

Human Interleukin-10 Naked DNA Delivery to Infarcted Pig Heart by Catheter Mediated Retrograde Injection in Coronary Sinus

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Abstract

Background: Interleukin-10 (IL-10) has proved to be important in recovery after acute myocardial infarction; increasing its expression in infarcted or bystander tissue therefore could be of great importance. Hydrodynamic DNA injection has been found to be very efficient in transferring genes to the liver of small animals, but the procedure is very aggressive and must be made compatible with clinical practice in a milder but not less efficient way. The present work evaluates the efficiency of noninvasive catheterization of the coronary sinus for human IL-10 gene transfer to infarcted and non-infarcted pig heart, with therapeutic production of the human protein.

Methods: Myocardial infarction was induced in pigs by a catheter-based approach to occlude the left anterior descending artery. After myocardial infarction verification, two catheters were placed in the coronary sinus, one of them to block blood circulation and the other to retrogradely inject 50 ml of a saline solution of DNA (20 µg/ml) containing the hIL-10 gene, and testing different flow rate conditions (control, 2, 5 and 10 ml/s).

Results: Therapeutic levels of hIL-10 protein were found in coronary sinus blood 2 and 72 hours after catheter-mediated hydrofection at 5 and 10 ml/s flow rate. Molecular analyses to evaluate the delivered DNA, its transcription to RNA and translation were also performed, and data were expressed as copies per cell.

Conclusion: Catheter-mediated gene transfer through the coronary sinus is a mild and well-tolerated procedure that achieves protective hIL-10 protein levels, which could minimize the inflammatory response after myocardial infarction.

Keywords: IL-10; Myocardial infarction; Gene therapy; Heart; Catheter; Naked DNA; Coronary sinus; Retrograde

Introduction

Several clinical trials with limited but encouraging results suggest that cardiac gene therapy is a promising therapeutic strategy for heart diseases such as ischemic events and heart failure [1-7]. Viral and non-viral vectors have been employed, with good results [8,9]. The classical lesser efficiency of non-viral procedures has been overcome by hydrodynamic gene transfer to mouse liver employing naked DNA, which has been shown to be an efficient and safe procedure in gene therapy [10-12]. This procedure involves the rapid injection of a large volume (2 ml) of solution containing the gene of interest through the tail vein. Important progress has been made in non-viral gene therapy since the hydrodynamic procedure for mouse liver gene delivery was first described [11,13-15]. However, the systemic hemodynamic stress caused after injection poses a serious limitation for clinical application, since it can even lead to circulatory collapse and death. To avoid this adverse effect, different approaches have been used to locally reproduce in pig liver the conditions mediated by tail vein hydrodynamic injection in mice, with a view to improving gene transfer efficiency [15]. Our group achieved important advances in the efficacy of naked DNA delivery to mouse liver and its transfer to larger animals employing noninvasive catheterization [16-18]. The promising results obtained with this procedure suggest that similar or even better results could be achieved in cardiac tissue, since it is a smaller organ with a muscular structure that could offer more resistance to injection-induced distension. In fact, this procedure was successfully transferred to the pig heart) through catheter-mediated retrograde injection into

the coronary sinus of 50 ml of a DNA solution containing the eGFP gene [19]. This procedure could find clinical application [20].

Once the expression of a marker protein was assured, evaluating the whole process of information decoding of a human gene was required. With this aim, we employed the mentioned procedure to inject a DNA solution containing the gene encoding for human interleukin-10 (IL-10) in pig heart after the induction of myocardial infarction. After 72 hours, tissue and blood samples were collected for further molecular analyses and evaluation of the whole gene decoding process (delivery, transcription, translation and expression).

In the present work we explore the potential therapeutic capacity of clinically applicable hydrodynamic gene delivery of hIL-10 to cardiac tissue, after induced infarction. The results show catheter-mediated retrograde injection of naked DNA bearing hIL-10 gene in the pig coronary sinus vein to result in whole cardiac gene delivery and efficient protein expression in plasma. These results contribute to

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introduce interventional catheterization as a noninvasive procedure for cardiac gene delivery in humans, with potential clinical application.

