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Regenerative therapies in electrophysiology and pacing

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Abstract

The prevention and treatment of cardiac arrhythmias conferring major morbidity and mortality is far from optimal, and relies heavily on devices and drugs for the partial successes that have been seen. The greatest success has been in the use of electronic pacemakers to drive the hearts of patients having high degree heart block. Recent years have seen the beginnings of attempts to use novel approaches available through gene and cell therapies to treat both brady- and tachyarrhythmias. By far the most successful approaches to date have been seen in the development of biological pacemakers. However, the far more difficult problems posed by atrial fibrillation and ventricular tachycardia are now being addressed. In the following pages we review the approaches now in progress as well as the specific methodologic demands that must be met if these therapies are to be successful.

Keywords

Gene therapy of arrhythmias; Cell therapy of arrhythmias; Treatment of bradycardias; Treatment of tachycardias

1 Introduction

Ventricular tachycardia (VT) and/or fibrillation lead to sudden cardiac death in 200,000–400,000 individuals/year in the USA, alone, accounting for up to 20% of all deaths in adults [1–4]. Atrial fibrillation (AF) afflicts about 2.3 million Americans [5], may reach 12–16 million by 2050 [6], and brings increased risk of stroke, heart failure, and death [7]. Despite these grim statistics, over a half-century of basic and clinical investigation with drugs and, more recently devices, have only modestly affected therapy and prevention. In the following pages, we review how this situation has come about, and new directions being followed to alleviate it.

2 The status of drug and device therapies: from empirical to mechanistic

From Withering's administration of a decoction of foxglove to his patients [8] through clinical use of quinidine, procaine and diphenylhydantoin in the first half of the twentieth century [9, 10] antiarrhythmic drug therapy was largely empirical. Singh and Vaughan Williams made the first widely accepted attempt to develop antiarrhythmic strategies based on an understanding of mechanism [11], defining drug actions largely via their electrophysiologic effects on isolated tissues. Their work was a major stimulus for developing Class 3 drugs as clinical antiarrhythmics. However, large clinical trials [12, 13] demonstrated virtually all antiarrhythmics tested to be proarrhythmic and that none was an acceptable primary or sole therapy for ischemic heart disease-associated. VT (reviewed in [14]). The picture has been marginally brighter for AF as flecainide, dofetilide, sotalol, amiodarone and other drugs have found therapeutic roles early in its evolution [7].

Exciting recent information regarding therapy includes (1) drugs that are not antiarrhythmic and (2) devices. Considering the former, the administration of *angiotensin II converting enzyme inhibitors* and of AT-1 receptor blockers has led to a reduction in AF recurrences in

several pathologic settings [7, 15–18]. This result emphasizes that approaches “upstream” to the usual ion channel targets may be of antiarrhythmic benefit through their modulation of channels as well as through the prevention or reversal of structural and/or electrophysiologic remodeling.

Yet it is device therapy that has seen the greatest antiarrhythmic advances. Whether it is biventricular pacing to reduce heart failure and its attendant arrhythmias, implantable cardioverter defibrillators to terminate life-threatening tachycardias (and less-effective atrial defibrillators for AF), ablation (which is time-consuming, often difficult to perform and has a moderate success rate) or (less-utilized now) surgical techniques for a variety of reentrant or focal atrial or ventricular arrhythmias, it is clear that devices that stimulate, shock or burn have significant antiarrhythmic efficacy (reviewed in [14]). For example, trials like MADIT, MADIT-II, ScD-HeFT and DEFINITE [19–22] convincingly show survival benefit in treating potentially lethal ventricular arrhythmias in the setting of ischemic heart disease.

However, device therapies are dependent on the application of invasive technology to deposit significant hardware in the body that has both physical and psychological impact. In addition, use of devices varies widely from community to community in the USA and Europe, and because of the cost (estimated as varying between \$28,000 [23] and \$210,000 [20] per year of life saved) alternative approaches would be welcomed. Hence, it would be desirable to have safe and effective alternatives.

