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Inhibitory action of the Interleukin 1ß over the cellular proliferation of smooth muscle cells cultivated from human saphenous veins

Ação inibitória da Interleucina - 1ß sobre a proliferação de células musculares lisas cultivadas a partir de veias safenas humanas

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Abstract

Objective: **The saphenous vein (SV) is an effective graft used in coronary artery bypass grafting, although, its patency can be affected by the development of atherosclerosis. We have developed an experimental study demonstrating the development of apoptosis in SV grafts cultivated under arterial hemodynamic conditions (AHC). The interleukin-1â expression was also elevated in these veins slices. The aim of this study is to evaluate the influence of interleukin-1â in the precocious proliferation of the cultures of primary smooth muscle cells (PSMC).**

Methods: **PSMC of 6 different human SVs were cultivated in Dulbecco's Modified Eagle Medium associated with bovine fetal serum. The control group was not treated with Interleukin-1â but treated groups were. Cellular proliferation (CP) was evaluated by measuring triple thymidine (3H), incorporated into the proliferated cells.**

Results: **The treatment with Interleukin-1â decreases cellular proliferation. The control group presented 100 ± 4.5% of CP. In the treated groups the quantity of Interleukin-1 administered and the respective levels of CP observed were: 0.1 ng/mL – 112 ± 0.7%; 1 ng/mL – 83 ± 4.7%; 10 ng/mL – 69.1 ± 3.8% and 100 ng/mL – 76.3 ± 10.9% (p < 0.01).**

Conclusion: **We can conclude that the administration of increasing quantities of Interleukin-1â inhibits the proliferation of PSMC cultivated from human SVs. This suggests that the precocious process of apoptosis observed in the SV grafts exposed to AHC can be related to the action of this Interleukin.**

Descriptors: **Cell Culture. Saphenous vein. Myocardial revascularization. Gene Expression. Interleukin-1**

Resumo

Introdução: **A veia safena é um enxerto coronário eficiente. Porém, sua patência pode ser limitada por desenvolvimento de aterosclerose. Estudos experimentais "ex vivo", por nós realizados anteriormente, demonstraram apoptose (ensaio de TUNEL) em veias safenas humanas cultivadas sob pressão arterial por 24 horas. Nessas veias safenas, a expressão gênica**

1 - Work performed in the Instituto do Coração – Incor/ FMUSP – São Paulo, SP.

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da Interleucina-1ß avaliada por RT-PCR em tempo real, também mostrou-se elevada. Não há ainda consenso sobre a ação moduladora das citocinas sobre proliferação/apoptose das células musculares lisas das veias safenas.

Objetivo: **Avaliar a influência da Interleucina -1ß na proliferação inicial de cultura de células primárias de músculo liso de veia safena humana.**

Método: **Foram cultivadas células primárias de músculo liso de seis diferentes veias safenas humanas (em triplicata). O meio de cultura foi o DMEM, suplementado com 10% de soro fetal bovino. O grupo controle não recebeu Interleucina - 1ß. Nos demais grupos, as células cultivadas receberam, respectivamente, 0,1; 1; 10 e 100 ng/mL de Interleucina - 1ß. A proliferação celular foi avaliada através da quantificação de timidina triciada [^{3}H], incorporada às células recém-proliferadas.**

Resultados: **O tratamento com Interleucina - 1ß diminuiu a proliferação celular, a saber: Grupo controle (sem Interleucina - 1ß): definiu-se esse grupo como apresentando 100±4,5% de proliferação celular. Nos demais grupos, a quantidade de Interleucina - 1ß administrada e a proliferação celular aferida foram, respectivamente, 0,1 ng/mL:112±0,7%; 1 ng/mL:83,8±4,7%; 10 ng/mL:69,1±3,8%; 100 ng/mL:67,3±10,9%; (p<0,01).**

