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Original article

Cytoprotective effect of low-dose tacrolimus on islets of Langerhans in cultures subjected to stimulation by acute rejection cytokines☆

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ABSTRACT

Introduction: The improvement in pancreatic islet transplantation results is due to immunosuppression protocols that include, among others, low-dose tacrolimus. Both anti-inflammatory and anti-oxidant effects of tacrolimus could be useful in preventing primary rejection.

Aim: To evaluate in vitro islet low-dose tacrolimus response after pro-inflammatory stimulation.

Material and methods: Isolated rat islets were cultured in RPMI medium in the presence of IL-1 (50 UI/ml) plus IF-γ (1000 UI/ml) and tacrolimus (5 ng/ml). The 24 h production of lipoperoxide (LPO) and nitric oxide (NO) were measured as oxidative stress markers. Determination of apoptosis markers (nucleosome content and Bcl-2) was also performed.

Results: Oxidative stress (LPO 10.1±1.16 pmol/isletx24; NO 19.1±3.28 pmol/isletx24 h) and apoptosis (nucleosome 0.24±0.04 UI/islet; Bcl-2 0.69±0.212 UI/islet) markers showed a very significant increase after cytokine stimulation (P<.01). Both effects improved by adding tacrolimus to the medium. Protective effect was complete when lipoperoxide (1.58 pmol/isletx24 h), nitric oxide (9.81 pmol/isletx24 h) and Bcl-2 (1.37±0.23 UI/islet) were determined.

Conclusion: In vitro cytoprotective effect of low-dose tacrolimus on isolated rat islets decreases both oxidative stress and apoptosis markers after stimulation of pro-inflammatory mediators.

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Efecto citoprotector del tacrolimus a dosis bajas sobre islotes de Langeerhans en cultivo sometidos a estímulos por citocinas del rechazo agudo

Palabras clave:
Trasplante de islotes pancreáticos
Apoptosis
Estrés oxidativo
Tacrolimus
Inmunosupresión

RESUMEN

Introducción: La mejoría de los resultados en el trasplante de islotes pancreáticos se debe en gran parte a la introducción de nuevos protocolos de inmunosupresión que incluyen, entre otros, tacrolimus a bajas dosis. Este fármaco tiene efectos antioxidantes y antiapoptóticos que podrían ser de utilidad en la prevención del rechazo primario.

Objetivos: Evaluar la respuesta in vitro a tacrolimus a bajas dosis en islotes de rata estimulados con citocinas proinflamatorias implicadas en el rechazo primario de islotes.

Material y método: Se cultivaron islotes de rata en medio RPMI determinándose producción de lipoperóxido (LPO) y óxido nítrico (NO) y marcadores de apoptosis (nucleosomas y Bcl-2) en presencia de IL-1 (50 UI/ml) e IF-γ (1000 UI/ml) y adición de tacrolimus (FK-506; 5 ng/ml).

Resultados: Tras la estimulación se apreció un aumento muy significativo ($p < 0,01$) de los marcadores de estrés oxidativo (LPO 10,1 ± 1,16 pmol/islote x 24; NO 19,1 ± 3,28 pmol/islote x 24 h) y apoptosis (nucleosomas 0,24 ± 0,04; Bcl-2 0,69 ± 0,212). Dichos efectos fueron contrarrestados de manera significativa tras añadir tacrolimus, siendo la reversión completa (p NS frente a controles) en el caso de la producción de lipoperóxidos (1,58 pmol/islote x 24 h) y óxido nítrico (9,81 pmol/islote x 24 h), así como en el descenso de Bcl-2 (1,37 ± 0,23 UI/islote).

Conclusiones: El efecto citoprotector in vitro del tacrolimus a bajas dosis sobre islotes estimulados con citocinas proinflamatorias consigue aminorar la generación de estrés oxidativo y la activación de la apoptosis, habitualmente implicados en el rechazo en las primeras 48 h postimplante.