3 Rationale for applying gene and cell therapy to the prevention/termination of arrhythmias

One obvious step to prevent/treat cardiac arrhythmias would be to repair or replace the myocardium responsible for them. This is both a dream and a direction that is being taken by a number of investigators, including our own group. But as attractive as myocardial repair/replacement may appear, we think it unwise to move uniquely in that direction. There are too many pitfalls, real and imagined, to accept this as the only rational approach to the problem of arrhythmias.

Another window that has been opened for developing new treatments is the genetic modification of targets. This relies on a technology that can target sites on proteins with a selectivity that neither antiarrhythmic drugs nor devices have approached to date. The technology derives from advances in genetics coupled with those in biophysics and molecular and structural biology. The sum of these has permitted us to relate the functional to the structural underpinnings of ion channels and connexins and has been applied to both the diagnosis and potential treatment of arrhythmias, especially those resulting from abnormalities of repolarization (reviewed in [14]). With regard to structural biology, this has identified a subset of targets for therapy as 3-dimensional constructs, and has identified the elements of the cytoskeleton and their role in the function and trafficking of intracellular components including ion channels (reviewed in [24]). In addition, studies of signaling pathways have demonstrated how components of the myocyte, the autonomic nervous system and various circulating and local factors influence the molecular determinants of impulse initiation and conduction (reviewed in [25]).

Application of these new technologies to arrhythmias is challenging as it depends on (1) understanding the mechanism for any arrhythmia, including spatial, structural and functional determinants in any one patient, and (2) identification of “vulnerable parameters” [25] whose modification would prevent/suppress an arrhythmia while having no untoward effect on a patient. Moreover, attempts at moving forward with gene and cell therapies must do more than target specific mechanisms for arrhythmias: equally important are (3) identifying appropriate constructs, (4) expressing these constructs adequately, and (5) satisfying the spatial complexities of the arrhythmogenic substrate to which constructs are delivered (6) developing a delivery system that can target the affected area leaving healthy myocardium unaltered.

4 The status of antiarrhythmic gene and cell therapies

Antiarrhythmic gene and cell therapies are a nascent field. Their limited application to arrhythmia research to date (1) has resulted in biological pacemakers for treating bradyarrhythmias [26–36]; (2) has in part relied on global delivery of viral constructs for treating VT or AF [37, 38]; and (3) for AF has also highlighted interventions that functionally or structurally ablate the *atrioventricular* (AV) junction [39–41]. There presently is need for considerable information about applicability of gene and cell therapies to specific arrhythmias, extent and duration of efficacy, safety, means for delivery, and comparison with standard device and drug therapies.

4.1 Therapy of bradyarrhythmias

The leading edge of research to date has focused on bradyarrhythmias using biological pacemaking and to a lesser extent AV bridging.

Biological pacemaker strategies have included over-expressing beta-2 adrenergic receptors [28], transfecting myocytes with a dominant negative construct to reduce I_{K1} [29], overexpressing the HCN (hyperpolarization-activated cyclic nucleotide gated) gene family to increase pacemaker current [30–32] and using mutagenesis to create designer pacemakers based on HCN or *potassium* (*K*) channel genes [32–34]. As for cell therapy, human embryonic stem cells have been coaxed into a pacemaker line [35], and adult human mesenchymal stem cells (hMSCs) have been used as platforms to carry pacemaker genes [31, 36].

Much of our own experience with viral vectors and hMSC platforms has derived from development of a biological pacemaker which we created by overexpressing HCN2 channels [30]. It is the HCN family of channels (four isoforms, HCN1, 2, 3 and 4) that is responsible for the I_f current that initiates the pacemaker potential (reviewed in [42]). Our initial *in vivo* studies demonstrated that we could administer an adenoviral HCN2/GFP construct into canine left atrium via intramyocardial injection [30] or into the left bundle branch via catheter injection [43] such that vagal stimulation to suppress sinus rhythm revealed good escape pacemaker function.

