



## Editorial

# Taking the Death Toll After Cardiomyocyte Grafting: A Reminder of the Importance of Quantitative Biology

Hans Reinecke and Charles E. Murry

*Department of Pathology, University of Washington, Seattle, WA, USA*

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## Introduction

Much of the morbidity and mortality from myocardial infarction results from the heart's inability to regenerate significant muscle mass in the infarct. Multiple groups, including our own, have studied the possibility of regenerating new muscle in infarcts by cellular implantation (cellular cardiomyoplasty). It seems intuitive that the most natural approach would be to replace dead cardiomyocytes with new, living cardiomyocytes. Indeed, initial studies with cardiomyocyte transplantation generated considerable excitement. Field's group, for example, showed that fetal cardiomyocytes formed stable grafts when implanted into the normal myocardium of mice and dogs, and that the graft cells formed intercalated disks with host myocytes [1–3]. Other groups, including our own, subsequently showed that fetal or neonatal cardiomyocytes could form stable grafts in infarcted or cryoinjured hearts [4–8], although the grafts typically were located at the lesion's periphery, close to host myocardium.

## Facing Up to Death

These qualitative studies painted a rosy picture, where it was possible to create new myocardium

in the infarcted heart. All that remained, we assumed, was to find the right dose of cells needed to completely repair the infarct and show functional improvement. Disillusionment soon followed, however, when quantitative, dose-response studies were undertaken. We observed no increase in graft size over an input range of 3 to 25 million cardiomyocytes [9]. (For reference, the adult rat left ventricle contains ~20 million cardiomyocytes [10].) Furthermore, grafts were all small, never exceeding 2% of the left ventricle at one week after implantation. This suggested that graft cell death was limiting the amount of new myocardium formed. Studies using TUNEL analysis showed that, although graft cells appeared viable shortly after implantation, by 24 hours ~32% had fragmented DNA. Cell death continued for at least 4 days after implantation, when the TUNEL index was 10%. By 1 week, the TUNEL index was ~1%, suggesting the wave of cell death was largely complete.

In the current issue of *The Journal*, Müller-Ehmsen and co-workers have extended these observations using a clever and highly quantitative molecular technique to measure graft cell number in the heart [11]. The authors implanted neonatal cardiomyocytes from male donors into normal hearts of syngeneic female rats. To quantify graft cell number, the authors used TaqMan real time quantitative PCR to detect the Y chromosome-linked gene, *sry*. The ratio of *sry* to a somatic gene,

\* Please address all correspondence to: C. E. Murry, MD, PhD, Department of Pathology, Box 357470, Room D-514 HSB, University of Washington, Seattle, WA 98195. Tel: 206-616-8685; Fax: 206-543-3644. E-mail: [murry@u.washington.edu](mailto:murry@u.washington.edu)

osteopontin (2 copies per diploid genome), was determined at various time points after injection of the cells for up to 12 weeks. This PCR-based approach was highly sensitive, detecting 2 male cells amongst 50 000 female cells. It also yielded a 0.993 correlation coefficient when compared with a known set of male:female DNA solutions, suggesting a high degree of accuracy.

Several important pieces of new information came from this study. The first was that graft cell seeding efficiency at 0–1 hour after injection averaged only 58%. Even more impressive was the variability of seeding, which ranged from 10–90%. This early variability cannot be due to death, since even cells killed by freeze-thaw showed a positive Y chromosome signal at 1 hour. The authors reasoned that such rapid loss was caused by a combination of cell retention in the syringe's dead-space (presumably correctable by using a Hamilton syringe), leakage from the injection site, and wash-out through the vasculature. Indeed, our own experience with cell grafting indicates that these factors certainly play a role, resulting in a highly variable graft size [12]. Given the variability occurs in laboratories with extensive experience with cell injection in the heart, it seems unlikely that more practice is needed to improve the results. Rather, the basic protocol for cell grafting may need further optimization to prevent cell loss. On a positive note, some of the technical obstacles may be caused by the use of small animals with high heart rates and would likely disappear in human patients.

