Precocious structural and molecular (cDNA) changes in the human saphenous veins cultivated under arterial hemodynamic conditions

Alterações estruturais e moleculares (cDNA) precoces em veias safenas humanas cultivadas sob regime pressórico arterial

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Abstract

Objective: The saphenous vein (SV) used in coronary artery bypass grafting is submitted to elevated and continuous shear stress. Occlusion of the grafts can occur in response to the new hemodynamic conditions. The aim of this study is to compare the precocious structural and molecular (cDNA) changes in saphenous veins grafts submitted to low pressure hemodynamic conditions versus systemic hemodynamic conditions.

Method: Forty sections of SV were cultivated "ex-vivo" under venous hemodynamic conditions (VHC) (without pressure, flow: 5 mL/min) and under arterial hemodynamic conditions (AHC) (pressure: 80 mmHg, flow: 50 mL/min). The following variables were analyzed: cellular viability (MTT assay) cellular density (Hoechst 33258 staining) and apoptosis (TUNEL assay), before the procedure and one, two and four days after the procedure. "cDNA microarray" analysis of the SV sections was used to determine the precociously changed molecular targets in the veins cultivated under arterial conditions. The identification of these targets was achieved using a RNA homogenized pool of these vein sections, interacting on slides with 16,000 pre-determined human genes (Agilent Technologies slide). The genes with changed expressions were verified by real time PCR in the veins of 16 patients.

Results: There was a gradual reduction in the cellular density and in the tissue viability in the saphenous veins cultivated under AHC, whereas no alterations were observed in the saphenous veins cultivated under VHC of up to four days. In the AHC group there were signs of a cellular apoptotic process (positive – TUNEL) from the first day after cultivation. In the VHC group these alterations were not observed. Although the cellular density was the same in the veins submitted to arterial conditions, after 24 hours of cultivation, many cells already showed signs of the apoptotic process. The Oncogene 3 and the Interleukin 1β were the most common sites with alterations identified in this research. The Oncogene 3 expression was elevated in 11 (68.7%) of the veins cultivated under AHC, and the Interleukin 1β expression was elevated in 9 (56.2%) of these vein sections (p<0.05).

Conclusion: The "ex-vivo" study model was able to mimic events that occur "in vivo" by SVs utilized in the coronary artery bypass grafting. In the AHC group precocious loss of cellular viability (apoptosis) and significant elevation in the Oncogene 3 and Interleukin 1β gene expressions were observed. The long-term follow up of these patients is important to determine the real effect of these immediate changes in the patency of the vein grafts.

INTRODUCTION

Coronary artery bypass grafting is one of the most common surgical procedures owing to its efficiency in the treatment of coronary insufficiency, improving the symptoms of angina and increasing the life expectancy of the patients.

Autogeneic segments of saphenous veins are the most frequent grafts utilized in coronary artery bypass grafting with the aim of re-establishing the coronary blood flow. Despite of the great effectiveness of the procedure, these vessels can, with the time, suffer from a degenerative process, especially of an atherosclerotic origin, compromising the surgical results. During the first year after coronary artery bypass grafting, as many as 15% of the saphenous vein grafts can become occluded. In the subsequent five years, this rate drops to 1% to 2% per year; at the end of 10 years, only 60% of the grafts remain patent, with only 50% of these free of atherosclerotic injury [1].

The high rates of evolution to atherosclerotic disease of the saphenous vein grafts and the native coronary arteries cause significant clinical repercussions. As many as 20% of the patients can present with anginal events during the first year after the operation and 4% of these present with recurrence of the symptom during the subsequent five years [2]. As a consequence, approximately 31% of the patients need to be submitted to new CABG procedures either using percutaneous or surgical procedures within 12 postoperative years of the first operation.

The principal factors related to the failure of the SV grafts are: surgical trauma during the dissection and preparation of the graft, an adaptation response of the graft to the arterial blood flow and the activation of inflammatory and coagulation responses [1].