We then hypothesized that biological pacemakers might compete effectively with electronic pacemakers to drive hearts in the setting of complete heart block and would be responsive to

physiologic demands [32]. Hence, dogs were injected into the left bundle branch with the adenoviral HCN2 construct or saline, complete heart block was induced via radiofrequency ablation, and an electronic pacemaker lead was implanted in the RV apex (set at VVI, 45 bpm). Data were collected for 14 days. All dogs receiving biological pacemakers and none receiving saline developed idioventricular rhythms pace-mapped to the injection sites. Mean heart rate with saline was slower throughout the study than with biological pacemakers (45 vs. 58 bpm, $p<0.05$). Overdrive pacing resulted in shorter escape times in dogs with biological pacemakers than controls ($p<0.05$). Electronic pacemaker-driven beats occurred 92% of the time with saline injection and only 31% of the time with the biological pacemaker ($p<0.05$), while epinephrine increased idioventricular rate by 53%, ($p<0.05$) in animals receiving biological pacemakers. We concluded that left bundle branch implantation of the HCN2 gene results in a stable rhythm that suppresses electronic pacemaker-driven beats, is subject to only transient overdrive suppression and is catecholamine responsive.

Hence, this biological pacemaker supported cardiac rhythm and physiologic demands effectively over the period of the study. In the same study we tested integration of biological and electronic pacemakers in tandem operation and found that tandem biological-electronic pacemaker therapy manifests (1) a seamless interface between biological and electronic components, maintaining heart rate above a preset baseline, (2) conservation of total electronic beats delivered (which should prolong battery life), (3) a more physiologic and catecholamine responsive heart rate than electronic pacemakers alone [32].

In research on hMSCs as a platform, we used electro-poration to load the cells with HCN2 [31]. We showed the hMSCs formed functioning gap junctions with canine myocytes and that they demonstrated >100-fold over-expression of pacemaker current in the electroporated hMSCs as compared to wild-type cells. We then injected these hMSCs into canine left ventricular free wall. They resulted in pacemaker function at significantly faster rates than controls (Fig. 1). This trial [31], plus a 6-week follow up study in dogs in complete heart block [36], have shown the HCN2 biological pacemaker functions well when delivered via a hMSC platform. In contrast to embryonic stem cells driven down a cardiac lineage [35], these hMSCs are not automatic. Rather the hMSC-myocyte cellular complex becomes the pacemaking unit.

The need to reestablish normal cardiac activation in the setting of sinus rhythm and complete heart block has also led investigators to engineer bypass tracts such that sinus impulses can gain access to the ventricles [44]. However this work is still in its infancy relative to biological pacemaking.

To summarize, treating bradyarrhythmias and bridging AV block benefit from the fact that current methods for gene delivery are adequate (albeit not optimal) for experimentation. In building biological pacemakers, one creates an automatic focus. Whether this is done via intramyocardially injecting viral vectors or engineered stem cells or stem cells incorporated in hydrogels or other carriers the result is a nidus of cells that initiate stable rhythms that propagate to the rest of the heart. To optimize heart rate requires further investigation of the effect of the site in the heart on the ability of biological pacemakers to generate a spontaneous rhythm. For example the greater I_{K1} in ventricle than ventricular conducting

system or atrium suggests that implantation in the latter sites will see faster rates than in the former. Additional research has tested mutant and chimeric channels in an effort to optimize rate [32–34].

In creating AV bridges the concept is also a localized therapy in which cells coupled to one another and to adjacent myocardium are housed in a carrier material. There is significant complexity to AV bridging, however: a favorable outcome requires the proper complement of ion channels, connexins and carriers to provide the type of slow propagation essential for sufficient AV conduction delay. Moreover, to completely mirror the function of the AV node, incorporation of an autonomic component is desirable.

4.2 Therapy of tachyarrhythmias

In contrast to the diverse strategies and relative simplicity of delivery for treating bradyarrhythmias, the treatment of tachyarrhythmias has seen a more constrained approach as well as greater delivery problems. This is not stated judgmentally with regard to the research performed to date but in recognition of the greater challenges posed by tachyarrhythmias. Accompanying the design of constructs to treat tachyarrhythmias is another methodological issue—the delivery of genes to the appropriate site. These two challenges, constructs and delivery methods will be considered together. The problem is further confounded by the need to determine whether an arrhythmia requires regional (administered to a chamber or chambers) or local (administered to a tightly defined zone) delivery.

