in the atrial and in the ventricle myocardium and so we referred to them without distinction.

Morphological, immunohistochemical and immunoblotting study of heart in control rats (group I)

The heart of control rats had a normal morphology consisting of striated muscle fibers and scarce connective tissue. The muscle fibers were made up of separate cellular units joined end to end in wide networks. They had one or two nuclei and abundant cytoplasm. The elongated nuclei of the cellular units were usually deeply located inside the fibers. The connective tissue was thin and well evidenced with sirius red staining (Fig. 1A). The collagen content expressed in volume fraction by quantitative morphometry of stained sections is reported in Table 1.

The immunohistochemical study showed very low levels of positivity for MMP1, MMP2 and MMP9 in the myocardial fibers (Fig. 2A).

The SDS-PAGE analysis of proteins in control hearts followed by silver staining of the gel showed different bands of various molecular weight in the range of 5-200 KDa . Specific anti-MMP1, anti-MMP2 and anti-MMP9 antibodies detected very weak bands with an apparent molecular weight of 57, 72 and 92 KDa. So, the levels of MMP1 MMP2 and MMP9 were very low or they were not constitutively expressed. Fig. 3 (line 2) shows, in control rats, the immunoblotting analysis for MMP2.

Table 1. Collagen volume fraction in control (14 samples) and CsA-treated (14 samples) rats.

CONTROL RATS	CsA-TREATED RATS
2.5±0.9	6.0±2.2*

The data are expressed as means±SD; *: p≤0.05.

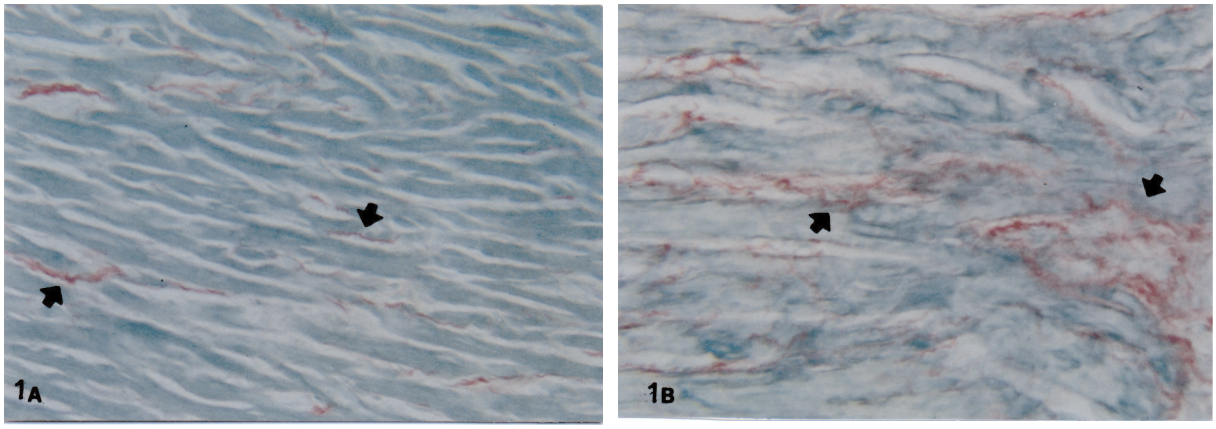

Fig. 1. A. Sirius red method in the heart of control rats (14 samples). The connective tissue (arrows) is evident amongst the myocardial fibers. x 160. **B.** Sirius red method in the heart of treated rats (14 samples). The connective tissue (arrows) is clearly increased. x 160

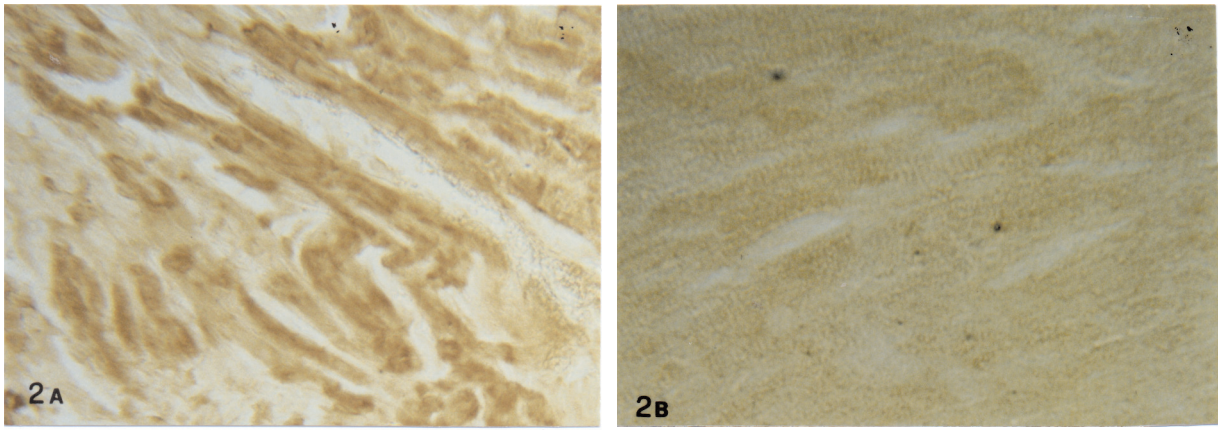

Fig. 2. A. Immunolocalization of MMP2 with polyclonal antibody in heart of treated rats (14 samples). A high positivity is evident in the myocardial fibers. x 250. **B.** Immunolocalization of MMP2 with polyclonal antibody in heart of control rats (14 samples). Low positivity is evident in the myocardial fibers. x 250

Morphological, immunohistochemical and immunoblotting study of the heart in rats treated with CsA (group II)

The heart of CsA-treated rats showed clear degenerative changes. The striated muscle fibers were disorganised and the networks disappeared. The fibers still had abundant cytoplasm and one or two nuclei inside them, similarly to control rats. On the contrary, the connective tissue was clearly increased and well highlighted with the sirius-red technique (Fig. 1B). The collagen volume fraction was significantly greater compared to control rats. The data are reported in Table 1.

