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VEGF gene therapy cooperatively recruits molecules from the immune system and stimulates cell homing and angiogenesis in refractory angina

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ABSTRACT

Background: New vessels are formed in response to stimuli from angiogenic factors, a process in which paracrine signaling is fundamental.
Objective: To investigate the cooperative paracrine signaling profile in response to Vascular Endothelial Growth Factor (VEGF) gene therapy in patients with coronary artery disease (CAD) and refractory angina.
Method: A cohort study was conducted in which plasma was collected from patients who underwent gene therapy with a plasmid expressing VEGF 165 (10) and from surgical procedure controls (4). Blood samples were collected from both groups prior to baseline and on days 3, 9 and 27 after the interventions and subjected to systemic analysis of protein expression (Interleukin-6, IL-6; Tumor Necrosis Factor-α, TNF-α; Interleukin-10, IL-10; Stromal Derived Factor-1 α, SDF-1α; VEGF; Angiopoietin-1, ANGPT-1; and Endothelin-1, ET-1) using the enzyme-linked immunosorbent assay (ELISA).
Results: Analysis showed an increase in proinflammatory IL-6 ($p = 0.02$) and ET-1 ($p = 0.05$) on day 3 after gene therapy and in VEGF ($p = 0.02$) on day 9. A strong positive correlation was found between mobilization of endothelial progenitor cells and TNF-α on day 9 ($r = 0.71$; $p = 0.03$). Furthermore, a strong correlation between β-blockers, antiplatelets, and vasodilators with SDF-1α baseline in the group undergoing gene therapy was verified ($r = 0.74$; $p = 0.004$).
Conclusion: Analysis of cooperative paracrine signaling after VEGF gene therapy suggests that the immune system cell and angiogenic molecule expression as well as the endothelial progenitor cell mobilization are time-dependent, influenced by chronic inflammatory process and continuous pharmacological treatment.

1. Introduction

In a cardiovascular ischemic event, the paracrine signaling effect of the immune system (leukocytes, monocytes) and progenitor cells recruits molecules capable of increasing the survival of cardiomyocytes and protect the heart [1,2]. This paracrine signaling is cooperatively expressed and includes cytokines such as interleukins, chemokines and growth factors that are involved in mobilizing cells and boosting the repair process after injury [3]. Thus, the immune response, angiogenesis and cellular homing, each of which is essential for its normal and effective functioning, are directly linked in this signaling process. Depending on the pathology or the therapy applied, the mechanisms involved in this cooperative signaling may undergo modification.

In atherosclerotic diseases, such as CAD, patients with refractory angina are those symptomatic that do not respond to medical treatment, coronary artery bypass graft (CABG) or percutaneous coronary intervention (PCI) [4–6]. In these circumstances, gene therapy represents a new alternative therapy that aims to stimulate vascularity in the ischemic myocardium in order to minimize angina symptoms and possibly increase life expectancy. The formation of new vessels is a response to the stimulation of angiogenic factors that regulate endothelial proliferation, migration,

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survival and proteolytic activity. Among these factors, VEGF and angiopoietins have emerged as critical regulators in the vascularization process [7–9]. These molecules promote angiogenesis and morphogenesis through a complex process of angioregulatory events [10,11], in which endothelial cells cooperate with the immune system, by encouraging the activity of cytokines and macrophages [12,13]. In fact, the endothelial progenitor cells are also recruited by a mechanism known as cell homing.

Due to its chemoattractant capacity, SDF-1 is one of the key molecules involved in stem cell homing, as it increases the adhesion and transmigration of circulating endothelial progenitor cells [14]. When coupled to its cell receptor, the CXC chemokine receptor type 4 (CXCR4), SDF-1 influences the expression of angiogenic molecules, interleukins and other factors implicated in endothelial function [15]. Thus, it is important to investigate the influence that other approaches might exert on this molecular network.