Marban et al. [45] summarized the potential benefits of local gene therapy as follows: “treating subsets of arrhythmias with localized gene delivery may suffice for long-term treatment while not subjecting the individual to the detrimental effects of disseminated treatment; autonomic responsiveness of cells that have received gene therapy persists (except to the extent that the therapy deliberately modifies autonomic signaling pathways); there is no need for long-standing implantation of hardware; trans-endocardial and intracoronary artery and venous routes of injection of constructs are available; in the event that rescue is needed, ablation would likely suffice.”

Delivery of local gene therapy has been especially productive in experiments on atrial fibrillation [45]. Here, G- α_{i2} overexpression in the porcine atrioventricular node has been achieved via injection into the atrioventricular nodal artery. The intent was to suppress basal adenylyl cyclase activity and via amplified vagal tone to indirectly reduce Ca current in the atrioventricular node [39, 45]. During sinus rhythm atrioventricular conduction slowing and ERP prolongation were reported and—in the setting of atrial fibrillation—there was a 20% reduction in ventricular rate [39, 45]. Other strategies include creating a region of Ca channel blockade in the node [40], or implanting the nodal region with fibroblasts to induce scarring and AV block [41]. While these approaches carry a likelihood of success with regard to local gene delivery, the therapeutic intent in every instance is to produce rate control (rather than rhythm control). As such, whether they will offer a useful alternative to radiofrequency ablation is uncertain.

Another potential atrial fibrillation therapy uses an ion channel gene mutation Q9E-hMiRP1, that contributes to long QT syndrome induced by I_{Kr} -blocking drugs. Levy and colleagues [46, 47] administered the construct in a plasmid via atrial epicardial injection into pigs: about 15% of cells manifested uptake. Clarithromycin then profoundly blocked I_{Kr} , leading the investigators to hypothesize that in AF they might achieve regional atrial I_{Kr} blockade and its attendant benefits, but without an effect on the QT interval. An additional benefit is that this is a “caged” therapy, expressed only during administration of an otherwise benign drug (except in LQTS patients). The therapy of ventricular arrhythmias offers additional challenges: Whereas infarcts can be thought of as requiring local therapy, variations in anatomy from patient to patient would likely require extensive mapping to determine sites at which to localize therapy in each patient. Of particular relevance to gene therapy of ventricular tachycardia is the recent report of delivery via vascular infusion to a peri-infarct zone of pigs of a dominant negative HERG mutant (HERG-G628S) [37]. Whereas a monomorphic ventricular tachycardia was consistently inducible in the infarcted animals before gene transfer, one week later all HERGG628S-transferred pigs showed no such arrhythmia. Ventricular septal MAP and ERP were increased in the HERG-G628S recipients, but no proarrhythmia was reported. Whether this reflected lack of a sufficient heterogeneity of repolarization to be proarrhythmic or other intrinsic and as yet unidentifiable changes in the treated substrate is uncertain. These results are exciting as they indicate the feasibility of a local approach to ventricular tachycardia therapy in the chronic infarct setting.

Congestive heart failure represents yet a different challenge. A major problem can be the prolonged repolarization associated with K channel down-regulation [48]. This has the potential to induce tachyarrhythmias in much the same way as a long QT syndrome. Nuss et al. [49] approached this issue by overexpressing Kir2.1 and found that while repolarization was accelerated there was an associated reduction of Ca cycling and loss of contractility. Their response to this conundrum was dual gene therapy administering both the Kir2.1 and SERCA1 genes to guinea pigs via multiple injections into the ventricular myocardium [50]. The result was an acceleration of repolarization without the attendant loss of contractility. This pair of studies nicely demonstrates the use of dual therapies to circumvent the potential toxicity of a single intervention.

5 Issues relating to gene/cell delivery and tracking

Major issues in gene/cell therapy include efficiency of delivery, utility of delivery systems, interactions of virus or cell platform and substrate, and the tracking of viral construct or hMSC.