Introduction

Currently, pancreatic islet transplantation (PIT) is a valid alternative for treatment of type 1 diabetes. The improvement in insulin independence results and better metabolic control are due largely to improvements made in donor and recipient selection, isolation, preservation and immunosuppression.[1] Along with the shortage of donors, immune problems are still the main obstacle to widespread islet transplantation.[2] The persistence of autoimmunity and rejection are the main aspects to consider.[3] However, the survival of transplanted islets is further threatened by a series of events causing a nonspecific inflammatory response. As well as the presence of the innate immune response, there is also the loss of an important part of the islets during the first 48 hours after implantation. Failure is observed therefore before most of the islets have had a chance to work.[4] This phenomenon is known as primary rejection/dysfunction, and is also observed in syngeneic and autologous transplants without observing the participation of adaptive immunity,[5] but with a massive release of pro-inflammatory mediators, such as TNF-α, IL-1 or IL-6.[6] The activation of pro-apoptotic signals and appearance of oxidative stress is triggered both by intraislet macrophages as well as ductal cells, endothelial cells and even beta cells themselves.[4,7] Moreover, mechanical, chemical and ischaemic stress originating during islet procurement and isolation contributes significantly to the loss of islets.[4] In addition, the introduction of treatments capable of reducing pro-inflammatory and pro-apoptotic phenomena observed in the primary rejection after PIT improves both the viability and survival of the transplanted islets.[8]

Most of the protocols currently used in PIT include low-dose tacrolimus.[9] Regardless of its long-term negative effects on beta cells, in certain circumstances it has a cytoprotective effect which is both non-specific and anti-inflammatory as well as blocking apoptotic phenomena.[10,11] Thus, such actions could be useful for PIT in reducing or offsetting pathophysiological phenomena associated with primary rejection.[12]

The aim of this study was to evaluate the possible cytoprotective effect of low-dose tacrolimus in rat islets subjected to stimuli similar to those involved in the primary rejection after transplantation.

Material and methods

Male Wistar rats of 250-350 g were used. They were kept under conventional housing conditions, fed with standard food (Panlab; Barcelona), subjected to automatic 12-hour light/dark cycles and a temperature of 22±2 ºC.

All experiments were performed by observing the Law 32/2007 for the care of animals in their use, transportation, testing and sacrifice, in compliance with animal welfare regulations in force in the European Union.

Isolation of islets

The islets were obtained after dissection and selective pancreatic duct cannulation in rats using an Abbocath 16Fr catheter. Subsequently, and after proximal and distal duodenal clamping, a solution of collagenase P 2 mg/ml (1213865, Roche Diagnostics, Laval, Canada) was infused to complete distension of the pancreatic capsule. The enzymatic digestion process was then carried out in closed chambers at 37 °C (Bio-Rep, Miami, USA), and stopped by the addition of Hank's solution at 4 °C (PAA Laboratories GmbH, Linz, Austria) for 4 consecutive cycles to obtain at least 100,000 islet equivalents (IE). A 20 mg/ml solution of dithizone (Sigma-Aldrich, St.Louis, USA) was then added to the medium to view the red staining of the islets, with their viability checked every 2 min. A Ficoll gradient (Sigma Chemical Co, St. Louis, USA) was then performed using a COBE cell processor (Denver, USA). The remaining islets were then collected manually under direct vision for storage in groups of 300 EI.

Islet culture and addition of pro-inflammatory stimuli

The islets were cultured for 24 h in RPMI-1640 medium (PAA Laboratories GmbH, Linz, Austria) at 37 °C in a micro-atmosphere of 95% O_2/5% CO_2. Both 11.11 mm glucose and 0.1% bovine serum albumin (Sigma Chemical Co, St. Louis, USA) were added to the medium. The pancreatic islets were stimulated by adding 50 IU/ml of interleukin-1, (IL-1, Boehringer Mannheim GmbH, Germany) and 1000 IU/ml of interferon-gamma (γ-IF, Boehringer Mannheim GmbH, Germany) to the culture medium. As treatment, tacrolimus (FK-506) was used at low doses (5 ng/ml, Fujisawa, Osaka, Japan) in the same culture medium.

Experimental groups

Experiments were conducted in groups of five different rats and 300 EI samples collected manually under microscopic vision were obtained from each. The experimental groups were: control, treated only islets, stimulated only islets and stimulated and treated islets.

Determinations performed

The 300 EI samples were homogenised by sequential centrifugation (600 gx2, 5,500x2, 2,400 g; 10,000 g) and cytosolic and microsomal material separated. To evaluate the occurrence of apoptosis, the nucleosome values (intra-islet content; IU/islet) and Bcl-2 protein (IU/islet) were determined by commercial kits for ELISA/spectrophotometry (Calbiochem, La Jolla, USA & Canada). To assess the impact on the generation of de novo oxidative stress, nitric oxide (NO) and lipoperoxides (LPO) values were determined. The NO determination (fmol/mlx24 h) was performed using the Griess reaction (absorbance at 550 nm). LPO level measurement (fmol/islet) was obtained by ad hoc spectrophotometric kits (absorbance at 533 nm, Calbiochem, La Jolla, USA and Canada).