Material and Methods

Animals

Female pigs (18-23 kg) were obtained from a farm supplying the Central Service of Research Support (SCSIE) of the University of Valencia (Valencia, Spain), and were housed in individual pigsties. The experiments were approved by the Animal Biological Research Ethics Committee of the Faculty of Medicine of the University of Valencia (Spain).

Anesthesia was induced with ketamine (Imalgene® 100, Merial France; 5-10 mg/kg, im), midazolam (Hospira® 1 mg/ml, Madrid, Spain; 0.3 mg/kg, im) and propofol (Lipuro® 2%, Braun, Melsungen, Germany; 4-6 mg/kg, iv), and was maintained with isoflurane (Isoflo®, Abbott laboratories, Madrid, Spain; 2.5% via the inhalatory route). Muscle relaxation was induced with vecuronium bromide (Norcuron® 10 mg; 0.08 mg/kg, iv). Morphine (0.4 mg/kg, iv) was administered for intraoperative analgesia, and buprenorphine (Buprex®, Schering-Plough, Madrid, Spain; 0.02 mg/kg, iv) was used for postoperative analgesia. Vital functions were monitored throughout the intervention. The pigs were sacrificed three days after the operation using potassium chloride (Braun 2 mEq, 20 mEq, iv), after sedation. Blood samples (2 ml) were collected from an ear vein at two and 72 hours after injection, before sacrifice. The heart was extracted, and representative tissue samples of each area (were collected for further analysis).

In vivo gene transfer in the pig

Arterial pressure and ECG were continuously monitored throughout the experiment using a 5 F sheath placed in the right coronary artery and a 6-lead ECG system. Myocardial infarction was induced by blocking the coronary blood circulation with a balloon catheter. After ECG confirmation of myocardial infarction, the balloon was deflated and other catheters were placed in the coronary sinus to inject the DNA solution. After right and left jugular puncture, two 7 F sheaths were introduced; a 7 F Swan-Ganz catheter (Arrow International, USA) and a 6 F multi-purpose catheter (Johnson & Johnson, USA) were placed in the coronary sinus vein. X-ray imaging and the perfusion of Ultravist 370 contrast solution (Iopramide, Schering) were used to guide placement of the catheters. In a typical experiment, the coronary sinus vein was sealed by inflating the Swan-Ganz catheter balloon, and closure was confirmed by the injection of contrast through the multi-purpose catheter. Then, 50 ml of saline solution containing the p2F-hIL-10 plasmid (20 µg/ml) was retrovenously injected through the multi-purpose catheter at 2 ml/s (n=1), 5 ml/s (n=3) and 10 ml/s (n=2) flow rates. A control was also employed, with induced infarction but no gene transfer catheterization. Five minutes after injection, the balloon was deflated and the catheters removed. Blood samples were taken from the coronary sinus before gene transfer, at "time 0", and two and 72 hours post-transfection. The animals were sacrificed 72 hours after transfection with an intravenous KCl injection; the heart was removed, and tissue samples for molecular analyses were taken from 6 different areas: right atrium, left atrium, anterior right ventricle, anterior left ventricle, posterior right ventricle and posterior left ventricle. In the groups where gene transfer was performed, additional samples from the infarcted area were also taken.