Conclusões: **Estes resultados indicam que a administração de quantidade crescente de Interleucina - 1ß inibe a proliferação de células primárias de músculo liso, cultivadas a partir de veias safenas humanas. Isso sugere que o processo de apoptose, observado já em fase precoce (um dia) de exposição do enxerto venoso a regime pressórico arterial, pode estar relacionado à ação dessa citocina.**

Descritores: **Veia safena. Revascularização miocárdica. Expressão gênica. Cultura de células. Interleucina-1.**

INTRODUCTION

Coronary artery bypass grafting using saphenous vein grafts is a surgical procedure still employed to reestablish the coronary blood flow. In spite of the effectiveness of the procedure, these vessels may undergo a degenerative process, in particular due to atherosclerosis, affecting future surgical results.

Recently, we developed an "ex-vivo" culture system which mimics the conditions in which the saphenous vein is submitted when implanted into the coronary arterial position [1]. Additionally, using a "cDNA microarray" platform, it was possible to identify a set of genes which may contribute to the degeneration of the saphenous graft and with this, to initiate studies about the molecular alterations involved in this process [1]. Among the identified genes, the functional role of Interleukin-1 â is being studied.

Similar to other cytokines, different types of action are being attributed to Interleukin-1 â, not only meditation in inflammatory reactions.

In this work, our aim was to evaluate the influence of Interleukin-1 â in the proliferation of smooth muscle cells of the saphenous veins.

METHOD

Primary culture of saphenous vein smooth muscle cells

Removal of the endothelial layer from the fragments of human saphenous vein was achieved by mechanical attrition. Subsequently, these fragments were cut into small pieces and placed on a culture plate treated with 0.3% gelatin. After approximately one week, growth of smooth muscle cells was observed, which were amplified and characterized with anti-â-actin antibodies. The cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium - Invitrogen) culture medium, supplemented with 10% bovine fetal serum.

Analysis of cellular proliferation

The smooth muscle cells of the human saphenous vein were cultivated in [^{3}H]thymidine. Subsequently, the "in vitro" cellular proliferation was identified by analyzing the incorporation of [^{3}H]thymidine in the DNA of the cell. The quantity incorporated was determined by the method of precipitation using trichloroacetic (TCA) acid.

The experiment was then performed on 24-well plates in which 2 x 10^4 to 4 x 10^4 cells were cultivated. The cells were kept for three days in 0.5% bovine albumin (BSA), with the

aim of synchronizing the cell cycle. Following this, the cells were treated with the desired stimuli (Interleukin-1 â for 48 hours). A control group did not receive Interleukin-1 â. In the other groups the cultivated cells received 0.1, 1, 10 and 100 ng/mL of Interleukin-1 â respectively. Twenty-four hours before the end of the experiment, 10 □Ci/□L of [^{3}H]thymidine was added to the culture. After precipitation with trichloroacetic acid, liquid scintillation was measured.

This study was approved by the Ethics Commission of the Heart Institute of the Hospital das Clinicas, Medical School of the São Paulo University.

Statistic analysis

The data were analysed by ANOVA and a p-value < 0.05 was considered significant.

RESULTS

Interleukin-1 â reduced the incorporation of [^{3}H]thymidine in the DNA of the cultivated cells depending on the dose as follow: Control group (without addition of Interleukin-1â) considered as base 100 ± 4.5% of cellular proliferation. In the other groups, the quantity of Interleukin-1 â administrated and the cellular proliferation recorded were: 0.1 ng/mL and 112 ± 0.7%; 1 ng/mL and 83.8 ± 4.7%; 10 ng/mL and 69.1 ± 3.8%; 100 ng/mL and 67.3 ± 10.9% respectively (N= 6; p-value<0.01) – (Figure 1).