The venous graft is submitted to high and continuous shear stress due to the effect of the systemic arterial blood pressure, leading to a greater radial deformation and a greater shear stress force. This hemodynamic variation acts on the endothelium function, influencing the vascular tonus, hormonal secretion and the gene regulation of several proteins [3].

In this work, a system that enables the SV to be mimetized the events iniciais sofridos “in vivo” pela VS utilizada na RM. No grupo CHA houve perda de viabilidade precoce das células (apoptose) e elevação significativa nas expressões gênicas do Oncogene 3 e da Interleucina 1B. O seguimento em longo prazo desses pacientes poderá esclarecer o real papel dessas alterações precoce na perviabilidade desses enxertos venosos.

pressures. The objective of this study was to investigate the occurrence of cellular and gene (cDNA) alterations owing to the different hemodynamic regimes in force.

METHOD

Saphenous vein
Forty segments of SV were obtained from patients who were electively submitted to CABG. Histological sections were taken and extraction of the RNA and proteins was achieved from these samples. This research was approved by the Ethics Committee of the Hospital das Clinicas, FMUSP, Brazil.

Culture of the saphenous vein
The method of culture of the SV was adapted from the artificial capillary system (Cellmax – Spectrum Laboratories), substituting the cartridge containing the capillaries for a cube containing the SV sample (Figure 1). This was attached to a pressure regulator that allowed independent control of the pressure and of the flow (Figure 2).

The 40 SV segments were cultivated ex vivo under venous hemodynamic conditions (without pressure, flow = 5 mL/min) and under arterial hemodynamic conditions (pressure = 80 mmHg, flow = 50 mL/min).

The cellular viability (MTT stain), cellular density (Hoechst 33258 stain) and apoptosis (TUNEL test) were analyzed before initiation of the trial and one, two and four days after the procedure. After this period, venous segments were taken for the histological analysis. The cDNA microarray was obtained utilizing RNA of the tissues cultivated for one day.

Histological analysis
The SV segments were prepared in 4% formaldehyde buffer for a period of 24 to 48 hours. After this period, the samples were embedded in paraffin and cut at 5 µm using a microtomy. The sections were then stained using hematoxylin-eosin, enabling evaluation of the tissue integrity with blue stain in the nucleus and pink in the cytoplasm and with Verhoeff-van Gienson (VVG) to see the elastic laminas.

The cellular nuclei were also studied using Hoechst stain to assess the cellular density and TUNEL test to verify apoptotic processes.

Nuclear staining with Hoechst
The cellular nuclei were stained with Hoechst 33258 (Sigma Chemical CO, St. Louis, MO). The sections of tissue were deparaffinized and treated with 0.5% triton X-100 for 15 minutes at 20 ºC. The staining reaction was achieved with 20 µg/mL of Hoechst 33258 diluted in a reaction buffer (mmol/L): NaCl 137, KCl 5, Na2HPO4 0.4, NaHCO3 4, glucose 5.5, MgCl2 2, EGTA 2 PIPES, pH 6.1. Visualization of the sections was performed utilizing a fluorescence microscope.

TUNEL test
The fragmentation of the DNA was observed using In Situ Cell Death Detection kit, AP (Roche Molecular Biochemicals, Mannheim, Germany) according to the instructions of the manufacturer. The sections were deparaffinized and permeabilized with 0.5% triton X-100 in 0.1% sodium citrate. The reaction with deoxynucleotide terminal transferase and the conversion of alkaline phosphatase were performed and the sections were studied under a light microscope.

Staining with MTT
To verify their viability, the SV sections were incubated for 1 hour at 37 ºC with 0.5 mg/mL of methyltiazol tetrazolio (MTT). MTT has the property of staining the active mitochondria of live cells dark blue.
cDNA microarray

The whole RNA was extracted from the samples using the method that utilizes the reagent Trizol (Gibco BRL). The sample was treated with DNase aiming at eliminating possible contamination of genomic DNA. From the RNA samples cDNA was produced, marked (Cy3 and Cy5) and hybridized on slides with a panel of human cDNAs. The fluorescent signals were read and the differences in intensities were calculated for the two populations of transcripts.