Reagents

Tissue extraction of DNA and RNA was performed employing the

commercial Maxwell 16 Mouse Tail DNA Purification Kit and Maxwell 16 LEV simply RNA Tissue Kit, respectively, from Promega (Barcelona, Spain). The specific kit for human IL-10 TaqMan quantitative PCR was obtained from Life Technologies (cat no. Hs00961622_m; CA, USA). The anti-hIL-10 antibodies kit was obtained from BD (cat. no. 555157; CA, USA). The plasmid p2F-hIL-10 (6.86 Kb), containing the human IL-10 protein cDNA driven by pCMV promoter, was constructed by cloning IL-10 into the Hind III site of pVITRO2 (Invitrogen). The total protein content of each sample was quantified using the NanoOrange protein quantitation kit (Life Technologies; CA, USA).

hIL-10 ELISA

Blood samples were collected at two and 72 hours, and tissue samples representing the whole heart were cut into small pieces 72 hours after gene transfer, and homogenized in homogenization buffer (Promega®, Barcelona, Spain) with an Ultra-Turrax homogenizer (Hielscher Ultrasonics GmbH, Teltow, Germany). Total protein amount was quantified using the NanoOrange protein quantitation kit (Life Technologies; CA, USA). ELISA was performed following the instructions of the manufacturer. Results of protein expression in tissue were calculated per cell assuming the total amount of protein to be 0.5 ng in a mammalian cell.

Quantitative PCR and RT-PCR

Tissue samples from the above mentioned territories, representing the whole heart, were cut into small pieces and homogenized in homogenization buffer (Promega®, Barcelona, Spain) with an Ultra-Turrax homogenizer (Hielscher Ultrasonics GmbH, Teltow, Germany). Further purifications with the Maxwell RNA and DNA purification from tissue kits (Promega®, Barcelona, Spain) were performed before spectrophotometric quantification. RNA retrotranscription to cDNA was carried out using 1 µg total RNA (DNA free), random hexamers and a High Capacity cDNA Archive Kit (Applied Biosystems). For quantitative real-time qPCR, TaqMan PCR master mix (Applied Biosystems) was employed according to the instructions of the manufacturer. The specific oligonucleotides for human IL-10 employed were a pre-mixed TaqMan kit from Life Technologies (cat no. Hs00961622_m).

Quantitative data were calculated as the number of DNA and RNA copies on a regression curve which was plotted employing the injected plasmid containing the hAAT gene. Data plotting was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

The data have been expressed considering the weight of the pig diploid genome (5.4 pg) for gene delivery, and the average content of RNA per cell in a mammalian hepatocyte (20 pg) for gene transcription [21].

Gold nanoparticle synthesis

All chemicals were purchased from Sigma-Aldrich (Madrid, Spain) and used as received, without further purification. Gold nanoparticles were synthesized following a modification of the Turkevich method [22]. Typically, 0.4 g of H₂AuCl₄·3H₂O was dissolved in 90 ml of water in a two-neck round flask (1.3 mM H₂AuCl₄). The resulting solution was heated to boiling and refluxed. Then, 9 ml of a 47.2 mM sodium citrate solution (0.125 g sodium citrate in 9 ml water) was preheated and quickly added. The solution turned from yellow to black and to deep red. After the color changed, the solution was refluxed for 20 min. Then, the heater was turned off and the solution was stirred until it reached room temperature. The resulting solution was diluted with water to a final volume of 500 ml. Gold nanoparticles were

characterized by Transmission Electron Microscopy (TEM) (Jeol 1010, Tokyo, Japan). The average diameter and the size distribution of the gold nanoparticles were determined from TEM images by counting at least 300 nanoparticles using Image Pro Plus software from Media Cybernetics (Bethesda, MD, USA).

Transmission electron microscopy

Pig hearts were injected (volume 50 ml) through the catheter placed in the coronary sinus with a solution of citrate buffer containing 10^{12} gold particles (4 nm and 15 nm in diameter) per ml to study the heart distribution of gold nanoparticles after injection, depending on the size of the particles. Then, small tissue pieces from different heart areas were removed and immersed in Sørensen phosphate buffer solution (pH 7.4) containing 2.5% glutaraldehyde. For TEM, multiple 1-mm³ pieces of heart were routinely processed and embedded in Epoxy resin. Ultrathin sections, stained with uranyl acetate, were examined under a Jeol JEM-1010 electron microscope. The sections were not stained with lead citrate as usual, in order to avoid the loss of contrast of the small gold nanoparticles and facilitate their observation.