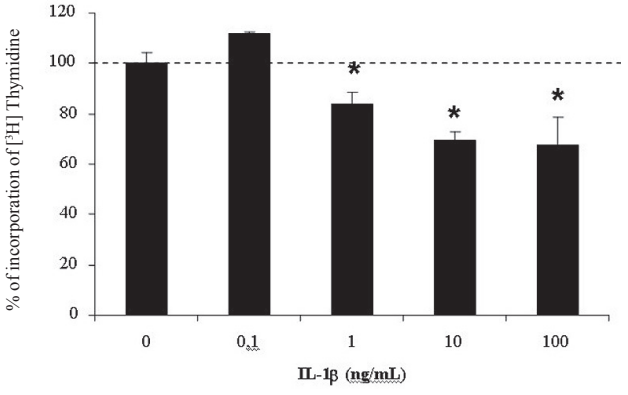

*Fig. 1 - Dose-response curve of the incorporation of [^{3}H]thymidine in the DNA of primary smooth muscle cells of human saphenous vein treated with Interleukin-1 â. The cells were incubated for 3 days with 0.5% BSA to synchronize the cellular cycle, followed by 48 hours of stimulus in the presence of 10% bovine fetal serum. The [^{3}H]thymidine (10 uCi/mL) was added in the last 24 hours. Each bar represents the mean ± standard deviation of 6 experiments in triplicate. The data were normalized in respect to the incorporation of [^{3}H] thymidine in cells without treatment (dotted line) – (*indicates p<0.05)*

COMMENTS

Interleukin-1 â is a recognized cytokine that actively participates in inflammatory processes [2]. Cytokines are observed together with growth factors in atherosclerotic lesions, interacting with vascular cells resulting in the inflammatory process [3]. The disease of venous grafts presents characteristics similar to the development of atherosclerosis in coronary arteries, but the role of cytokines in the pathogenesis of the graft lesion is not very clear.

Recently, Christiansen et al. [4] reported that cytokine levels, including Interleukin-1 â, are higher in saphenous vein grafts when compared to atherosclerotic coronary arteries. This suggests that a more pronounced inflammatory process occurs in the grafts than that that is seen in coronary arteries, contributing to a faster evolution of atherosclerosis.

In experimental models, it was demonstrated that Interleukin-1 â is increased both in the endothelium and in the medial layer during the two first two days after grafting epigastric veins into the femoral arteries of rats [5]. This agrees with the data which we recently reported [1] where the expression of Interleukin-1 â increased by l.95 times in human saphenous veins cultivated for 24 hours in arterial pressure conditions when compared with saphenous vein segments cultivated in venous conditions (Figure 2).

Immunohistochemical analysis demonstrated that this increase in expression is accompanied by a tendency of enhanced staining for Interleukin-1 â in saphenous veins cultivated in arterial hemodynamic conditions suggesting that the increase of the expression of Interleukin-1 â are reflected in the increase of its protein (Figure 3).

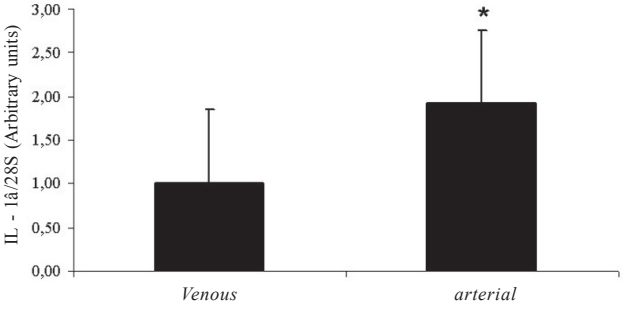

*Fig. 2 - Expression of Interleukin-1 â in human saphenous vein cultivated in venous and arterial regime for 24 hours. Measurement was performed by RT-PCR in samples of 16 people. (*indicates p<0.05 in relation to the venous condition)*