Next, Muller-Ehmsen *et al.* showed that over the first 24 hours there was a considerable loss of graft cells, down to 24% of the injected cell number (41% of the initial seeding number). Over the ensuing weeks the number of graft cells remained relatively constant, with just a borderline further reduction to 15% of those injected (26% of initial seeding number) at 12 weeks. Although these data indicate that substantial cell death occurred after grafting, the authors are careful to emphasize that this technique likely underestimated cardiomyocyte death. Like all cardiomyocyte isolations, theirs contained a fraction of non-myocytes, which they estimated at 20% prior to implantation. The Y chromosome PCR assay, of course, cannot distinguish between myocytes and non-myocytes. It is likely that the myocytes were preferentially killed after implantation, whereas the fibroblasts likely proliferated. Thus, cardiomyocyte survival after grafting was likely to be substantially lower than the 15% value reported at the end of the study. It would be useful to utilize a Y chromosome *in*

*situ* hybridization, coupled with cell type-specific antibody staining, to determine the relative number of graft cardiomyocytes and non-myocytes remaining at the end of the study.

It is noteworthy that the survival reported by Muller-Ehmsen *et al.* likely reflects a best-case scenario, since the cardiomyocytes were grafted into normal hearts representing the least hostile environment. Indeed, our group found that death of grafted neonatal cardiomyocytes after 24 hours is lowest in normal hearts, intermediate in vascularized 2 week-old injuries, and highest in acutely injured hearts [9]. Grafting into an established injury probably resembles an eventual clinical situation more closely, where cardiomyocytes likely would be transplanted some time after the infarct.

Müller-Ehmsen *et al.* also showed the initial number of cells grafted in the heart could be increased by simply increasing the concentration of the cell suspension. On the other hand, increasing the number of cells seeded had no effect on the final number surviving. This result agrees well with our previous dose-response study and suggests that the cells are competing for limited resources for survival. In their study, the survival curve plateaued at ~1 million cells, indicating perhaps an optimal number of cells to implant in the normal heart.

Why do so many cardiomyocytes die after grafting? Müller-Ehmsen found no benefit to survival when the cardiomyocytes were treated with the broad spectrum caspase inhibitor AcYVADcmk prior to grafting. They interpreted this to mean that caspase-independent mechanisms are responsible for most of the death. It is important to note, however, that the inhibitor used is a "suicide substrate" for caspases, which will inhibit the enzymes only when they are active. If the caspases were in their pro-form *in vitro* and activated after cell implantation, *in vitro* pre-treatment would not be expected to have an effect. As mentioned above, our own studies showed markedly improved survival when cells were grafted into vascularized tissues, suggesting ischemia was a major source of injury [9]. We observed modestly protective effects by transfecting the cells with a constitutively active form of the cytoprotective protein kinase, Akt, indicating there might be a role for apoptosis in inducing cell death. We saw dramatic protection when the graft cells were heat shocked one day prior to implantation. Although providing a practical means to enhance survival, heat shock is not a precise molecular intervention and, unfortunately, sheds little light on the mechanism of death. It is interesting to speculate on a possible role for non-myocytes in myocyte death. Standard protocols for

cardiomyocyte isolation are designed to minimize the content of fibroblasts and other cells. It is possible, however, that the non-myocytes could provide important paracrine factors that actually might enhance myocyte survival. This hypothesis seems worthy of further exploration.

## Summary

At the moment, cardiomyocyte grafting presents a “good news/bad news” scenario. The good news is that we definitely can make new myocardium in injured hearts by cell grafting. The bad news is that we cannot make very much of it. Cell death after transplantation severely restricts the size of eventual grafts that form. This cell death, coupled with the inability of cardiomyocytes to proliferate after grafting, present the two major experimental obstacles to regenerating the infarcted heart by cardiomyocyte grafting.

These studies also show the importance of quantitative analysis in biology. It is quite a different thing to show that something can happen (e.g. formation of new myocardium) *v* showing that it occurs to a physiologically meaningful extent. Our own studies serve as a good example. In our first study of cardiomyocyte transplantation, we concluded that fetal and neonatal cardiomyocytes “survived” because we could detect viable grafts at later time points. Although some cell death was noted in the initial days after transplantation, its magnitude was grossly underestimated, simply because we did not quantify it. Once quantitative studies were undertaken, the same neonatal cell transplantation model served as the platform for cell death analysis. Although quantitative analysis requires substantially more work, we believe it adds an element of rigor to the experiment and forces more intellectual honesty in data interpretation. In this light, Müller-Ehmsen *et al.* have provided a good, quantitative system for analyzing the mechanism of cell death after cardiomyocyte grafting.

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