Adiponectin expression in epicardial adipose tissue after percutaneous coronary intervention with bare-metal stent

Expressão gênica de adiponectina no tecido adiposo epicárdico após intervenção coronária percutânea com implante de stent metálico

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Abstract

Background: The classical view of adipose tissue as a passive reservoir for energy storage is no longer valid. In the past decade, adipose tissue has been shown to have endocrine functions and the most abundant peptide secreted by adipocytes is adiponectin. Pericardial adipose tissue (PAT) is distributed around coronary arteries and endovascular injury, caused by the presence of intracoronary bare-metal stent (BMS), could promote inflammatory changes in the periadvential fat, contributing to vascular restenosis.

Objective: We sought to determine gene expression of inflammatory mediator in pericardial adipose tissue after bare-metal stent implantation and vascular restenosis that had been referred to operative treatment.

Methods: Paired samples of PAT were harvested at the time of elective coronary artery bypass surgery (CABG) in 11 patients (n=22), one sample was obtained of the tissue around BMS area and another sample around coronary artery without stent. Local expression of adiponectin was determined by real-time polymerase chain reaction (RT-PCR) using Taq DNA polymerase.

Results: In two samples, there was no gene expression of adiponectin. We are able to identify adiponectin in 20 samples, however, the pattern of gene expression were heterogeneous. We did not notice specificity when we compared PAT obtained near BMS area or far from BMS area.

Conclusion: There were no correlation between adiponectin gene expression and presence of BMS.

Descriptors: Adiponectin. Coronary restenosis. Angioplasty, transluminal, percutaneous coronary.

Resumo

Introdução: A visão clássica de tecido adiposo como um reservatório passivo para o armazenamento de energia não é mais válido. Na última década, o tecido adiposo tem demonstrado funções endócrinas, sendo o peptídeo mais abundante secretado pelos adipócitos a adiponectina. O tecido adiposo epicárdico (TAE) é distribuído em torno das artérias coronárias e, a lesão endovascular causada pela presença de stent metálico intracoronário, poderia promover alterações inflamatórias na gordura periadventicial, contribuindo para reestenose.

Objetivo: Determinar a expressão gênica de mediadores inflamatórios no tecido adiposo epicárdico após implante de stent metálico com reestenose que haviam sido encaminhados para tratamento cirúrgico.

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Métodos: **Amostras pareadas de TAE foram colhidas no momento da cirurgia de revascularização miocárdica (CRM) em 11 pacientes (n = 22), uma amostra foi obtida do tecido em torno da area com stent e outra amostra em torno da artéria coronária sem stent. Expressão local de adiponectina foi determinada por reação em cadeia de polymerase em tempo real utilizando Taq DNA polimerase.**

Resultados: **Em duas amostras, não houve expressão do gene da adiponectina. Fomos capazes de identificar**

adiponectina em 20 amostras, no entanto, o padrão de expressão gênica foi heterogêneo. Não percebemos especificidade quando comparamos TAE obtido próximo à área de stent ou distante da área de stent.

Conclusão: **Não houve correlação entre a expressão do gene de adiponectina e a presença de stent intracoronário.**

Descritores: **Adiponectina. Reestenose coronária. Angioplastia transluminal percutânea coronária.**

INTRODUCTION

The results of several studies carried out in the last decade indicate that inflammatory mechanisms play a central role in the process of intimal neoproliferation responsible for restenosis after percutaneous implantation of intracoronary metallic stent. The cellular damage caused by stent implantation seems to be the trigger factor for the release of chemotactic molecules, such as the tumor necrosis factor-alpha (TNFá) and interleukin (IL) by endothelial cells and leukocytes, causing the recruitment of inflammatory cells into the stent [1].

Although the mechanisms proposed for restenosis after stent implantation usually considered inflammatory factors of vascular origin, it is reasonable to assume that extravascular sources can significantly contribute to the occurrence of this phenomenon, which is the case of the epicardial adipose tissue (EAT) located around the coronary arteries. It is postulated that the EAT is a deposit of lipid energy reserves for the heart and possibly offer some degree of mechanical protection for the coronary arteries [2-4]. In addition to these classical functions, the adipose tissue is now recognized as an endocrine and active paracrine organ that secretes various bioactive molecules, collectively known as adipokines. These molecules have a modulating effect of the inflammatory process, a common characteristic of the EAT, which is able to secrete pro-and anti-inflammatory adipokines, and possibly take on an important role in the process of restenosis after angioplasty with stent implantation [5,6].