Statistical analysis

Statistical analysis of the results was carried out using the software package Statgraphics Plus 5.1 (Statpoint Inc, USA) for Microsoft Windows NT. Comparison of the group averages was conducted using factor analysis of variance (ANOVA) and Scheffé's test post hoc. The results were expressed as averages±SEM. Values of $P<.05$ were considered significant and highly significant at $P<.01$.

Results

The addition of low-dose tacrolimus to the culture medium of rat islets stimulated with IL-1 and IF-γ significantly reduced the increase in markers of cell damage observed after exposure to cytokines.

A. Generation of oxidative stress
   Stimulation with IL-1/IF-γ correlated with a very significant increase ($P<.01$) in the concentrations of oxidative stress markers (LPO and NO). In both experiments, the addition of tacrolimus completely reversed this effect, such that the values of these markers decreased to values comparable with the controls. There were no significant variations in the concentration of LPO and NO in islets treated only with drugs (Figures 1 and 2).
B. Activation of apoptosis
   Stimulation with pro-inflammatory mediators resulted in an increase in apoptosis markers, such that it very significantly increased the presence of nucleosomes and reduced the concentration of Bcl-2 ($P<.01$). In both cases, the addition of tacrolimus to the culture medium reduced this effect. The decrease was very significant for nucleosomes compared with islets in the stimulated group ($P<.01$), but did not reach levels comparable with the controls ($P<.05$). When levels of Bcl-2 were determined, the drug completely reversed the decrease observed after the addition of pro-inflammatory cytokines, such that the values were comparable to the controls. As with the oxidative stress markers, there were no significant variations in the levels of nucleosomes or Bcl-2 in islets treated only with drugs (Figures 3 and 4).

Discussion

PIT is a good therapeutic alternative in some DM1 cases.[1] Among the different adverse events, that have made it difficult to achieve favourable clinical outcomes on a regular basis, primary rejection is still a source of dysfunction and a very important cause of loss of islets.[13] This means that new prevention strategies are required using mainly cytoprotective agents capable of neutralising the associated physiopathologic events. The use of tacrolimus in PIT has always raised concerns due to its diabetogenic and pro-apoptotic properties, known beforehand. However, its inclusion at low doses in most immunosuppression protocols, based on the experience of the Edmonton group in 2000,[14] suggests a beneficial effect on one of the rejection types involved in PIT. Its cytoprotective

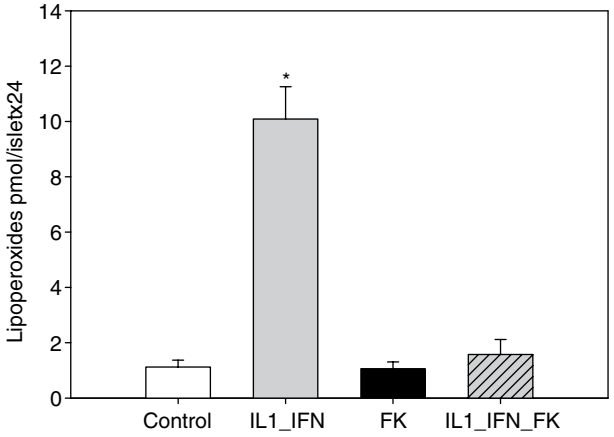

Figure 1 – The increase in the production of lipoperoxides in islets exposed to pro-inflammatory cytokines is counteracted by the addition of tacrolimus at low dose. The graph shows the production of lipoperoxides (mean+SEM, pmol/isletx24 h) in rat islets cultured in RPMI media and stimulated with IL-1 (50 IU/ml) and IF-γ (1000 IU/ml) in the presence of low-dose tacrolimus (5 ng/ml). The addition of cytokines induces a significant increase in the production of lipoperoxides, which is counteracted by adding the drug (*P<.01 versus all other groups).