5.1 Efficiency of delivery and utility of delivery systems

In exploring methods for transfecting atria with viral gene constructs, we used the surfactant, Pluronic 127 as a vehicle for loading adenovirus into myocardial cells. This was based on the work of Kikuchi et al. who had reported both efficacy in delivering Kir 2.1 and 2.2 genes to atrium while having no untoward effects in atrium, and no effect at all in ventricle [38]. The method involves painting a 20% solution of pluronic containing 0.5% trypsin containing adenovirus as a vector directly onto canine atrial myocardium (trypsin, in low concentrations

allows penetration of viruses into the interstitium). As shown in Fig. 2, using GFP in an adenoviral vector one can consistently show uptake of GFP by myocytes obtained from atria treated with pluronic (Fig. 2(d)). However, the procedure is complicated by a fair degree of persistent inflammation (Fig. 2(b)). For this reason, we do not think of pluronic as a primary tool for virus administration and have gravitated to more standard means of virus and cell administration, namely endocardial catheter injection, open chest direct intramyocardial injection or intracoronary injection.

For catheter delivery of viral gene therapy our primary mode of administration is injection using a custom made 8F steerable catheter incorporating a bipolar recording electrode at its tip and a retractable (3 mm) 29 gauge needle in its lumen, as reported by us previously [32, 43]. While this has been useful for electrophysiologically guiding administration of viral vectors, the lumen is too narrow to permit delivery of hMSCs without shear damage. We have also worked with intracoronary versus direct intramyocardial administration of viral constructs. As shown in Fig. 3 we find far more favorable infection of atrial (Panel D) and ventricular (Panel F) myocytes via the intramyocardial route than via intracoronary injection (Panels A and B). Inconsistency of various routes of administration has been reported by others as well [45, 51–56].

For hMSC administration we favor the use of direct trans-epicardial administration via a 23 gauge needle which allows surface mapping and permits 80–90% cell survival [31, 36]. Catheter administration transendocardially is possible as well.

5.2 Interaction of hMSCs or virus with substrate

The literature suggests hMSCs are immunoprivileged (summarized in [57, 58]) but because much animal research involves xenotransplantation, we believed it important to test the presence/absence of cell rejection/loss. Hence we studied 12 dogs injected intramyocardially into the LV anterior wall with 150,000 to 1.2 million hMSCs. At 6 weeks (Fig. 4) there was no edema, the hMSCs appeared normal and stained positively for GFP and the human antigen, CD44. In addition, there is no evidence of cellular or humoral rejection or apoptosis. However, these observations still must be considered preliminary: far longer durations of study are required.

With respect to reactions to viral constructs the use of adenovirus is associated with 1–3 days of inflammatory response, but experiments to date have found the gene effect of over 2 weeks to far outlast it (e.g. [32]). Tempering excitement regarding some viral vectors are concerns about inflammation and induction of chronic illness or neoplasia. There are major issues with retrovirus and lingering concerns exist regarding the use of lentiviruses. Additional concerns about some viruses are episomal or limited genomic expression of genes (although these concerns are unimportant for proof-of-concept experiments).

5.3 Tracking of viral constructs or hMSCs

GFP appears to be an adequate tracking agent in many viral experiments to date [32], but will not provide a long-term solution. Moreover, GFP itself has biological effects that can confound experimental results [59]. Tracking of hMSCs and stem cells in general has been an issue of major concern. Both scientists and regulatory authorities have voiced concern

that cells might migrate, exhibit untoward effects at other sites in the body, and might have a neoplastic potential [reviewed in 26, 57]. Attempts to track stem cells *in vivo* have used a variety of approaches. These include transfection *in vitro* with either fluorescent [31] or nonfluorescent [60] proteins, the use of fluorescence in situ hybridization (FISH) to identify delivered cells by their chromosomal content (usually by delivering male cells to a female host) [61], the use of species-specific surface markers to identify delivered cells in a xenographic host [31, 36] (see Fig. 4), and labeling cells with inorganic particles such as Feridex [62, 63] or radiotracers [64–66].