Recently, our group conducted a clinical trial (NCT 00744315) with gene therapy using a plasmid expressing the VEGF 165 isoform for patients with refractory angina [16,17]. Additional analysis of the mobilization of endothelial progenitor cells from this sample was recently published, with follow-up at baseline and 3, 9 and 27 days [18]. As a complement to that, the aim of this study was to investigate the cooperative profile of paracrine signaling in response to VEGF gene therapy in patients with angina, as well as a surgical procedure control group, by correlating the release of cytokines, angiogenesis and factors implicated in stem cell homing.

1.1. Materials and methods

The Research Ethics Committee of the IC/FUC, Porto Alegre/RS, Brazil approved the study (number 4413/09) and the investigation conforms to the principles outlined in the Declaration of Helsinki. All the patients signed the Free Informed Consent Form in accordance with law number 196/96.

1.1.1. Study design and sample

We conducted a cohort study in which the sample consisted of two surgical intervention groups. The first is a group of 10 patients with refractory angina who underwent gene therapy with 2 mg of plasmid containing the VEGF 165 gene (clinical assessment parameters in Kalil et al., 2010; Giusti et al., 2013) [16,17]. The second (control group) consists of four cardiac patients that underwent pacemaker-related surgery, three of whom underwent cardiac pacemaker implantation and one cardiac pacemaker-generator replacement. In all cases, complete atrio-ventricular block (CAVB) was the primary condition for the pacemaker implantation surgery. The control group was chosen because the surgical incision (in centimeters) is similar to the mini-thoracotomy employed in the gene therapy group, reducing the possibility of bias due to the surgical procedure.

A venous blood sample was collected immediately prior to initiating gene therapy (baseline) and 3, 9 and 27 days after treatment initiation. The control group sample was collected prior to pacemaker surgery (baseline) and 3, 9 and 27 days after surgery. After centrifugation at 1.000 rpm for 10 min at 4 °C, the plasma was separated and stored at −80 °C until assayed.

1.1.2. Systemic analysis of proteins released in response to VEGF gene therapy

Serum levels of IL-6, TNF-α, IL-10, SDF-1α, VEGF, ANGPT-1 and ET-1 were determined by the ELISA test, based on antigen-antibody interaction, with the aid of commercial kits (Quantikine, R&D Systems; Ebiosciences; RayBiotech), used in accordance with the manufacturers' instructions. All samples were dosed in duplicate. The data are expressed as protein picograms per milliliter (pg/mL).

The frequency of endothelial progenitor cells ($CD34^+/KDR^+$) in the patients who underwent gene therapy had been previously analysed by our group and the results have been recently published [18]. In this study, that frequency was correlated with the data on the release of VEGF, SDF-1α, ANGPT-1 and TNF-α.

1.1.3. Statistical analysis

The continuous nonparametric data are expressed as median and interquartile intervals. Friedman's nonparametric test, followed by Friedman's Multiple Comparison test were used to analyse the protein expression at different times in both the intervention and control groups. The nonparametric Mann-Whitney *U* test was used to compare protein expression at each moment in each group. Furthermore, Fisher's exact test was performed to calculate the probability of the existence of any association between the characteristics of the groups (independent characteristics). In the intervention group, the nonparametric correlation measure of expression between the proteins at different times as well as with the pharmacological treatment was analysed was using the Spearman correlation coefficient.

Statistical significance was set at $p < 0.05$. The analyses were performed using the Statistical Package for Statistical Program the Social Sciences (SPSS) (version 19.0) with additional analyses being made with the aid of the BioEstat statistical program, (version 5.0).

The limited sample size is derived from our original study [16,17], and can be justified by the complexity of both the gene therapy procedure and the patient selection and inclusion criteria.

1.2. Results

1.2.1. Temporal expression of the proteins involved in cooperative signaling in response to a gene therapy intervention: intra-group and inter-group analysis

Table 1 presents the demographics of the sample groups, Table 2 shows the description of the medication used by the patients and Fig. 1 details the temporal expression of the proteins. Analysis of the molecules determined by ELISA revealed there was a significant increase in plasma levels of proinflammatory IL-6 on day 3 in the intervention group (3.5–11.3 pg/mL, $p < 0.001$), followed by reductions on days 9 (5.2 pg/mL, $p < 0.001$) and 27 (3.7 pg/mL, $p < 0.001$). The control group showed higher baseline levels, followed by a significant drop on day 9 (8.0–5.3 pg/mL, $p = 0.02$). Intergroup analysis of the expression of factors involved in the

Table 1
Demographics of the sample groups.