The 3DNA™ Submicro™ EX Array Detection Kit (Genishere Inc.) was utilized to obtain the marked cDNAs according to the instructions of the manufacturer. First, reverse transcription of 4 µg of whole DNA was made utilizing dT oligo containing a specific capture sequence for each fluorescent 3DNA molecule. The resulting cDNAs were hybridized on a slide with two arrays with 16000 human cDNAs (Agilent Technologies) at 55 ºC for 15 hours. Subsequently, hybridization of the fluorescent 3DNA was performed for the formation of the cDNA/3DNA complex.

Measurement of the gene expression utilizing RT-PCR

RT-PCR is a combination of PCR with fluorescent techniques. Its real time determination was achieved utilizing the SYBR® Green PCR Master Mix kit (Applied Biosystystems). Thus, the quantity of the PCR product in the reaction is directly proportional to the generation of a fluorescent signal. The intensity of the fluorescence is monitored after each PCR cycle, which enables the analysis during the exponential phase of the reaction. To obtain accurate reproducible results, the efficiency of the reaction should be close to 100%, that is, the quantity of DNA duplicates during each cycle of the exponential phase. The standard curve was constructed and the slope of the straight line enabled the calculation of the efficiency of the reaction. Additionally, a dissociation curve was drawn to verify the purity of the product formed, that is, if there was more than one type of amplification formed within the reaction. The dissociation curve evaluated the dissociation of the double ribbon of DNA with the increase of the temperature and the product present in the reaction is considered pure when only one temperature of dissociation is evidenced.

Aiming at identifying molecular targets altered at an early stage in the cultivated veins under arterial conditions, a microarray analysis of the cDNA of the SV segments cultivated for one day was performed. Identification of these targets was achieved using a homogenized pool of RNA of these venous segments, interacting by homology on a slide containing 16000 predetermined human genes (Agilent Technologies slide). The most altered expressions of the cDNA of the veins of each group were then identified by pre-established staining and researched in the veins of 16 different individuals.

RESULTS

Characterization of the ex vivo system of the human saphenous vein culture

Staining by hematoxylin-eosin showed that the number of stained nuclei gradually diminished in the SV cultivated for one to four days in the arterial conditions. On the other hand, no alteration was seen in the segments cultivated in the venous conditions (Figure 3). This same result was verified by staining of the nuclei with Hoechst 33258. The cellular density gradually reduced in the segments cultivated for one to four days in the arterial conditions, whilst the cellular density remained unaltered in those cultivated in the venous conditions (Figure 4).

Aiming at evaluating the tissue viability, staining with MTT was performed as a control in fresh tissue segments (taken before the start of the culture process) and in dead segments (exposed to low temperatures). The fresh segments were stained completely and the dead fragments did not present any staining by MTT whatsoever (Figure 5).

Based on this property of MTT staining, our SV samples were stained to test the tissue viability. The SV segments cultivated for one, two and four days in venous conditions were stained.
hemodynamic conditions presented viable cells over the entire vascular wall. The segments cultivated for four days in arterial conditions, however, presented with an absence of staining by MTT in some portions of the vascular wall, suggesting cellular death in these regions (Figure 6).

Finally, the TUNEL test was performed to verify if the reduction in cellular density and cellular viability observed in the SV samples cultivated for four days in arterial conditions occurred due to the apoptotic process. Significant fragmentation of the DNA of the SV cultivated in venous conditions was not confirmed. The veins cultivated in arterial conditions, however, presented with a large quantity of apoptotic cells when cultivated for one or two days (Figure 7).