The epicardial fat of individuals with coronary artery disease shows increased expression of TNF-alpha, IL-6, leptin, visfatin and decreased adiponectin in comparison with epicardial fat of individuals without coronary disease [7-10]. The levels of messenger RNA (mRNA) of adiponectin and IL-6 in EAT are directly associated with the extension of coronary disease, therefore, it is observed that the greater the number of diseased vessels, the lower the level of adiponectin is and increased the level of IL-6 [9].

Considering the peculiar characteristics of the EAT and its similarities with visceral fat, the development of hypotheses is natural where this type of fat plays an important role in the evolution of cardiovascular disease, and possibly, in the mechanism of restenosis after stent implantation (probably due to inflammatory response).

The objective of this study is to determine the gene expression profile of adiponectin in the EAT of patients with metal stent undergoing coronary artery bypass grafting (CABG) and assess the impact upon the inflammatory process in the presence of this intracoronary device.

METHODS

After institutional approval by the Research Ethics Committee, two samples of EAT were obtained by through biopsy in 11 patients undergoing coronary artery bypass grafting (CABG), with angiographic in-stent restenosis diagnosed by hemodynamic study. A sample was taken near the coronary artery treated with stent and the other one from an untreated artery. The tissues were immediately frozen in liquid nitrogen and then transported to the laboratory for storage in a freezer at -70 ° C until processing for RNA extraction.

The patients agreed to participate in the study by signing the informed consent.

RNA extraction

The tissue frozen in liquid nitrogen was weighed on an analytical balance and macerated using a mortar and pestle. The fragments were transferred to a 1.5 mL tube, in which was added 1.0 mL of Trizol (Invitrogen) and then vigorously homogenized. They were centrifuged at 4°C, 12,000 g for 10 minutes. The intermediate phase was transferred to another tube, discarding the precipitate and the superior phase (triglycerides). Two hundreds mL of chloroform was added and slowly homogenized for 15 seconds, after a 3-minute rest. They were again centrifuged at 4° C, 12,000 g for 15 minutes and the other tube started the aqueous phase. Five

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hundreds of isopropanol were added and left to precipitate for 10 minutes at room temperature. They were centrifuged at 4°C, 12,000 g for 10 minutes and the supernatant was disregarded.

One mL 75% ethanol was added to the pellet and centrifuged at 7500 g for 5 minutes. The supernatant was poured then off and disregarded. The samples were placed in the vacuum and centrifuged at 4°C, 12,000 g for 2.5 minutes. The pellet was resuspended with 25 mL of deionized water treated with diethyl pyrocarbonate (DEPC) homogenizing with its own pipette. Forty U of RNase inhibitor (RNaseOUT, Invitrogen) were added, homogenizing again. The RNA preparation was stored at -70°C.

Determination of quality of RNA preparation

The quantification of RNA concentration in each sample was performed by means of the protocol of Qubit quantitation platform, which is composed of Qubit fluorometer device (Invitrogen) and Quant-it kit reagents (Invitrogen). The working solution was prepared at 200 ìl buffer and 1 ìl dye. For instrument calibration, 2 standard samples with 190 ìl working solution and 10 ìl negative or positive standards were prepared. One ìl was used for each RNA sample, adding 199 ìl of working solution. All the tubes were homogenized in a vortex and incubated at room temperature (25°C) for 2 minutes. The samples were then read on the Qubit fluorometer.

Complementary DNA Synthesis

Total RNA was converted into cDNA via reverse transcription reaction, using reagents supplied by Invitrogen. One μL of oligo DT12-18 (0.5 mg / mL) was added in a new and sterile tube, 1 μgmg of RNA, 1 μg of dNTP (10 mM) and 9 1 μg of deionized water treated with diethyl pyrocarbonate (DEPC), a total volume of 13 μg. The solution was warmed to 65 ° C for 5 minutes, and incubated on ice for 1 minute. It was quickly centrifuged. Four 4 μL of 5X First-Strand buffer were added (250 mM Tris-HCl pH 8.4, 375 mM KCl, 15 mM MgCl2), 1 μL of DTT (0.1 M), 1 μL of RNase inhibitor (RNaseOUT, Invitrogen) (40 U / 1 μL) and 1 μL of Superscript III RT enzyne(200 U / μL) and were mixed by pipetting. They were Incubated at 50 ° C for 60 minutes and then at 70 ° C for 15 minutes.