Results

Catheterization

In anesthetized pigs, two types of catheters with (Swan-Ganz) and without a balloon (multi-purpose) were employed, and correct perfusion of the coronary sinus was monitored by fluoroscopy after the injection of contrast solution. Both catheters were placed in the coronary sinus through the jugular vein. The multi-purpose catheter was placed in a distal position in the coronary sinus, and was used for plasmid DNA or contrast injection. The Swan-Ganz catheter was placed in the proximal position and was inflated to guarantee vein sealing in order to avoid retrovenous flow escape during injection. Figure 1 shows the positioning of the two catheters reaching the coronary sinus on the posterior side of the heart, with fluoroscopy imaging in a typical experiment in which 5 ml of contrast was injected just after balloon inflation. We confirmed that the contrast persisted as long as the balloon remained inflated, and the contrast mark observed confirmed that the whole coronary sinus was full of solution, since the contrast flowed out to the azygos vein, which is known to lead to the coronary sinus in pigs. No significant arrhythmias, ECG changes or hemodynamic destabilization occurred during the pig preparation or injection phase, and no significant events occurred over the subsequent 72-hour period before sacrifice. Due to the good tolerance of the procedure, inotropic agents were not administered.

hIL-10 gene transfer: ELISA protein detection and molecular analyses

Following the experimental procedure described, after inflation of the balloon of the proximal catheter, contrast was injected through the distal catheter to confirm the appropriate position and conditions for injection. Then, 50 ml of saline solution containing plasmid p2F-hIL-10 (20 µg/ml) was injected through the distal catheter at a flow rate of 5 ml/s (n=3 pigs) and 10 ml/s (n=2 pigs). Two additional pigs were employed: one injected under milder conditions (2 ml/s) and other one without hydrodynamic gene injection, as controls. Five minutes after the injection, the balloon was deflated, the catheters were removed, and the animals were allowed to recover from anesthesia. The pigs were sacrificed 72 hours after transfection, and the hearts were removed to collect the 6 non-infarcted cardiac tissue samples from the heart atria (left and right), right ventricle (anterior and posterior) and left ventricle

(anterior and posterior). One additional sample was also collected from the infarcted area for evaluating the efficacy of the procedure. In the control group, all the samples from non-infarcted and infarcted areas were analyzed together, since no gene transfer was performed in this group.

Figure 2 shows the hIL-10 protein levels detected in the blood samples obtained directly from coronary sinus, two and 72 hours after gene transfer. The control and 2 ml/s groups showed undetectable levels of the human protein, while the 5 ml/s and 10 ml/s groups had approximately 80-90 pg/ml and 40-60 pg/ml, respectively. The levels of

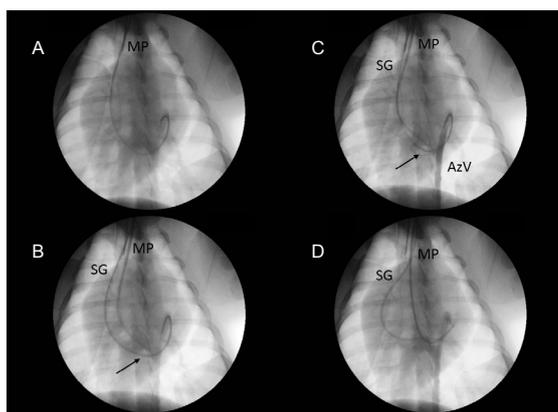


Figure 1: Catheterization of the coronary sinus. In anesthetized pigs, two catheters were introduced through the jugular vein into the coronary sinus. One of them, the Swan-Ganz (SG) catheter equipped with a balloon, was placed in a proximal position. The multi-purpose (MP) catheter, placed in a more distal position, was used to retrogradely inject contrast solution immediately after the balloon (black arrow) of the former catheter was inflated. Upper left: fluoroscopic view of MP catheter placement in the coronary sinus; Lower left: fluoroscopic view of MP and SG catheter placement in the coronary sinus with inflated balloon (black arrow); Upper right: fluoroscopic view after balloon inflation (black arrow) and injection of contrast solution into the coronary sinus and azygos vein (AzV); Lower right: contrast solution disappearance after balloon deflation.