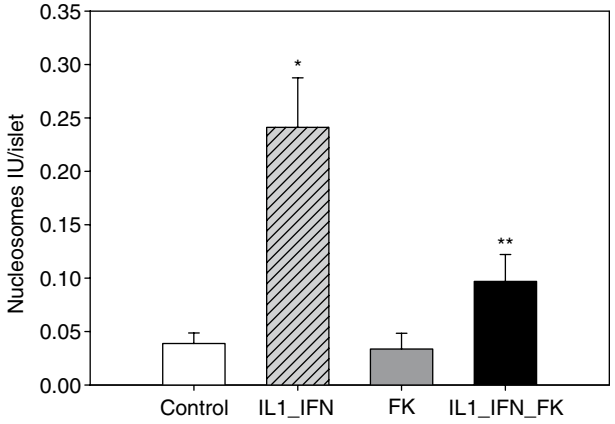

Figure 3 – Increased nucleosomes in islets stimulated with pro-inflammatory mediators is partially offset by the addition of tacrolimus to the media. In rat islets cultured in RPMI media, the addition of IL-1 (50 IU/ml) and IF-γ (1000 IU/ml) results in a significant increase in nucleosome levels (mean+SEM; IU/islet) that significantly decreases in the presence of low-dose tacrolimus (5 ng/ml). The anti-apoptotic effect of the drug is partial, since the values do not fall to levels similar to the control group (*P<.01 versus all other groups, **P<.05 versus controls). IU indicates international units.

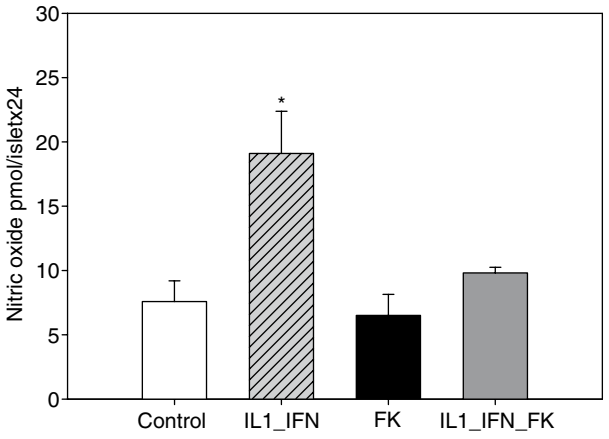

Figure 2 – Stimulation of pancreatic islets with L-1+IF induces an increase in nitric oxide production neutralised in the presence of low-dose tacrolimus. The bar graph reflects the significant increase in nitric oxide production (mean+SEM, pmol/isletx24 h) in rat islets cultured in RPMI media exposed to IL-1 (50 IU/ml) and IF-γ (1000 IU/ml). This is neutralised on adding tacrolimus (5 ng/ml) to the media, restoring values comparable with the control group (*P<.01 versus all other groups).

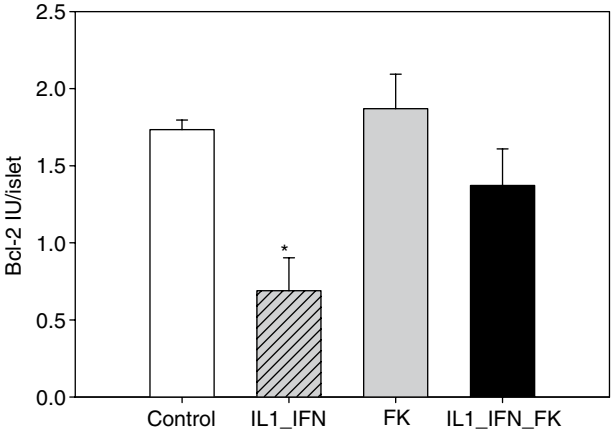

Figure 4 – Tacrolimus reverses the decrease in Bcl-2 in rat islets after stimulation with IL-1 and IF-γ. The levels of the anti-apoptotic protein Bcl-2 (mean + SEM; IU/islet) in rat islets cultured in RPMI media decrease significantly in the presence of IL-1 (50 IU/ml) and IF-γ (1000 IU/ml). This effect is completely neutralised in the presence of low-dose tacrolimus (5 ng/ml; *P<.01 versus all other groups). IU indicates international units.

effects (anti-inflammatory, antioxidant and anti-apoptotic) in various experimental models[15,16] could explain the beneficial effect due to its interference with the sequence of events involved in primary rejection.