Characteristics	Intervention group (n = 10)	Control group (n = 4)	p
Age (years)†	58.7 ± 5.31	64.8 ± 2.06	0.051
Male	9/10	2/4	0.176
Comorbidities			
Hypertension‡	9/10	3/4	0.505
Diabetes‡	5/10	3/4	0.580
Dyslipidemia‡	7/10	4/4	0.505
Prior vascular diseases			
Myocardial infarction‡	10/10	1/4	0.011*
Stroke‡	2/10	0/4	1.000
Peripheral vascular disease‡	1/10	1/4	0.505
Myocardial revascularization			
Surgical‡	8/10	1/4	0.092
Percutaneous‡	10/10	1/4	0.011*

* Statistical tests: Fisher's Exact test ($p < 0.05$).
† Variable described in mean and standard deviation.
‡ Variables described in frequency.

Table 2
Description of the medication.

Drug class	Intervention group (n = 10)	Control group (n = 4)	p
β-blocker†	10/10	3/4	0.505
Statin†	9/10	1/4	0.041*
Diuretic†	1/10	3/4	0.041*
Calcium channel blockers†	6/10	2/4	1.000
Antiarrhythmic†	1/10	1/4	0.505
Antiplatelet†	10/10	2/4	0.066
Angiotensin converting enzyme inhibitor†	5/10	2/4	1.000
Antidiabetic†	3/10	2/4	0.580
Vasodilator†	9/10	0/4	0.005*
Hypothyroidism†	1/10	0/4	1.000
Angiotensin receptors antagonist†	2/10	1/4	1.000

* Statistical tests: Fisher's Exact test ($p < 0.05$).
† Variables described in frequency.

secretion of cytokines by immune system cells in the patients treated with gene therapy showed the levels of IL-6 tended to increase on day 3, while the control group showed higher baseline levels of this cytokine, which were significant (3.5–8.0 pg/mL, p = 0.03).

TNF-α remained relatively constant in the intervention group, with levels showing a fall on day 3 (3.9–1.4 pg/mL, p = 0.01), followed by a rise on day 9 (4.4 pg/mL, p = 0.01) and another decrease on day 27 (2.3 pg/mL, p = 0.01). In the group control, levels remained constant on each day of analysis, with a small decrease on day 27 after the procedure. In the intervention group, TNF-α expression was lower at all times, with a significant difference 3 days after intervention when compared to the control group (1.4–17.6 pg/mL, p = 0.02).

In relation to the intra-group analysis of the anti-inflammatory cytokine IL-10, the intervention group showed a small increase in the levels of this cytokine on day 3, while the control group showed higher levels as from the baseline, with peak expression occurring on day 3 after the procedure. In the control group, there was a significant increase on day 27 (8.5–15.5 pg/mL, p = 0.02).

Plasma levels of SDF-1 in the intervention group were high, thus representing the recruitment of stem cells as from baseline (3041 pg/mL), with a subsequent reduction on day 3 (2799 pg/mL) and a slight increase on day 9 (2842 pg/mL). The control group, despite an increased expression of SDF-1 on day 9 after the procedure (1183–1394 pg/mL, p = 0.112) showed lower expression of this chemokine on each day of analysis throughout the period of the experiment when compared to the intervention group (baseline 3041–1183 pg/mL, p = 0.002; day 3 2799–1145 pg/mL, p = 0.003; day 9 2842–1394 pg/mL, p = 0.008).

In relation to the angiogenic process, plasma levels of VEGF peaked on day 9 after the initiation of gene therapy (baseline 121.4 pg/mL; day 3 146.8 pg/mL; day 9 331.1 pg/mL, p = 0.02). The control group showed a constant expression on each day of analysis throughout the period of the experiment. Intergroup analysis of VEGF protein expression showed a tendency to increase on day 9 in the intervention group (331.1–196.6 pg/mL, p = 0.08).