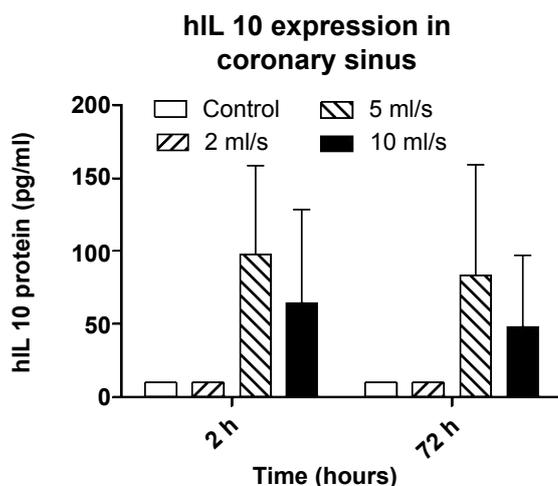


Figure 2: hIL-10 protein expression in plasma. After infarct induction, 50 ml of saline solution containing hIL-10 plasmid was injected retrovenously at 2, 5 or 10 ml/s into the coronary sinus. Blood samples from the coronary sinus were collected at two and 72 hours, and protein was quantified by ELISA. Protein concentrations (pg/ml) in blood after injection under different conditions were compared.

this protein under normal healthy conditions are lower than 3 pg/ml in humans, approximately.

The molecular studies on the samples collected for PCR and RT-PCR were evaluated on the basis of the number of molecule copies per cell, as already described by our group.

Briefly, after DNA and RNA purification, quantitative PCR was carried out employing a standard curve prepared with the same plasmid injected to quantify the copy number. Results are expressed as copies per cell DNA and RNA based on the total amount of these per cell in pig cells.

Figure 3 shows the results of the molecular analyses for non-infarcted cardiac tissue, in the samples taken 72 hours post-transfection, after sacrifice. It includes part of the data of Figure 2, regarding protein production in coronary sinus blood at 72 hours, to facilitate the comparisons. As expected, the control group did not show any record at any of the analyzed times. The group injected at 2 ml/s showed a small amount of DNA copies per cell (0.01 copies/cell) that were transcribed to RNA (0.01 copies/cell) in the tissue, but were not detectable in coronary sinus blood. The group injected at 5 ml/s achieved higher DNA and RNA copies per cell. Finally, although the group injected at 10 ml/s had a level of DNA copies/cell similar to that of the 5 ml/s group, lower levels of RNA copies were obtained, with similar protein levels in tissue that in turn become lower in the sinus blood samples.

Figure 4 shows the corresponding data from infarcted cardiac tissue in the same four study groups. Under mild conditions of 2 ml/s, no significant changes were detected between non-infarcted versus infarcted tissue, whereas DNA delivery at 5 ml/s and 10 ml/s was more than 10-fold higher in infarcted tissue than in normal tissue. However, the final expression of the protein in tissue was lower.

Electron microscopy

The ultrastructural study was made to evaluate the tissue distribution of nanoparticles according to their diameter, in order to know how the natural barriers of the heart tissue limit nanoparticle access to cardiomyocytes. The observation of small nanoparticles required an increase in tissue contrast; we therefore had to avoid the use of lead-stained samples, and consequently the morphological structures are not perfectly defined.

Gold nanoparticles measuring 4 and 15 nm in diameter were injected under the same conditions of injection as the plasmid. The tissue and/or intracellular location of the particles was observed by electron microscopy. The diameters of the nanoparticles were chosen to observe selective passage through tissue membranes and barriers. The images show an inverse proportional relationship between particle size and penetration capacity (Figure 5). The panel at left offers a wide view of a cardiomyocyte and its nucleus. Detail A corresponds to the amplified square area of this cell, where the particles can be identified. We systematically found smaller diameter (4 nm) particles in the cytoplasm, always close to Z-band areas, suggesting these to participate in their distribution. However, we failed to identify larger nanoparticles within the cardiomyocytes.