Study of the molecular alterations of the saphenous vein cultivated in arterial hemodynamic conditions – expression of interleukin-1 beta (IL-1β) and of the GRO3

Fig. 4 - Staining of the saphenous vein with Hoechst 33258 for 1 (A,B) and 4 (C,D) days in venous (A,C) and arterial (B,D) hemodynamic conditions (magnification 100x). Note the maintenance of the cellular density in the saphenous veins of the group submitted to venous conditions (A,C), while there is a reduction of this density in the group submitted to arterial conditions (B,D)

Fig. 5 - Control staining of the saphenous vein with MTT to establish the tissue viability: A – fresh saphenous vein that acquires homogenous staining. B – Dead tissue of the saphenous vein (after refrigeration) without staining by MTT

Fig. 6 - Study of the cellular viability by MTT staining of saphenous vein segments cultivated for 1 (A,B) and 4 (C,D) days. Note the presence of viable cells in all the vascular wall when cultivated in venous conditions (A,C). The veins of the group cultivated in arterial conditions (B,D) present with portions without stain suggesting cellular death in these regions

Fig. 7 - TUNEL test for the study of cellular apoptosis in saphenous veins cultivated for 1 (A,B) and 4 (C,D) days in venous (A,C) and arterial (B,D) hemodynamic conditions (magnification 100x). Only the veins cultivated in arterial conditions presented with a great amount of cellular apoptosis
The molecular alterations of the SV cultivated for one day under arterial hemodynamic conditions observed using cDNA microarray are presented in Table 1.

Among the genes the present with a differentiated expression in the SV cultivated for one day in arterial conditions, basically the cytokines are the most evidenced, oncogene expression in 11 (68.7%) of these vein segments (p<0.05).

Table 1. Genes selected in the cDNA microarray experiment performed on saphenous veins cultivated in venous (flow = 5 mL/min) and arterial (flow = 50 mL/min; pressure = 80 mmHg) conditions for 1 day. The slide had two arrays (A and B) containing 16,000 human cDNAs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>“Array” A (A/V)</th>
<th>“Array” B (A/V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>colony stimulating factor 3 (CSF3)</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Interleukin 1, beta (IL 1ß)</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Interleukin 8 (IL 8)</td>
<td>2.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Oncogene GRO3</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Oncogene GRO2</td>
<td>2.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Oncogene GRO3</td>
<td>2.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Interleukin 8 (IL 8)</td>
<td>2.7</td>
<td>2.2</td>
</tr>
<tr>
<td>tumoral necrosis factor, alpha-induced protein 3</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Oncogene GRO1</td>
<td>2.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Interleukin 6 – interferon, beta 2</td>
<td>2.2</td>
<td>1.99</td>
</tr>
<tr>
<td>Interleukin 1, beta (IL 1ß)</td>
<td>2.4</td>
<td>2.5</td>
</tr>
<tr>
<td>serine (or cysteine) proteinase inhibitor</td>
<td>1.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Interleukin 1, beta (IL 1ß)</td>
<td>2.1</td>
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<td>tumoral necrosis factor, alpha-induced protein 3</td>
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</tr>
<tr>
<td>tumorgenicity suppressor 16</td>
<td>1.9</td>
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(A/V) = Fluorescence of the vein cultivated under arterial conditions/ Fluorescence of the vein cultivated under venous conditions

all of which are involved in tumoral processes.

COMMENTS

Failure of the SV grafts utilized in CABG involves many factors. Early occlusion, within the first 30 days after the operation is normally the result of thrombosis, which could be caused by factors such as: reduced diameter or unfavorable distal bed of the revascularized coronary artery. This generates a reduction in the blood flow and an increase in the risk of thrombosis of the graft [4].

The development of diffused and concentric hyperplasia of the intimal layer is observed in SV grafts of patients who evolve with relapse of the anginal symptoms after CABG and require reoperations. RATLIFF & MYLES [5] detected the presence of hyperplasia in 99% of SV grafts analyzed in a series of 100 patients submitted to reoperation of CABG.

The surgical manipulation during the dissection and preparation of the venous graft may cause endothelial injury and alterations of the thromboregulation. The loss of endothelial integrity may cause aggregation of the sanguineous cells and platelets and alter the homeostasis of the vascular wall of the graft. The injury of the endothelial cells leads to a reduction in the local liberation of nitrous oxide and prostacyclins, increasing the adhesion and the platelet aggregation. The platelet activation leads to the
liberation of pro-coagulant factors and vasoconstrictors, which stimulate the proliferation of the medial vascular smooth muscle cells (VSMC) and the cellular migration to the intimal layer of the graft [6].