The intragroup analysis of ANGPT-1 revealed the intervention group had high levels of the protein at baseline (27,110 pg/mL), with a subsequent reduction on day 3 (24,715 pg/mL) and an increase on day 9 (29,027 pg/mL), p = 0.115. ANGPT-1 levels were higher at all times in the intervention group compared to the control, with a significant difference being detected on day 9 after gene therapy (29,027–17,480 pg/mL, p = 0.008).

The intragroup analysis of ET-1 expression revealed the intervention group, compared with the baseline (0.8 pg/mL), had an initial increase on day 3 (5.2 pg/mL, p = 0.05), which was followed by a drop in protein levels on days 9 (2.5 pg/mL) and 27 (1.5 pg/mL), while in the control group, ET-1 expression peaked on day 9 after the procedure (2–5.2 pg/mL, p = 0.183).

1.2.2. Correlation between the elements involved in cooperative signaling after gene therapy

Table 3 shows the correlations between the proteins involved in the process of angiogenesis in the intervention group on the different days of analysis. There was a moderate positive correlation between VEGF and SDF-1α on day 3 (r = 0.47; p = 0.21), a moderate negative correlation between VEGF and ANGPT-1 on day 3 (r = −0.55; p = 0.13) and a moderate positive correlation between VEGF and SDF-1α on day 9 (r = 0.53; p = 0.12). Additionally, there was a strong positive correlation between endothelial progenitor cell mobilization and TNF-α on day 9 (r = 0.71; p = 0.03) and between ANGPT-1 and SDF-1α on day 27 (r = 0.70; p = 0.04). On each evaluation day, there were both positive and negative correlations, demonstrating that cooperative paracrine signaling, driven by the polarization of the anti-inflammatory/inflammatory response, was essential in both progenitor cell homing and the angiogenic process.

Correlation between pharmacological treatment and molecule expression

Table 4 shows the correlations between the drugs in use (β-blocker, antiplatelet, and vasodilator) and molecule expression on different days of analysis in the patients submitted to gene therapy. Since the analysed medications act on the endothelial layer, they could have influenced the vasoconstriction/vasodilatation activity.

The correlation between SDF-1α and the drugs in use can be seen to diminish over time: at baseline, there was a strong positive correlation (r = 0.74; p = 0.004); on day 3 there was a moderate positive correlation (r = 0.55; p = 0.05); while on day 9, there was a weak positive correlation (r = 0.40; p = 0.2). A slight variation was found in the correlation between ANGPT-1 and the drugs in use, which was moderate positive at baseline (r = 0.62; p = 0.02) and on day 9 (r = 0.57; p = 0.04), but weak on day 3 (r = 0.48; p = 0.09). No correlation was found between the drugs in use and VEGF, ET-1 or the analysed inflammatory proteins.

These findings seem to suggest the pharmacological treatment with β-adrenergic blockers, antiplatelet and vasodilators may have influenced the cell homing and angiogenesis pathways and competed with the proposed gene therapy.

1.3. Discussion

The present study examined the expression of molecules involved in cooperative paracrine signaling in response to gene therapy with a plasmid expressing a pro-angiogenic factor in patients with refractory angina. It was found that the gene therapy with VEGF provided a transitional clinical improvement in these patients, in phases I and II [16,17]. Interestingly, the analysis of the temporal expression of cytokines and other factors involved in the recruitment of stimuli and responses generated by signal transduction and gene expression, to mobilize cells and induce the secretion of pro-angiogenic factors, revealed that, in fact, the gene therapy was able to mechanically and temporally modulate these phenomena (Figs. 1 and 2). Thus, our data suggest that the response generated by patients was actually due to the gene therapy, and not a response to surgical trauma. The same response was not found in the control group that underwent a similar surgical procedure. Hence, the gene therapy caused the release of angiogenic factors, modulation of chemokines involved in cellular homing and, consequently, increase in the frequency of progenitor cells and mobilization of pro and anti-inflammatory interleukins.