Amplification of cDNA (RT-PCR)

The specific Adiponectin cDNA (ADIPOQ), TNF-alpha, CD45, Interleukin-6 (IL6), MCP-1 and GAPDH (control) were amplified by PCR (polymerase chain reaction) using Taq DNA polymerase (Invitrogen) and primer, described in the literature or chosen according to their respective mRNA sequences deposited in GenBank. Two μL of 10x PCR buffer were added in a new and sterile

tube without magnesium (200 mM Tris-HCl pH 8.4 and 500 mM KCl), 0.4 μL of dNTP (10 mM), 0.6 μL of 50 mM MgCl2 (this amount is used for final concentration of 1.5 mM magnesium), 0.1 μL of Taq DNA polymerase (5U/ìL) (Invitrogen), 1.0 μL of cDNA, and 1.0 μL of primer, and completed the volume to 20 μL of autoclaved deionized water. They were incubated at 94°C for 5 minutes, followed by a cycle of 94°C for 1 minute, 55-60°C for 1 minute, 72°C for 1 minute, which was repeated 35 times. It was then incubated at 72°C for 10 minutes. The annealing temperature in PCR cycle was adjusted to the specific primers as follows: 60°C (adiponectin), 56°C (GAPDH) or 55°C (IL-6, CD45, TNF-alpha, MCP-1) For standardization of the technique, each amplification reaction was performed three times, varying the number of PCR cycles (25, 30 and 35 cycles) in order to find amplification condition where there has been no saturation reaction and permit the preliminary quantitation of PCR products.

PCR analysis by agarose gel electrophoresis

The PCR products were separated by electrophoresis in 2% agarose gel in EAT buffer at 100 V, and visualized by fluorescence with 1.5 μL ethidium bromide. The gel image was captured by a digital camera. The band relative intensity was quantified by Scion Image software.

Analysis of results (calculation of relative expression)

The analysis of the amplification curves was performed by using the Rotor-Gene 6000 Series Software 1.7 (Qiagen ®). Due to the relativity of these enzymes, the calculation was determined by the number of cycles (Cts). The final result was obtained from the average of cycles in the control group (sample size), and the value of the gauge sample was the same for all samples, including the control group calculation.

RESULTS

For this study, 22 samples of approximately 1 g were collected from the EAT in two distinct regions of each patient (one sample was taken near the coronary artery treated with stents and the other one from the untreated artery).

In 21 samples, there was expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), showing the viability of RNA. In one sample, there was variation in intensity of amplification, and the repetition of these reactions will be necessary (withdrawn from the study). All samples expressed adiponectin. The corrected adiponectin expression was correlated with the clinical data of patients (Table 1) and its variation depending on the presence or absence of the stent, which was individually analyzed (Figure 1).
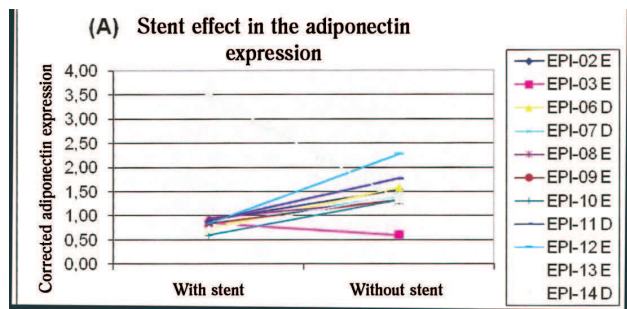
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Fig. 1 - Variation of adiponectin due to the presence or absence of stent

DISCUSSION

The results of several studies carried out in the last decade indicate that inflammatory mechanisms play a central role in the process of intimal neoproliferation responsible for restenosis after implantation of intracoronary stent. The cellular damage caused by stent implantation seems to be the trigger factor for the release of chemotactic molecules, such as the tumor necrosis factor-alpha (TNFα, IL-1 and IL-6) by endothelial cells and leukocytes, causing the recruitment of inflammatory cells, chiefly into the stent [1].

In relation to the subcutaneous adipose tissue of the heart disease carrier, there was greater expression in EAT of these TNF-alpha, IL-6, IL-1beta patients and reduced

adiponectin expression, CD45 and leptin [11,12]. Thus, the gene expression profile of the EAT approached the visceral fat compartment, in which the accumulation is associated with increased risk of atherosclerotic disease [11,13,14]. Recently, Baker et al. [15] demonstrated the potential contribution of macrophage infiltration through high levels of expression of CD45 and CD68 in EAT patients with coronary artery disease, supporting the concept that macrophages play a crucial role in inflammatory activity that leads to atherosclerosis.