The elevated hydrostatic pressure experienced during the preparation of the graft may cause necrosis of the VSMC [7]. These hemodynamic factors are dependent on the endothelial function and among the hemodynamic factors shear stress is the main force acting on the endothelium [3]. The mechanism by which the endothelium detects shear stress is not completely understood, but there is evidence demonstrating that the shear stress actively participates in vascular homeostasis through the regulation and liberation of endothelial proteins. The vascular behavior is very dependent on the endothelial function and the gene regulation of diverse proteins. The vascular flow pattern is altered by turbulence and with a reduction in the flow of blood [4].

An interesting fact that occurs in the venous graft interposed in an artery is the induction of the apoptotic process. The decrease of blood flow in the venous graft may be caused by an accentuated reduction in the caliber of the coronary artery in relation to the diameter of the venous graft and by the unfavorable distal bed of the revascularized coronary artery, not allowing adequate outflow of blood [4].

Several studies have shown the induction of cellular apoptosis in venous grafts imposed in arteries. The interposition of the saphenous vein in a pig’s carotid artery presented with an apoptotic peak after eight hours of grafting. The rate of cellular proliferation of the medial layer, however, seems to be greater during a study period of 48 hours. Initially, the rate of apoptosis in the venous graft seems to be greater than the rate of proliferation, as the proliferation peak occurs after five to seven days [19]. This process of cell death, which occurs soon after the interposition of the graft, is a result of the stress in which the wall of the vein is submitted when placed in arterial conditions. These hemodynamic conditions of greater blood flow may cause necrosis of the VSMC and the cellular migration to the intimal layer of the graft [6].

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flow and higher pressure cause a stretching of the wall of the vein, whose structure is not prepared to receive such a tension. This can be confirmed when a polytetrafluoroethylene membrane is positioned surrounding a venous graft, reducing the tension and the stretching on the wall of the vessel, with a significant reduction of the rate of apoptosis in the graft [20].

In the ex vivo system presented in this work, the cellular density and the tissue viability gradually diminished when the saphenous vein is cultivated in the arterial hemodynamic conditions, whilst no morphological or viability changes were observed in the vein cultivated under venous conditions for up to 4 days. It is interesting to note that the saphenous vein cultivated in arterial conditions for 1 day presented with cellular density similar to the fresh fragments, although several of these cells presented with fragmentation of the DNA, indicating the presence of the apoptotic process (Figures 3 and 5). This event may explain the cellular loss observed in the fragments of saphenous vein cultivated in the arterial conditions.

Vascular remodeling results from the balance between cellular death and proliferation. In the venous graft in an arterial bed, apoptosis occurs in response to the increase in mechanical stress and is counterbalanced by a subsequent process of cellular proliferation. In our ex vivo system, the absence of cellular proliferation in the saphenous vein in arterial conditions may be explained by the necessity components originating in the blood circulation, that are not present in the culture medium, or maybe, by the necessity of a greater time than four days of culture. Even though the proliferation event was not observed, we verified that the phenomena that occur in vivo were being reproduced in our ex vivo culture system, demonstrating that this system is a good model to study the changes that occur in the saphenous graft.

It is evident that the stretching of the wall of the venous graft in the arterial bed provokes a series of structural and molecular alterations that contribute to hyperplasia of the intima, which frequently evolves to an atherosclerotic process. This process may be related to the regulation of growth factors such as PDGF and TGF-ß components of the MAP kineses pathway such as p38, transcription factors such as E2F and proteins that compose and break down the extracellular matrix such as tenascin-C and MMPs (matrix metalloproteinases) respectively [21]. The occlusion process that occurs in the saphenous grafts involves the participation of several proteins and the study of how these proteins jointly participate is an important approach to understanding the vascular physiology and physiopathology.