The intervention group showed a high level of the IL-6 on day 3, which demonstrates its protective action against an introduced

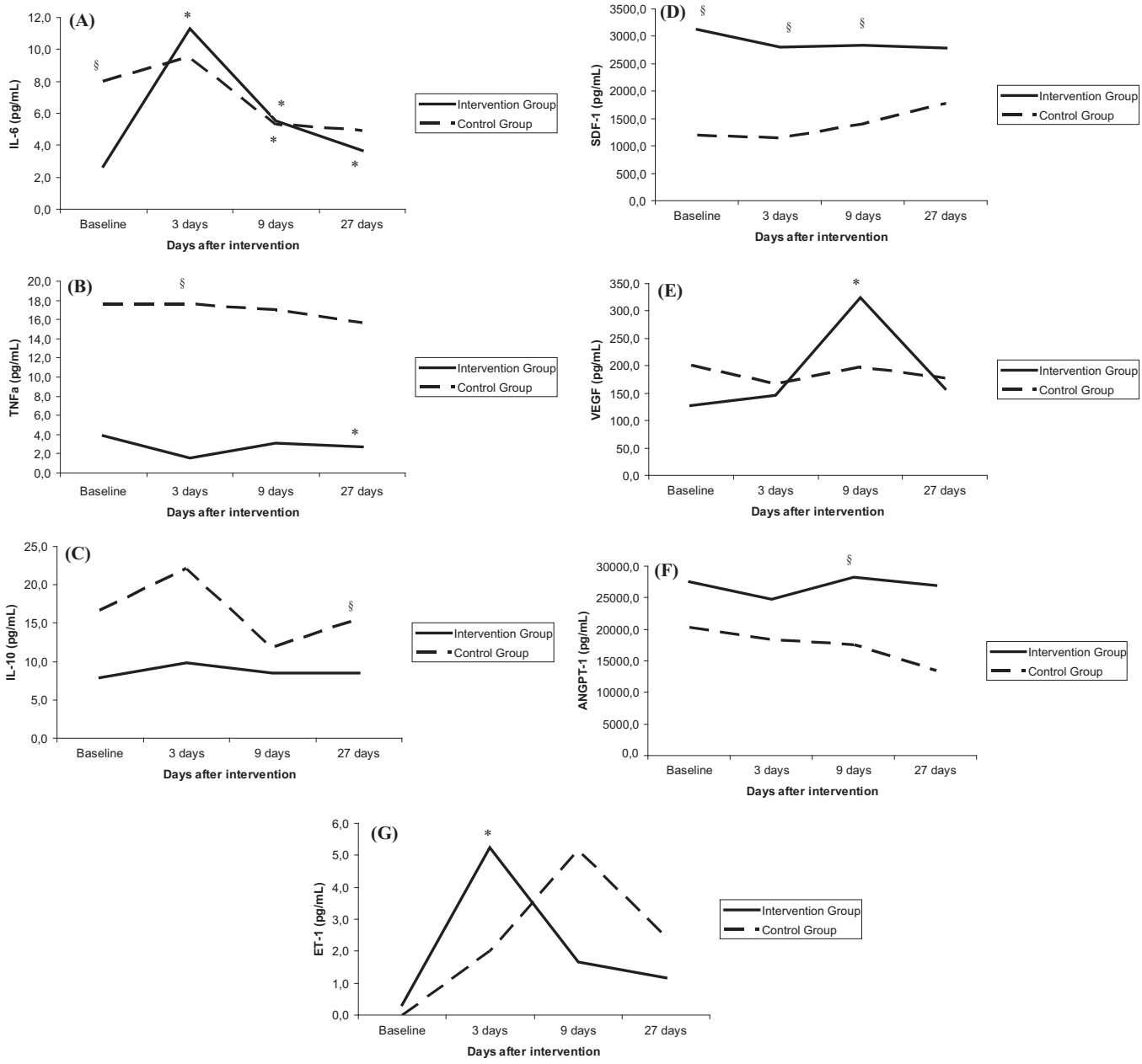

Fig. 1. Temporal analysis of protein release in the intervention and control groups. Values obtained by ELISA test (pg/mL), demonstrating the analysis of protein release in the intervention group (I) and control group (C) by time of evaluation: Interleukin-6 (IL-6); Tumor Necrosis Factor-α (TNF-α); Interleukin-10 (IL-10); Stromal Cell-Derived Factor-1 (SDF-1α); Vascular Endothelial Growth Factor (VEGF); Angiopoietin-1 (ANGPT-1); Endothelin-1 (ET-1). Values expressed as median and interquartile interval. Statistical tests: Friedman's, followed by Friedman's test of multiple comparisons (*p < 0.05) - intra-group analysis; Mann-Whitney U (§ p < 0.05) - intergroup analysis.