Despite the individual advantages characterizing each approach, a general problem has been that all focus on identifying the location and fate of a small population of the delivered cells. Additional problems are (1) the maps of *in vivo* stem cell distribution tend to be of low resolution and that imaging labeled cells in the heart is confounded by myocardial autofluorescence [67, 68]; (2) with regard to inorganic labels, cells may die and the particles left behind can remain in the tissue and be interpreted falsely as a positive signal, or they can be taken up by other cells and remain in situ or migrate to other loci; (3) the threshold of detection is such that small yet significant numbers of cells may migrate and not be detected. Because of these issues and the fact that an ideal tracking agent is not yet available, there has been no success in following the fate of the overall population of stem cells administered and doing this at the resolution of the single cell.

For these reasons we and others have explored cell marking and detection using quantum dots [69, 70]. When we load hMSCs with these nanoparticles they exhibit a diffuse pattern in the cytosol and are readily visualized for long periods after cell injection. As a result we can track the cells in a three-dimensional pattern and understand their geometry with respect to that of the injection site. The details of our approaches for cell tracking with quantum dots are described elsewhere [69]. They are loaded into hMSCs efficiently, do not appear to alter hMSC function, and do not exit the cells via gap junctions or other means. In addition, when there is cell death the quantum dots are removed by the reticuloendothelial system from the heart in less than 1 hour. Figure 5 shows results using quantum dots to track the location of hMSCs delivered to the rat heart by injection. Using customized algorithms the fluorescence images are filtered and thresholded to generate binary maps of quantum dot-positive zones from all of the tissue sections (Fig. 5(b)). The spatial locations of quantum dot-loaded hMSCs are identified from the series of binary maps and visualized in three-dimensions (Fig. 5(c,d)). Our algorithms permit enumeration of the total number of hMSCs in the whole heart from these models (in Fig. 5 approximately 50,000 at 1 h and 30,000 at 1 day). We can also compute a distance parameter to characterize the distribution of cells, based on the distance between individual cells and the centroid of the total stem cell mass. In Fig. 5(f) most of the cells are clustered in close proximity (85% of cells within 1.5 mm at 1 h and 95% within 1.5 mm at 24 h).

6 Future issues: translational potential and clinical applicability

While there is clear therapeutic potential of gene and cell therapies for arrhythmias, they are at present distant from translation to the clinic. Nonetheless, it is useful to consider the problems and obstacles that must be faced if eventual human administration is a goal.

6.1 Delivery of constructs

One issue of concern is delivery of constructs. This is important to large animal research and to future applicability to human subjects. It also is of immediate impact in other areas of gene and cell therapy being delivered to human subjects. As mentioned above, catheters are available for delivery in specific settings but the goal of a flexible catheter with electrode recording capability at its tip and a large enough bore to not inflict damage on stem cells is not yet a reality. In the interim a combination of endocardial mapping and use of existing catheter delivery systems is the state of the art.

Another issue regarding delivery is whether this should be focal (to a region of interest, perhaps identified by mapping) or global, to the entire heart or much or all of a chamber. We have not attempted to and do not plan to work with global administration, as we believe the therapeutic approach for arrhythmias (as opposed to, say, cardiac failure) should be one that uses appropriate techniques to identify regions/sites of interest and administers constructs to those regions rather than applying a construct with whatever degree of uniformity to a larger region.

6.2 Viral vectors

Additional important issues relate to optimization of vectors and integration into the cardiac syncytium. The adenoviral vectors used by many of us in proof of concept experiments are only episomal in their expression. While these are adequate for proof of concept, or for transient gene delivery in specific settings (for example, delivery of VEGF to stimulate vascular growth over a short period [54]) they cannot be seen as providing a lasting therapy. Adeno-associated virus provides more long-term expression, although in large part this appears episomal as well. An additional limiting factor is the size of the gene that can be inserted into adeno-associated virus, which makes it impossible to incorporate a full range of gene constructs one might want to consider. Lentivirus does not have the latter limitation and does result in genomic incorporation. At present it would appear to be the vector of choice for long-term maintenance of an effect.