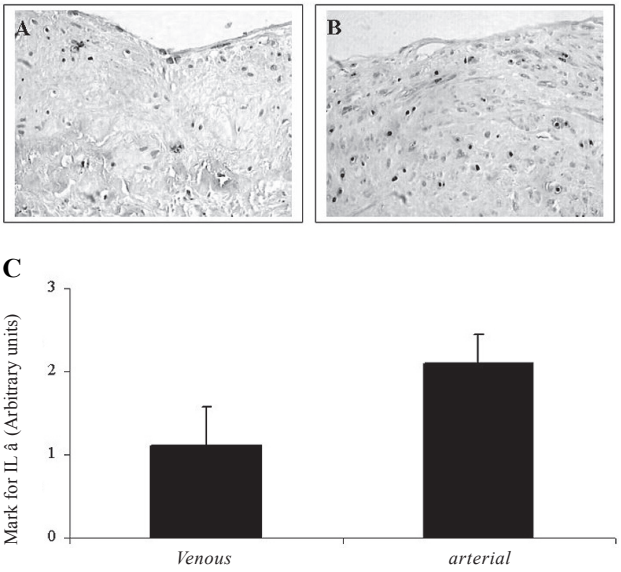

Fig. 3 – Immunohistochemical for Interleukin-1 â in segments of human saphenous vein cultivated in venous (A) and arterial (B) conditions for 24 hours. (C) Measurement of the markers of Interleukin-1 in samples from 9 patients. Analysis was made by an observer blind in respect to the cultivation conditions, with points of from 0 to 3 given proportionally to the amount of the marker. (Magnification 200x)

It is already well known in experimental models that, during the process of the 'arterialization' of venous grafts, two important phases occur. Initially, a precocious degenerative process is seen, in particular involving the apoptotic process and consequently, a loss of cellular density. This is the result of the stress to which the wall of the vein is submitted when placed under arterial conditions. This hemodynamic regimen of greater blood flow and higher pressure causes stretching of the grafted vein wall, a structure that is not prepared for these conditions [6,7]. The second stage is a proliferative process that occurs in response to the first stage [8]. Cellular loss and the mechanical stress on the wall of the vein promote alterations so the smooth muscle cells start to proliferate to strengthen their structure and to adapt to the new imposed hemodynamic conditions.

Our previous data [1] showed an increase in apoptotic events in cultivated human saphenous veins under arterial pressure conditions for 24 hours (Figure 4). This increase is followed by an increase in Interleukin-1 â (Figures 2 and 3). These data highlighted the necessity to test the hypothesis that Interleukin-1 â may be participating in the process of apoptosis and the loss of cellular density observed in the first stage of the 'arterialization' process of the venous graft.

Thus, the primary culture of smooth muscle cells of the human saphenous vein were treated with increasing concentrations of Interleukin-1 â and the effect on the cellular density was measured (Figure 1). These data indicate that Interleukin-1 â interferes in the density of smooth muscle cells of the human saphenous vein, reducing the cellular proliferation, suggesting that this may be related to the precocious degeneration observed in veins submitted to arterial hemodynamic conditions. Future studies in this research line will include new tests which will evaluate the direct relation between Interleukin-1 â and the apoptotic process.

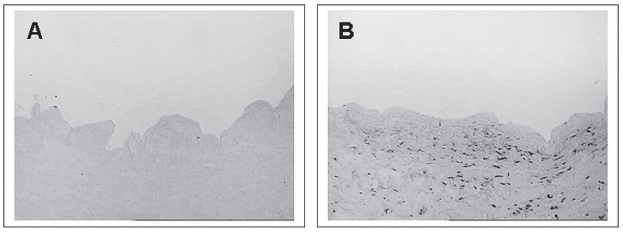

Fig. 4 – TUNNEL trial to evaluate the apoptotic process in saphenous vein segments cultivated for 24 hours in venous (A) and arterial (B) hemodynamic conditions. (Magnification 200x)

CONCLUSIONS

These results indicate that the use of increasing quantities of Interleukin-1 â inhibits the proliferation of primary smooth muscle cells cultivated from human saphenous veins. This suggests that the process of apoptosis, previously observed in the early period (one day) of exposure of the venous graft to arterial pressure conditions, may be related to the action of this cytokine.