Table 3
Correlation between molecules involved in the cooperative signaling in the group underwent gene therapy.

	R	P
VEGF 3 days × SDF-1α 3 days	0.47	0.21
VEGF 3 days × ANGPT-1 3 days	-0.55	0.13
VEGF 9 days × SDF-1α 9 days	0.53	0.12
EPC 9 days × TNF-α 9 days	0.71	0.03*

Non-parametric correlation measurement of the expression between the proteins and expression of endothelial progenitor cells (EPC) in different times analysed in the intervention group. Vascular Endothelial growth factor (VEGF); Stromal Cell-Derived Factor-1 (SDF-1α); Angiopoietin-1 (ANGPT-1); Tumor Necrosis Factor-α (TNF-α); r = correlation.
* p < 0.05. Statistical test used: Spearman correlation coefficient.

Table 4
Correlation between pharmacological treatment and molecules expression.

	R	P
Drugs in use × SDF-1α baseline	0.74	0.004*
Drugs in use × SDF-1α 3 days	0.55	0.05*
Drugs in use × SDF-1α 9 days	0.40	0.2
Drugs in use × ANGPT-1 baseline	0.62	0.02*
Drugs in use × ANGPT-1 3 days	0.48	0.09
Drugs in use × ANGPT-1 9 days	0.57	0.04*

Non-parametric correlation measurement of the medication in use by patients (β-blocker, antiplatelet, vasodilator) and molecules expression, in the group undergoing gene therapy on different days of analysis. Stromal Cell-Derived Factor-1 (SDF-1α); Angiopoietin-1 (ANGPT-1); r = correlation.
* p < 0.05. Statistical test used: Spearman correlation coefficient.