6.3 Cell persistence

We have shown that hMSCs used to create biological pacemakers persist for at least 6 weeks when xenotrans-planted into canine heart, with no evidence of rejection or apoptosis, and with persistent expression functionally and immunohistochemically of the constructs administered [36]. We are currently testing the ability of hMSCs to persist and to express the desired function over the long-term, in this case one year. Experiments by others have shown that hMSCs can be administered allogeneically to human subjects with no adverse reactions, although long-term persistence is still an issue [58].

6.4 Optimization of constructs

An initial interpretation of many approaches to gene/cell therapy might be that they use constructs in such a way that they appear to be “off-the-shelf” drugs. Yet, this would be a misinterpretation. Our own group starts by working with a library of ion channels and connexins to identify those whose characteristics appear to well-serve the need to prevent/treat the arrhythmias in question [30, 71, 72]. However, in so doing we are using “the shelf”

only as a starting point. For example, we are making changes in amino acid sequences and creating mutant and chimeric channels as means to further our understanding of why a particular construct has a specific effect. Examples of this are seen in our work with biological pacemakers, first with an E324A mutant of HCN2 [32] and even more specifically in our creation of an HCN212 chimera, having the amino and carboxyl termini of HCN2 and the transmembrane spanning domain of HCN1 [73] (we had reasoned that the HCN1 pore forming unit would provide more favorable activation kinetics than HCN2 while the HCN2 cAMP binding site (near the carboxyl terminus) would provide more robust catecholamine responsiveness than HCN1). Information obtained from the preparation and testing of these mutant and chimeric channels has shown the benefits and the risks of each intervention and is now being used to aid in design of additional constructs.

6.5 Choice of promoter and regulation of nucleic acids

Another issue to consider is the ideal promoter to be used. To date many of us have worked with a CMV promoter. However, given that there can be temporal changes in the expression of promoters (see [76]). CMV is not necessarily the best long term choice. Moreover, because of safety concerns, the idea of a cardiac-specific (and maybe even cardiac region-specific) promoter would be of importance.

In considering what the duration of effect as well as the robustness of expression of effect will be for any gene, we need to be aware of regulation of protein expression. For example, we would have to consider the possibility that a microRNA may reduce expression and/or that alterations in protein degradative processes in cells would alter the expression of the channel protein of interest. In addition there are other issues impacting on RNA/DNA expression, DNA regulatory sequences and the modulation of expression. All of these as well as issues regarding RNA and DNA that influence expression and duration of effect impact on development of constructs and will be the subject of a major research effort across laboratories for years to come.

7 Conclusions

Clearly the field of gene and cell therapy is young, and significant challenges remain. Yet the need to more effectively cope with arrhythmias causing major morbidity and mortality is sufficiently great that attempts to find solutions through these and other novel approaches are necessary. Given the rate of advancement in the less than one decade of investigation to date, it is anticipated that a subset of these therapies should become available for clinical trial within the next decade.

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Fig. 1.

Example of loading hMSC with a gene of interest, studying it biophysically and determining its effect on the heart. Functional expression of I_f in hMSCs transfected with the mHCN2 gene (b) but not in nontransfected stem cells (a). (c) Fit by the Boltzmann equation to the normalized tail currents of I_f gives a midpoint of -91.8 ± 0.9 mV and a slope of 8.8 ± 0.5 mV ($n=9$). I_f was fully activated around -140 mV with an activation threshold of -60 mV. *Inset* shows representative tail currents used for activation curves. Voltage protocol: hold at -30 mV, hyperpolarize $\times 1.5$ s to -40 to -160 mV in 10-mV increments followed by 1.5 s voltage step to $+20$ mV. (d, e) Ten days after injection into LV anterior wall in dog in complete heart block. (d) Dog injected with placebo. *Left* pacing from injection site at the time of placebo administration. *Right* idioventricular rhythm having a slow rate (about 42 bpm) and completely different QRS vector from paced beats at the injection site. (e) Dog injected with 1.2 million hMSCs loaded with HCN2. *Left* paced beats at injection site at time of hMSC administration. *Right* 10 days later rhythm has same QRS vector and rate of 60 bpm. Modified with permission from [75]