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DISCUSSION

Commentator – Prof. Domingo M. Braile
São José do Rio Preto, SP

1- This work apparently contradicts published findings, as Interleukin-1 â is an inflammatory mediator which favors cellular proliferation, as suggested by references cited in the work (References 2 and 4). The authors of Reference 5 even confirmed that the expression of Interleukin-1 â was seen at a very early stage (peak on the second day) and was thought to be involved in the process which causes cellular proliferation. How does the author explain the fact to have found a reduction of the cellular proliferation with progressively higher concentrations of Interleukin-1 â? I also ask if the evaluation, as it was made after only 48 hours, may also have been badly interpreted taking in consideration that in Reference 5 the highest peak of cellular proliferation was seen in two weeks?

2 - In the methods section, the authors inform us that the statistical study was performed using ANOVA (variance analysis). This method is only recommended to compare the means of 3 or more groups. Thus, it can only be utilized with variables that allow calculation of the mean and standard deviation. For this, the variable needs to be continuous and have a Gaussian distribution. This is not the case of the variables utilized in the current study. The authors compared the percentages of the incorporation of thymidine as indicative of the level of cellular proliferation in respect to a control group. The variables utilized (cell count or estimation of the level of proliferation) would be, in the maximum, a small quantitative variation and, thus, a non-parametric test should be utilized, as is the case the Kruskal-Wallis with the post-test of multiple comparisons of Dunn, if some difference were detected. It is important to remember that even if it were possible to use ANOVA, the groups show very different standard deviations as can be verified using Bartlett's method, again invalidating the utilization of a parametric method and indicating the necessity of a non-parametric test. The authors must agree that the conclusions may be different using an appropriate statistical test for the study.

Answers to the commentator

1 - Certainly, Interleukin-1â is classically known as an inflammatory mediator that leads to cellular proliferation. On the other hand, it is known that cytokines have multiple actions. Hence, Interleukin-1 â is cited as an apoptotic factor for vascular cells [1]. As discussed in the work, the 'arterialization' process of venous grafts occurs in two stages: the first one, an apoptotic process with a loss of cellular density is observed and the second stage corresponds to the proliferative process which happens in response to the first stage. Probably, this decellularized vein becomes vulnerable in the initial stage and it is possible that it deteriorates over the next months or years. In our previous study, it was observed that the quantity of Interleukin-1 â is higher in the saphenous vein after 24 hours of cultivation in arterial hemodynamic conditions; additionally, the presence of apoptosis was verified, as has already been demonstrated in the literature. The fact that the treatment of the saphenous vein smooth muscle cells with Interleukin-1 â has led to a reduction in the cellular proliferation suggests that Interleukin-1 â may participate directly in the initial degenerative process of saphenous vein grafts. It is known that the prevention of the initial apoptosis, observed in the venous grafts in the arterial bed, leads to a regression of hyperplasia observed later [2]. Our hypothesis is that, if Interleukin-1 â is directly participating

of the apoptotic process, its inhibition may prevent the subsequent hyperplasia process, as this is a consequence of the first stage. The work of Faries et al. (Reference 5 in our work) used as an example by the reviewer reinforces our hypothesis. These authors demonstrated that Interleukin-1 â is higher within the first few days of grafting and the hyperplasia is a later event with a peak after two weeks. Thus, our aim was to directly test this hypothesis, verifying if there is any relation between the increase of Interleukin-1 â and apoptosis. As this hypothesis is true, the inhibition of Interleukin-1 â in saphenous vein grafts prior to the revascularization process, may be an important intervention to improve the patency of these grafts.

2 - As the Commentator said, the statistical analysis used in our work is recommended for a comparison of the means of three or more groups. In our study the means and standard deviations of six experiments with five groups are being compared. The data are presented as percentages because the level of [^{3}H]thymidine incorporation were adjusted in relation to the values obtained in the control group (without treatment with Interleukin-1 â). Thus, the data are continuous and have a Gaussian distribution.

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