Discussion

The present work shows that the retrograde injection of naked DNA encoding for the human interleukin-10 gene in the coronary sinus results in whole cardiac gene transfer, since hIL-10 DNA, RNA and protein were identified by quantitative PCR, RT-PCR and ELISA

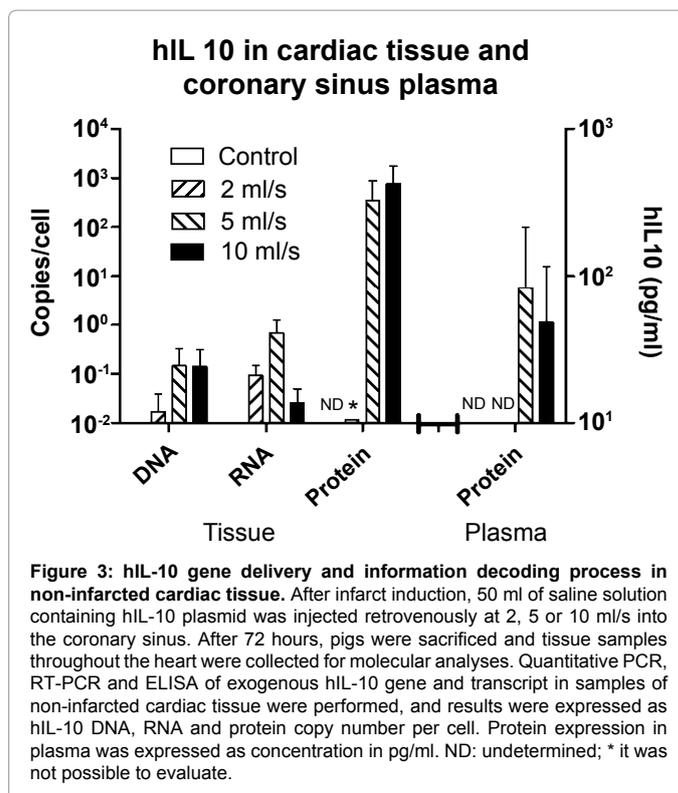


Figure 3: hIL-10 gene delivery and information decoding process in non-infarcted cardiac tissue. After infarct induction, 50 ml of saline solution containing hIL-10 plasmid was injected retrovenously at 2, 5 or 10 ml/s into the coronary sinus. After 72 hours, pigs were sacrificed and tissue samples throughout the heart were collected for molecular analyses. Quantitative PCR, RT-PCR and ELISA of exogenous hIL-10 gene and transcript in samples of non-infarcted cardiac tissue were performed, and results were expressed as hIL-10 DNA, RNA and protein copy number per cell. Protein expression in plasma was expressed as concentration in pg/ml. ND: undetermined; * it was not possible to evaluate.