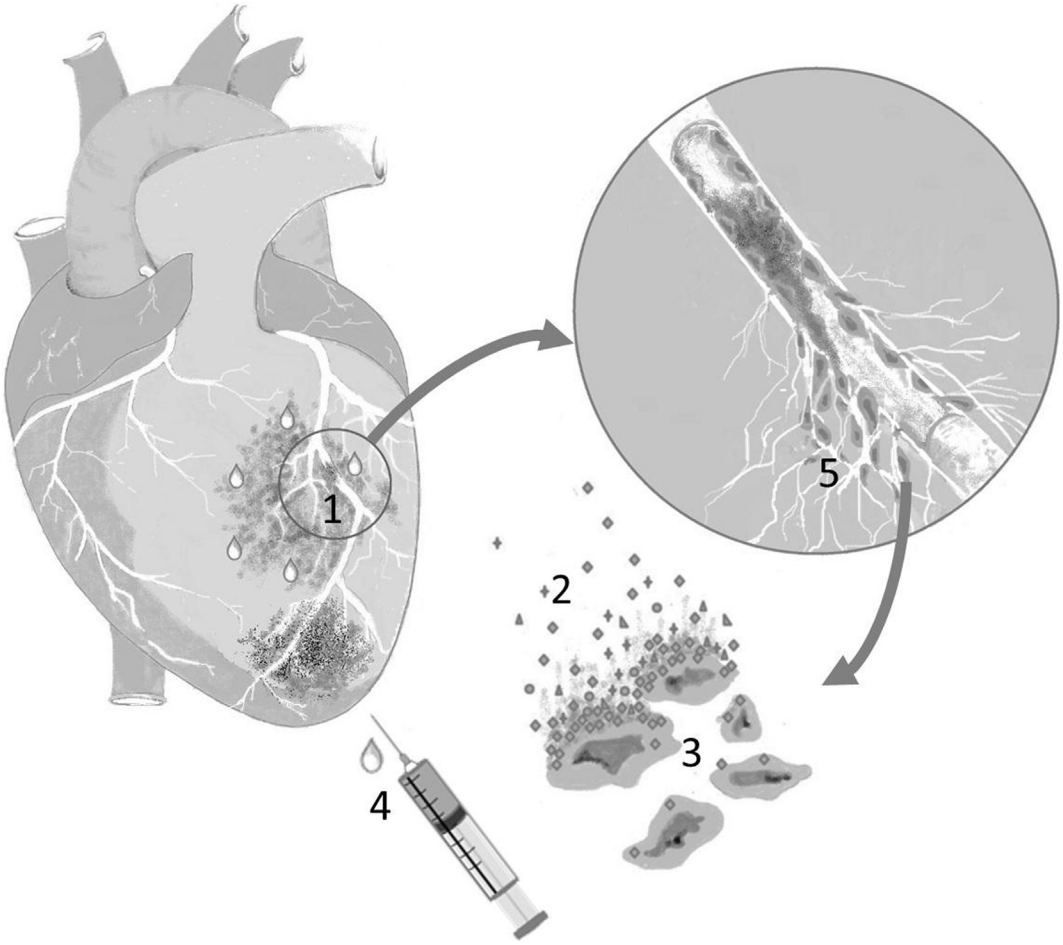

Fig. 2. Cooperative paracrine signaling in response to gene therapy with VEGF in patients with refractory angina. Coronary Artery Disease (CAD) causes an ischemic process where factors such as hypoxia, endothelial dysfunction and generation of reactive oxygen species (oxidative stress) causes the expression of proinflammatory molecules (IL-6, TNF-α) by cells of the immune system (1). In addition to these proteins, chemokines such as SDF-1, which is a chemoattractant gradient (2) for other immune cells (macrophages and leucocytes) and progenitor cells (such as endothelial or mesenchymal cells) which are able to regenerate tissue (3). The process is slow and limited due to the poor healing capacity of the weakened body. If a molecular intervention occurs, for example, by gene therapy with proangiogenic factors such as VEGF (4), progenitor cells will be able to respond more easily to the chemoattractant gradient, restoring endothelial function equilibrium (balance ET-1/eNOS) and secrete more angiogenic factors (VEGF and ANGPT-1), promoting the formation of new vessels by feedback from the injured region (5). This cooperative signaling is time-dependent. The molecules are expressed according to need and respond positively to gene therapy.

external factor. TNF-α, the first cytokine released after injury, produces a dyslipidemic state and activates endothelial cells [19], resulting in vasoconstriction and homeostasis. At all times, its expression was lower in the intervention group than in the control group, demonstrating that the pro-inflammatory action was not exacerbated by the gene therapy. Furthermore, published data suggests that β-blockers (mainly carvedilol) have been shown to decrease serum concentrations of the inflammatory cytokine TNF-α in patients with ischemic and nonischemic dilated cardiomyopathy [20]. This may explain the findings for this cytokine in our intervention group, since almost all the patients included were using this class of drug (Table 2).

Similarly, the plasma levels of the anti-inflammatory cytokine IL-10 were lower at all times when compared to the control group. In the intervention group, there was a slight increase in IL-10 levels on day 3, which is consistent with its role in suppressing the inflammatory process, whereby it balances the pro-inflammatory effects generated by IL-6 and TNF-α [21]. Experimentally, it has been shown that IL-10 is capable of protecting endothelial function after acute inflammatory stimulus by limiting increases in vascular superoxide generation within the inner wall of the vessel [22]. The polarization of the immune response and the cytokines released by different cellular mechanisms influenced the response to injury, including the cell homing involved in tissue regeneration.

Progenitor cell homing occurs both in myocardial infarction, due to the high release of SDF-1, as well as in chronic diseases [23]. Additionally, it has been suggested that SDF-1 not only acts as a chemotactic factor, but also on the retention of pro-angiogenic cells in the perivascular region [24]. Finally, short-term monitoring does not provide comparisons for tissue fixation and the long-term function of cells applied. However, without the initial homing, therapies that aim to manage growth factors would not be effective, because the cells would not be able to engage in paracrine and regenerative activities.