Fig. 2. Use of the surfactant, Pluronic 127 (poloxamer, 5 ml of a 20% solution) containing 0.5% trypsin as a vehicle for loading adenovirus into myocardial cells. **(a)** Control H and E stain showing normal epi- and myocardium. **(b)** H and E stain showing inflammatory cell infiltrate following administration of pluronic and trypsin with viral construct. **(c)** Control peroxidase staining for GFP in normal tissue, showing no peroxidase reaction. **(d)** Positive peroxidase stain for GFP at site of administration of pluronic plus viral construct. **(a, b)** $\times 100$; **(c, d)** $\times 400$

Fig. 3. Administration of GFP-adenoviral construct. **(a, b)** Administration via catheterization of SAN artery: there is some perivascular and neural staining, but little overall incorporation; **(c)** H and E and **(d)** (GFP) LA intramyocardial injection shows uniform GFP staining. **(e)** H and E and **(f)** (GFP) LV intramyocardial injection show uniform GFP staining

Fig. 4. hMSCs 6 weeks after LV anterior wall intramyocardial injection. H&E identifies basophilic cells (a) that are CD44 positive (b) and GFP positive (c: peroxidase stain). hMSCs do not display labeling/binding of dog-IGG to their surface, (d: evidence against humoral rejection). CD3 positive T lymphocytes are rarely noted in association with clusters of hMSCs (e: paucity is evidence against cellular rejection); staining is negative for activated CASPASE 3, (f: evidence against apoptosis; original magnification: $\times 400$). Reprinted with permission from [74]

Fig. 5.

Quantum dots (QDs) are used to identify single hMSCs after injection into the rat heart and further used to reconstruct the 3-D distribution of all delivered cells. Rat hearts were injected with QD-hMSCs. Fixed, frozen sections were cut transversely [*plane shown in (b)*, inset] at 10- μ m and mounted onto glass slides. Sections were imaged for QD fluorescence emission (655-nm) with phase overlay to visualize tissue borders. QD-hMSCs can be visualized at (a) low power, and [(a), inset] high power (Hoechst 33342 dye used to stain nuclei blue). In [(a), inset], endogenous nuclei can be seen adjacent to the delivered cells in the mid-myocardium (*arrows*). Serial low power images were registered with respect to one another and (b) binary masks were generated, where white pixels depict the QD-positive zones in the images. The **vertical line in [(b), inset]** represents the z axis, which has a zero value at the apex of the heart. The binary masks for all of the QD-positive sections of the heart were compiled and used to generate the 3-D reconstruction of delivered cells in the tissue. QD-hMSCs remaining in the tissue adhesive on the epicardial surface (and not within the cardiac syncytium) were excluded from the reconstruction. (c) QD-hMSC reconstruction in an animal that was terminated 1 hour after injection. (d) Reconstruction from an animal euthanized 1 day after injection with orientation noted in inset. Our reconstructions in (c) and (d) do not account for all of the approximately 100,000 hMSCs delivered through the needle. Some of these cells undoubtedly leaked out of the needle track, while others may not have survived the injection protocol. The views of both reconstructions (c) and (d) are oriented for optimal static visualization (and also have different scales and are situated at different positions along the z-axis depending on the distance of the injection site from the apex of the heart); (e) One day after injection into the heart, the pattern of QD-hMSCs is well-organized and appears to mimic the endogenous myocardial orientation (dotted line highlights myofibril alignment). Complete representations of the spatial localization of QD-hMSCs in the heart permits further quantitative analyses. (f) One parameter that can be computed is the distance of individual cells from the centroid of the total cell mass. The plots show the percentage of cells at a distance less than or equal to x for both the 1-hour

and 1-day rats. At both time points, most of the cells are within 1.5 mm of the centroid.
Scale 500 μm , inset=20 μm , scale bar on **(b)**, inset=1 cm, scale bar on $e=\bar{e}$ on a=500 μm .
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