Adiponectin is a protein synthesized chiefly by adipocytes, although there are reports of other production sites (such as the cardiomyocytes) [16]. It has a protective role in atherosclerosis processes, with reduced concentration in situations of insulin resistance (such as obesity and type 2 diabetes) as well as in patients with coronary artery disease [17-20].

Gomes et al. [21], observed a severe acute inflammatory process in the intima of arteries associated with myocarditis in a histological study after adjacent biopsy of the heart muscle and the coronary artery around the stent.

The results of our study showed lower adiponectin expression in EAT obtained from areas close to the stent when compared with those obtained in places without the device with samples of the same patient. Despite the nonexistence of other studies analyzing adiponectin gene expression in EAT after percutaneous angioplasty with stent, Iacobelli et al. [2] observed a group of 16 patients in

Table 1. Correlation between clinical data and experimental results.

Case	Gender	Associated diseases and habits					Samples	Stent	Gene Expression
		DM	SAH	PEC	COPD	Smoking			Adiponectin
EPI-2	M	-	+	+	-	+	D	-	0.90
							E	+	0.84
EPI-3	M	+	+	+	+	+	D	-	0.73
							E	+	0.81
EPI-6	M	-	+	+	-	+	D	+	0.95
							E	-	0.83
EPI-7	M	-	+	+	-	+	D	+	0.58
							E	-	0.88
EPI-8	F	+	+	+	-	+	D	-	0.84
							E	+	0.71
EPI-9	M	-	+	+	-	+	D	-	3.49
							E	+	1.53
EPI-10	F	-	+	+	-	-	D	-	0.60
							E	+	1.58
EPI-11	M	-	+	-	-	+	D	+	1.39
							E	-	1.30
EPI-12	M	+	+	+	-	+	D	-	1.32
							E	+	1.32
EPI-13	M	-	+	+	-	+	D	-	1.77
							E	+	2.27
EPI-14	M	-	+	-	-	-	D	+	1.42
							E	-	1.34

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which the EAT in ischemic heart disease carriers synthesizes less adiponectin compared with control group (without ischemic heart disease). Moreover, a rapid increase in plasma concentration of this protein was observed soon after myocardial revascularization, thus, we could extrapolate a deleterious effect associated with the presence of intracoronary stent, as demonstrated by decreased adiponectin expression in the EAT of the analyzed patients, however, this utterance requires confirmation by other studies [22]. Clinical studies also suggest that the presence of metallic stent can irreversibly impair the endothelium of the coronary artery and aggravate the inflammatory process [23-25].

This study will discuss whether the treatment of coronary artery disease by percutaneous angioplasty with metallic stent promotes specific changes in the epicardial fat that, eventually, may determine different directions in the evolution of the disease.

The limitations of this study are associated with the need for more samples and statistical confirmation of the obtained results. Moreover, other primers are being developed at our laboratory that will allow expression analysis other markers of inflammatory response present in the EAT (IL-6, CD45, TNF-alpha).

In conclusion, during preliminary analysis of the results, it is possible to note a tendency, not statistically confirmed, that the adiponectin expression is decreased in the sample with stent in relation to the sample without stent from the same patient. The presence of metallic intracoronary stent did not change the gene expression of adiponectin in EAT, analyzed in a semiquantitative form in patients with intrastent restenosis undergoing CABG.

REFERENCES

1. Gaspardone A, Versaci F. Coronary stenting and inflammation. Am J Cardiol. 2005;96(12A):65L-70L.

2. Iacobellis G, Pistilli D, Gucciardo M, Leonetti F, Miraldi F, Brancaccio G, et al. Adiponectin expression in human epicardial adipose tissue in vivo is lower in patients with coronary artery disease. Cytokine. 2005;29(6):251-5.

3. Sacks HS, Fain JN. Human epicardial adipose tissue: a review. Am Heart J. 2007;153(6):907-17.

4. Rabkin SW. Epicardial fat: properties, function and relationship to obesity. Obes Rev. 2007;8(3):253-61.

5. Ahima RS, Flier JS. Adipose tissue as an endocrine organ. Trends Endocrinol Metab. 2000;11(8):327-32.

6. Hutley L, Prins JB. Fat as an endocrine organ: relationship to the metabolic syndrome. Am T Med Sci. 2005;330(6):280-9.