In the case of angiogenesis, the results from Yamaguchi et al. [25] indicate that SDF-1 augments vasculogenesis and subsequently contributes to ischemic neovascularization *in vivo* by recruiting endothelial progenitor cells in ischemic tissues. Although SDF-1 has been shown to increase angiogenesis in multiple disease models and in different tissues, this chemokine may be acting as a chemoattractant for endothelial progenitor cells rather than a growth factor on endothelial cells [26]. Our findings showed mobilization of EPCs regardless of increasing of SDF-1, but correlated with the increased of VEGF expression (Fig. 1 and Table 3).

Importantly, our results suggest a reduction, although insignificant, in levels of SDF-1 after the intervention of gene therapy. By contrast, in the control group there was a trend towards increased of this chemokine on day 27, there is no difference with respect to the group underwent application of a plasmid containing the gene that encodes the protein VEGF. While SDF-1 levels may be higher depending on the inflammatory status of patients with severe CAD, it seems that the gene therapy could assist in the regulation of cytokine release. SDF-1 specifically binds to a membrane receptor, CXCR4, G protein-coupled, and this interaction results in activation of signaling pathways for chemotaxis of various cell types, such as lymphocytes, stem cells and neurons [27–29]. The CXCR4, as well as the β-adrenergic receptors, undergoes regulation by GRKs proteins that inhibit G protein, also desensitizing this class of receptors [30,31]. Knowing that patients in the intervention group were making use a range of drug classes, including β-blockers (Table 2), the communion of specific signaling pathways can lead to desensitization of the receptors, inhibiting the physiological restorative functions. In this way, the release of the factors implicated in stem cell homing probably has suffered an influence from parallel pharmacological treatment used by the group undergo gene therapy (Table 4), resulting in inhibition of proliferation, migration and differentiation of endothelial cells [32].

In relation to neoangiogenesis, the study showed a transitional increase in VEGF and ANGPT-1 levels on days 3 and 9 after the gene therapy intervention with exogenous VEGF. VEGF is reported to be a potent activator of endothelial cells and to stimulate the formation of new vessels, whereas ANGPT-1 is required for the release of VEGF and the maturation, integrity and development of such vessels [33]. Due to this capacity, VEGF gene therapy has been employed in a large number of clinical studies [8,9,34,35]. Tao et al. [36] showed that cardiac-specific and hypoxia-induced co-expression of VEGF and ANGPT-1 improves the perfusion and function of the heart in porcine myocardial infarction by inducing angiogenesis and cardiomyocyte proliferation, activating pro-survival pathways and reducing cell apoptosis.

The cell recruitment process and angiogenesis are directly related to an improvement in endothelial function. In our study, we analysed the expression of the ET-1 protein to evaluate the vasoconstrictor endothelial function [37,38] in these patients. At baseline, the intervention group showed a low ET-1 protein expression and high variance between the evaluation days, which is certainly due to the fact they had CAD and consequently endothelial dysfunction. This result suggests a vasoconstrictor response and indicates a possible combined effect of multiple vasoconstrictor and vasodilator endogenous factors. Additionally, the patients presented an increased systemic inflammatory response. Thus, the expression of inducible nitric oxide synthase (iNOS) is upregulated in response to inflammatory cytokines that are elevated in many diseases, including CAD [39]. Also, the factors that regulate cell maintenance, repair and angiogenesis should be further investigated in order to optimize gene therapy, such as, begin to consider the influence of the parallel medication in use (β-blocker, antiplatelet, vasodilator).

1.4. Conclusion

Analysis of the cooperative paracrine signaling after VEGF gene therapy, in this group of patients, suggests that the expression and recruitment of molecules are time-dependent, influenced by the chronic inflammatory process (CAD) and medication in use. Moreover, the gene therapy stimulates the expression of the immune system cells and angiogenic molecules, providing the angiogenic process.

Conflict of interest

The trial was designed and conducted independently by the authors, with financial support from the government agencies cited above (FAPERGS and CNPq) and FAPICC. No competing financial interests exist.

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