The immunohistochemical study showed very low levels of MMP1 and MMP9 in the myocardial fibers whereas MMP2 was notably increased. The MMP2 increase is evident in Fig .2B.

SDS-PAGE analysis of cell proteins and silver staining of the gels showed a similar protein fractionating pattern as in control. However, the immunoblotting analysis with specific anti-MMP2 antibody showed an increase in the MMP2 levels, compared to that observed in control rats. However the anti-MMP1 and anti-MMP9 antibodies showed very low levels or none of these proteins as in control rats. Fig. 3 (line 1) shows, in treated rats, the immunoblotting analysis for MMP2.

Discussion

Our study showed that the treatment with CsA determines both structural myocardial alterations and enzymatic changes in the extracellular matrix. Two main findings have to be underlined: a) the myocardial fibrosis; and b) the high increase of the MMP2 proteins only. We found that the disorganisation of striated muscle fibers was accompanied by a significant increase in connective tissue content. This myocardial fibrosis seemed to be the consequence of a CsA cardiotoxic effect which would alter the balance between synthesis and degradation of ECM collagen to extracellular collagen. The following abnormal accumulation of collagen which is a potential cause of developing fibrosis, could be due to the activation of fibroblasts, which are the principal collagen-producing cells in the heart. The activation and in particular the proliferation of fibroblasts have been shown to be determined by several growth factors or mechanical stimuli (Butt, 1995; Butt and Bishop, 1997). It is known that in rat kidneys CsA releases cytokine and peptide growth factors from injured cells (Dumelinck et al., 1998). These factors are also released from myocardial fibers damaged by CsA (Karch and Billingham, 1985; Sanchez et al., 2000) and probably, in turn, they stimulate a cascade of signalling molecules, including phospholipases A and C, protein kinase C and mitogen-activated protein kinases (MAP). An important effect of this signalling is the stimulation of the expression of immediately-early genes, such as c-fos and the proliferation of their gene products (Sadoshima and Izumo, 1993). The activation of these genes triggers the stimulation of gene expression encoding contractile (Sadoshima et al., 1992), structural (But and Bishop, 1997) and cell-cycle regulatory proteins (Predel et al., 1992). The activation of such pathways, ultimately, increases protein synthesis and fibroblast proliferation.

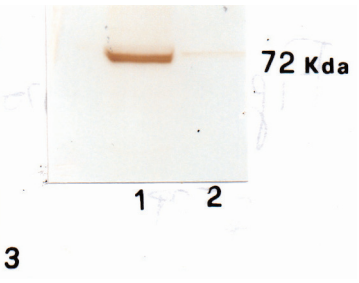

Fig. 3. Western blot analysis using anti MMP2 polyclonal antibody in heart of treated (line 1) and control (line 2) rats.

The other interesting point of this study regards the analysis, by immunohistochemical and immunoblotting procedures, of the matrix metalloproteinases, because they contribute to fibrillar collagen degradation (Tyagi et al., 1993). These proteins are the members of a family of metal-dependent enzymes, classified on the basis of their substrate specificity, in interstitial collagenase (MMP1) and gelatinase (MMP2 and MMP9). Our results demonstrated that, after CsA treatment, the myocardial fibers showed a clear increase in MMP2 whereas MMP1 and MMP9 levels remained almost unchanged. These findings suggest that MMP2 may play a different role to that of MMP1 and MMP9. In particular we showed that the alteration of MMP2 and MMP9 can differently occur even if these enzymes act on the same collagen components. We could suggest that the MMP2 modification is due mainly to: 1) protective action of enzyme against myocardial damage; and 2) different genic regulation. The first point can indicate that the MMP2 increase might represent a compensatory mechanism against the excessive accumulation of collagen. This hypothesis agrees with Lipke et al. (1993) who showed that the accumulation of type IV collagen induced by hypertension was associated with the increase in MMP2 only.

As regards the second point, it is known that the regulation of MMPs occurs at the level of gene expression, but their activities are also controlled at post-translational level by different factors involving tissue inhibitors of metalloproteinases, plasminogen activators and inhibitors (Matrisian, 1990; Woessner, 1991). Since MMP1 and MMP9 do not increase after CsA treatment it is possible that the synthesis of these proteins has only a transcriptional regulation that is blocked by CsA action. Instead the MMP2 increase suggests that this protein could be controlled at post-translational level similarly to that reported in the infarcted rat heart (Cleutjens et al., 1995) and thus the drug would not interfere with the MMP2 synthesis.

Moreover, in this work we could underline the role of calcium in the context of CsA and MMP proteins. CsA, in fact, has been shown to induce myocyte injury (Owunkanne et al., 1993) and to decrease left ventricular developed pressure in isolated rat hearts (Kingma et al., 1991). This fact has been related to the abnormal calcium movements in the sarcoplasmic reticulum (Banijamali et al., 1998). CsA inhibits calcium efflux in isolated adult rat cardiac myocytes,by closing the permeability of the transitional pore (Bowers et al., 1991). The increased intracellular calcium amount produces an increase in MMP proteins and, in particular of MMP2, which are calcium-dependent enzymes (Borkakoti, 1998).

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