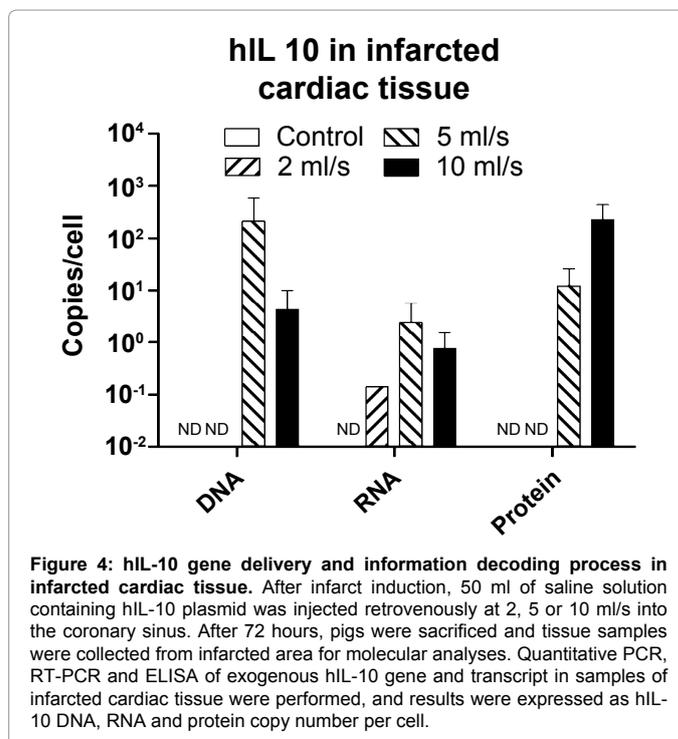


Figure 4: hIL-10 gene delivery and information decoding process in infarcted cardiac tissue. After infarct induction, 50 ml of saline solution containing hIL-10 plasmid was injected retrovenously at 2, 5 or 10 ml/s into the coronary sinus. After 72 hours, pigs were sacrificed and tissue samples were collected from infarcted area for molecular analyses. Quantitative PCR, RT-PCR and ELISA of exogenous hIL-10 gene and transcript in samples of infarcted cardiac tissue were performed, and results were expressed as hIL-10 DNA, RNA and protein copy number per cell.

in the samples collected from both non-infarcted (left and right atria, left and right ventricles) and infarcted areas. The human IL-10 protein was present in blood from the coronary sinus for at least 72 hours, as demonstrated by ELISA.

In the last decade, more than 20 gene therapy trials have been

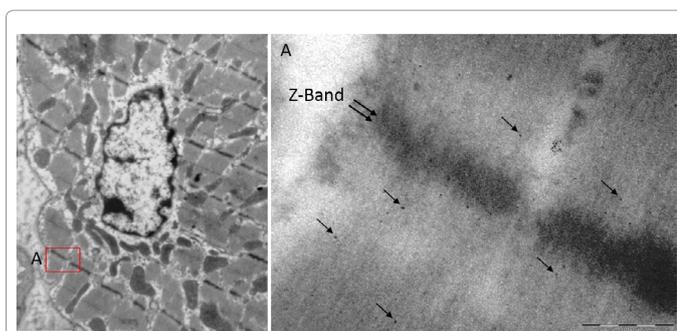


Figure 5: Gold nanoparticle distribution in hepatocytes. A 50 ml volume of citric buffer solution containing gold nanoparticles with diameters of 4 nm and 15 nm (10^{12} nanoparticles of each size per ml) was injected into the heart at 5 ml/s. The image at left shows a cardiomyocyte with its nucleus. Box A corresponding to the squared area is enlarged (image at right). Only 4 nm diameter particles are present within the cell, especially surrounding the Z-band area. The 4 nm particles are widely distributed within the cytoplasm, while the larger nanoparticles appear to be less abundant.

performed, with limited but promising results [1-4]. However, vector efficiency and safety remain as the principal unresolved issues in application to clinical therapy [23]. Viral vectors offer higher transduction efficiency, but this does not necessarily imply greater efficacy; furthermore, their capacity to produce toxicity and immunological responses persists [24,25]. Naked DNA remains the safest procedure for transferring exogenous gene to the myocardium, and the results obtained in clinical trials have demonstrated potential interest. However, the transfection efficiency, stability and long-term expression of the therapeutic genes need to be improved [26]. In the present study we show that catheter-mediated retrovenous injection into the coronary sinus is a well-tolerated procedure for DNA delivery to cardiac tissue which leads to efficient expression of foreign protein in sinus blood for at least three days.