The present study shows an in vitro protective effect for tacrolimus at low doses on rat islets stimulated with the pro-inflammatory cytokines normally involved in the primary rejection after PIT. Both the generation of oxidative stress

and apoptosis were effectively neutralised in the presence of the drug. Our group obtained comparable results by using human islets stimulated with IL-1 and IF-γ as well as other inflammatory mediators such as LPS.[17] These in vitro results must be interpreted with caution as they are murine islets, and it may not be possible to extrapolate them for effects on primary rejection after actual PIT. Similarly, in vivo assessment of the tacrolimus effect via insular transplant would allow the study of the overall effect of the drug in rejection after PIT.

The contribution of oxidative stress to the development of primary rejection after PIT is a well known phenomenon that has its origin in very different stimuli involving the very process of obtaining the donor, isolation and the inflammatory response of the receptor endothelium.[4] In this study, tacrolimus has a clearly antioxidant effect on the islets exposed to pro-inflammatory cytokines. Not much is known about the role of tacrolimus as an antioxidant, however, it has shown beneficial effects in ischaemia reperfusion and cerebral damage models.[18,19] The survival in ischaemia reperfusion models in mice improves after the use of tacrolimus after decreasing the inflammatory response, as it slows the production of reactive oxygen species, neutrophil infiltration and the in situ production of IL-1 and TNF-α.[20] In this case, the production of both NO and LPO was effectively neutralised. These results are a secondary phenomena blocking both the early and late inflammatory response as well as amplification. The release of NO is a second generation phenomenon which also has a potential mediator effect, with the lipid peroxidation and apoptosis occurring at the end of the cellular dysfunction pathophysiological sequence in the non-specific immune response. Regarding this fact, the use of blocking agents in the production of NO, such as NG-methyl-L-arginine (L-NMA) could help in reducing the oxidative stress associated with primary rejection after PIT.

In our experiments, the tacrolimus managed to reduce the pro-apoptotic response in conditions similar to the primary rejection. The drug significantly, but partially, reversed the negative effect of exposure to mediators after assessing the nucleosome and total values, regarding the decrease of anti-apoptotic factor Bcl-2. The presence of nucleosomes is a late apoptosis marker, reflecting morphological changes. The expression of Bcl-2 protein indicates the blocking of the apoptotic cascade in the cell. Constitutively, it is expressed in equilibrium with the other pro-apoptotic subfamily (Bax, Bad…),[21] such that the decline in levels as a result of the inflammatory mediators indicates a clear correlation with the loss of islets during the non-specific inflammatory response associated with the primary rejection in PIT.[22] The alterations in the proteins on the Bcl-2 group are related to the activation of the apoptosis mitochondrial pathway, usually in response to the arrival of "cell death signs", ie, it is an early phenomenon in programmed cell death. Thus, one might conclude that tacrolimus acts preferentially on apoptotic events where the mitochondrial pathway is the main one. This is because the effect on a late genetic marker, such as the nucleosomes (and the result of activating all apoptotic pathways), is partial. This differential effect was not observed in human islets exposed to similar stimuli.[17] The effects of tacrolimus on apoptosis are variable and are hardly studied in pancreatic islets. They are sometimes dose- and tissue-dependent, without affecting physiological patterns of apoptosis in animal models, and may even stimulate cellular regeneration.[23,24] While some of its immunosuppressive action is due to a pro-apoptotic effect on the T cells,[25] anti-apoptotic effects have been reported in the presence of pro-inflammatory stimuli.[10] Other authors have reported results different to ours in non-stimulated human islets using much higher doses of the drug on decreasing the levels of anti-apoptotic markers Bcl-2, Bcl-XL or XIAP26 gene transduction.[26] The anti-apoptotic effect of tacrolimus may have an ultrastructural explanation, since the proteins involved in its mechanism of action (FK-BP) interact with Bcl-2 protein by relocating it next to the mitochondria, where it exerts its anti-apoptotic effect.[27,28]

Given the various actions in apoptosis that the drug causes, it may be that such effects are not just tissue- and dose-dependent, but also dependent on the stimulation. So that the presence of the immediate immune response favours the cytoprotective and anti-apoptotic effects on non-immune cells at low doses.

The prevention of rejection in PIT requires consideration of pathophysiological events that are usually not as important in other types of transplants and, compared to those, cytoprotection strategies are necessary. Therefore, the in vitro antioxidant and anti-apoptotic effect of low-dose tacrolimus is a useful finding that could be influential when designing effective immunosuppression protocols in clinical PIT.

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Conflicts of interest

The authors declare they have no conflict of interest.

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