7. Iacobellis G, Corradi D, Sharma AM. Epicardial adipose tissue: anatomic, biomolecular and clinical relationships with the heart. Nat Clin Pract Cardiovasc Med. 2005;2(10):536-43.

8. Cheng KH, Chu CS, Lee KT, Lin TH, Hsieh CC, Chiu CC, et al. Adipocytokines and proinflamatory mediators from abdominal and epicardial adipose tissue in patients with coronary artery disease. Int J Obes (Lond). 2008;32(2):268-74.

9. Eiras S, Teijeira-Fernández E, Shamagian LG, Fernandez AL, Vazquez-Boquete A, Gonzales-Juanatey JR. Extension of coronary artery disease is associated with increased IL-6 expression and decreased adiponectin gene expression in epicardial adipose tissue. Cytokine. 2008;43(2):174-80.

10. Iacobellis G, Cotesta D, Petramala L, De Santis V, Vitale D, Tritapepe L, et al. Intracoronary adiponectin levels rapidly and significantly increase after coronary revascularization. Int J Cardiol. 2010;144(1):160-3.

11. Mazurek T, Zhang L, Zalewski A, Mannion JD, Diehl JT, Arafat H, et al. Human epicardial adipose tissue is a source of inflammatory mediators. Circulation. 2003;108(20):2460-6.

12. Iglesias MJ, Eiras S, Piñeiro R, Lópes-Otero D, Gallego R, Fernández AL, et al. Gender differences in adiponectin and leptin expression in epicardial and subcutaneous adipose tissue. Findings in patients undergoing cardiac surgery. Rev Esp Cardiol. 2006;59(12):1252-60.

13. Baker AR, Silva NF, Quinn DW, Harte AL, Pagano D, Bonser RS, et al. Human epicardial adipose tissue expresses a pathogenic profile of adipocytokines in patients with cardiovascular disease. Cardiovasc Diabetol. 2006;5:1.

14. Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr Rev. 2000;21(6):697-738.

15. Baker AR, Harte AL, Howell N, Pritlove DC, Ranasinghe AM, da Silva NF, et al. Epicardial adipose tissue as a source of nuclear factor-kappaB and c-Jun N-terminal kinase mediated inflammation in patients with coronary artery disease. J Clin Endocrinol Metab. 2009;94(1):261-7.

16. Piñeiro R, Iglesias MJ, Gallego R, Raghay K, Eiras S, Rubio J, et al. Adiponectin is synthesized and secreted by human and murine cardiomyocytes. FEBS Lett. 2005;579(23):5163-9.

17. Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokyne regulating glucose and lipid metabolism. Trends Endocrinol Metab. 2002;13(2):84-9.

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18. Diez JJ, Iglesias P. The role of the novel adipocyte-derived hormone adiponectin in human disease. Eur J Endocrinol. 2003;148(3):293-300.

19. Beltowski J. Adiponectin and resistin: new hormones of white adipose tissue. Med Sci Monit. 2003;9(2):RA55-61.

20. Fantuzzi G. Adipose tissue, adipokynes, and inflammation. J Allergy Clin Imunnol. 2005;115(5):911-9.

21. Gomes WJ, Gianotti Filho O, Catani R, Paez RP, Hossne NA, Buffolo E. Alterações inflamatórias das artérias coronárias e do miocárdio induzidas por stents coronários. Rev Bras Cir Cardiovasc. 2002;17(4):292-8.

22. Gomes WJ, Braile DM. Estudo Syntax: análise e implicações clínicas. Rev Bras Cir Cardiovasc. 2008;23(4):III-IV.

23. Gomes WJ, Buffolo E. Cirurgia de revascularização miocárdica após intervenção coronária percutânea. Podemos obter os mesmos resultados? Rev Bras Cir Cardiovasc. 2006;21(4):V-VIII.

24. Fragomeni LSM, Falleiro RP, Hoppen G, Krahl G. Revascularização cirúrgica do miocárdio em pacientes com stents coronários. Rev Bras Cir Cardiovasc. 2005;20(4):371-6.

25. Gomes WJ, Buffolo E. Stent coronário e inflamação. Rev Bras Cir Cardiovasc. 2003;18(4):III-VI.