With the purpose of evaluating the efficiency of gene delivery and the steps of the decoding process, pigs were sacrificed 72 hours after transfection, and tissue samples from different parts throughout the heart were collected for molecular studies. The expression of hIL-10 protein in sinus plasma was quantified by ELISA at two and 72 hours, before sacrifice, and under the best conditions the concentrations reached were close to 100 pg/ml (normal values: <3 pg/ml). It has been reported that a blood hIL-10 concentration of 124 pg/ml is able to inhibit by half the effects of the proinflammatory protein TNF α [27]. Accordingly, the IL-10 expression achieved by this procedure could mediate local immune modulation in the heart that would not affect other organs. In order to study the efficiency of each step involved in the information decoding process, hIL-10 DNA, RNA and protein in tissue were quantified in all cardiac tissue samples (infarcted and non-infarcted), and the results indicated that transgene expression was present and extensively distributed in the whole cardiac tissue. This result is in agreement with the presence of human protein in plasma and with recent observations indicating that retrograde injection of the coronary sinus vein is efficient [28].

Human IL-10 gene delivery has been expressed as gene copies per cell, considering the weight of one diploid genome in pig to be 5.4 pg [21]. The results indicate that injecting at 5 and 10 ml/s achieved higher (10-fold) gene delivery than 2 ml/s. The index of gene delivery (gene copies/cell) markedly increased in infarcted tissue to more than 100 gene copies per cell, maybe due to the state of cell membrane disrepair. When the transcription index was evaluated, we found that whereas 5

ml/s and 10 ml/s achieved similar delivery indexes, the RNA level was more than 10-fold higher at 5 ml/s and was slightly higher in infarcted tissue. We hypothesize that injection injury could affect the cells, limiting the efficiency of transcription. Protein translation in tissue was also evaluated, and surprisingly, the levels of hIL-10 protein reached were very similar with the 5 ml/s and 10 ml/s flow rates, despite the difference in RNA. This might occur if an excess of transcribed RNA causes the translational machinery to operate at saturation in both cases. However, in this case this does not appear to be the cause, and therefore other mechanisms of downregulation must be involved. The high translation index in cardiac tissue supports the good efficiency of the procedure, and is correlated to expression efficacy, since the hIL-10 gene encodes for a secreted protein that is released into the bloodstream. The data from different areas were averaged, but little dispersion was observed among them.

These results are interesting, since it has been reported that after infarction, the areas surrounding the necrotic tissue are also affected by the elicited inflammatory response. Thus, IL-10 transgene expression could modulate the local inflammatory response and play an important role in protecting the heart tissue from the negative effects induced by the inflammatory environment. In this respect, naked DNA gene delivery by retrograde injection could offer interesting advantages for safe clinical application. The effective plasma expression of IL-10 during three days suggests that the final efficacy of the gene transfer procedure could be of functional relevance in different pathologies, depending on the gene employed. Further long-term studies would be needed to evaluate the persistence of gene expression. The choice of the correct gene construct could improve the final efficiency of the procedure in terms of intensity and/or expression persistence, avoiding the main problems facing cardiac gene therapy (inefficient gene transfer, host responses to the procedure, and a lack of sustainable therapeutic transgene expression) [29,30].

Regarding the gene of interest, hIL-10 has been widely evaluated in the infarcted heart setting. The prognosis of patients suffering from myocardial infarction is mainly determined by infarct size, which in turn depends on the inflammatory response of the myocardium [15]. Several studies have reported that a local increase in IL-10 reduces infarct size and attenuates tissue injury by modulating the inflammatory response, although this point remains subject to controversy [15,31-37]. The transfer of IL-10 or any other anti-inflammatory gene such as TGF- β could exert a similar tissue protection effect against pro-inflammatory cytokines, and could be of great interest in the solid organ transplantation setting with a view to exploring ways to improve graft tolerance [38]. It has also been reported that the retrograde transplantation of cells into the heart via the coronary vein is an efficient and scantily invasive angiogenic therapeutic procedure [7,39,40].

In conclusion, we provide evidence that catheter-mediated retrovenous gene transfer into the coronary sinus could be of potential interest in the clinical setting, since it is a harmless procedure for gene transfer that achieves therapeutic plasma levels of protein encoded for by the delivered gene.

Acknowledgments

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