Currently, we arranged the strategies for the global analysis of thousands of genes simultaneously. Among these 'differential display', random sequencing of the cDNA libraries, SAGE (serial analysis of gene expression) and cDNA microarray stand out. The greater knowledge in respect to the genes involved in the atherosclerotic process of grafts has improved the possibility of gene therapy development. In this work we developed an ex vivo system for the culture of saphenous veins that enables the analysis of vascular behavior in controlled hemodynamic conditions for periods of up to four days. The study was performed cultivating the saphenous vein in arterial hemodynamic conditions to simulate the condition that the vein is submitted after the process of coronary artery bypass grafting. This system was adequate as a model for the study of the changes that occur in the saphenous graft, as seen by the fact that the phenomena that occur in vivo were being reproduced. Additionally, using the technology of the cDNA microarray we identified two genes, IL-1ß (interleukin-1ß) and GRO3 (oncogene 3), that may be participating in the transformation process of venous grafts to arteries. A future action on the genes could contribute to the greater patency of the graft.

Molecular alterations of the saphenous veins cultivated for one day under arterial hemodynamic conditions, observed using the cDNA microarray are presented in Table 1. Among the genes that present with a differentiated expression in the saphenous vein cultivated for one day under arterial hemodynamic conditions are the cytokines, all of which are involved in the tumoral process. In this context, the response to cellular proliferation on the vessel wall imitates common aspects to those observed in the development of neoplasias with activation of the growth factors due to the increased stress. Unhappily, this is a process which is little understood and that evolves to the development of atherosclerosis and occlusion of the graft in a significant number of patients.

Elucidation of the physiopathologic basis of the atherosclerotic process of saphenous grafts is of fundamental importance. Studies in respect to the histologic and molecular changes involved may provide the development of alternative therapies efficacious at reducing the number of reoperations after coronary artery bypass grafting.

Greater knowledge in respect to the genes involved in the atherosclerotic process of the venous grafts favors the development of gene therapy aiming to control this process. The first reports on the transference of exogen gene material to the vascular system were described at the end of the 1980s [22]. There are two alternative approaches to gene therapy. One of them consists of the direct introduction of a gene in the cell, making it produce a determined protein. The other is the induction of anti-sense oligonucleotides, which act as decodifiers and blockers in the production of proteins [1].
The success of gene therapy depends on the following factors: the correct choice of the therapy to act on the vascular wall, the availability of a safe and efficient vector for the transference of the gene and the availability of an adequate mechanism for the gene transference.

There are studies under way in respect to gene therapy for saphenous vein grafts. MANNION et al. [23] obtained an 82% reduction in the proliferation of cells of the medial layer of porcine saphenous veins submitted to gene therapy with antisense c-myc oligonucleotides. GEORGE et al. [24] performed a study with the culture of saphenous veins in humans, utilizing tissue inhibitors of metalloproteinase (TIMP-1). The authors reported a 54% reduction in neointima and a 78% reduction in the migration of smooth muscle cells after 14 days of therapy.

MANN et al. [25] utilized CDC2 and PCNA antisense oligonucleotides in rabbit jugular veins interposed in carotid arteries. The vector used was the herpes virus. The authors demonstrated a reduction in the hyperplasia of the neointima and an increase in the resistance of the venous grafts against atherosclerosis induced by diet.

In our study, confirmation of the gene expression was achieved using a measurement method known as RT-PCR. Until now, an increase in the expression of the IL-1ß and GRO3 genes has been confirmed. The role in the atherosclerotic process of venous grafts and the therapeutic potential of these two genes need to be evaluated. For this, future models of venous grafts in the arterial bed of experimental animals and interference methods such as fusion protein and interference by RNA will be used.

CONCLUSIONS

The ex vivo study model associated with analysis using the cDNA microarray system of saphenous graft segments utilized in coronary artery bypass grafting allowed the identification of the apoptotic process and a greater gene expression of interleukin 1ß and oncogene 3 in veins cultivated under arterial hemodynamic conditions.

Elucidation of these gene alterations in saphenous vein grafts utilized in coronary artery bypass grafting is of fundamental importance for the development of new alternative therapies such as gene therapy for the prevention of the atherosclerotic process in venous grafts.

BIBLIOGRAPHIC REFERENCES


