LOAD DEPENDENCE OF CARDIAC FIBROBLAST MMP ACTIVITY IN FIBROBLAST-POPULATED COLLAGEN GELS

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INTRODUCTION

As heart failure develops, the extracellular matrix (ECM) undergoes significant remodeling. Changes in collagen synthesis and degradation lead to disruption of the myocardial collagen network, extensive left ventricular (LV) remodeling, increased wall stress, and consequent pump dysfunction. One of key factors in the ECM remodeling of the LV is a group of enzymes called matrix metalloproteinases (MMPs). MMPs have been shown to be responsible in matrix remodeling of many tissues during normal as well as pathological processes.

Lambert et al. [1] used collagen lattices seeded with dermal fibroblasts in two configurations: freely contracting lattices, which impose little or no load on the fibroblasts, and bound lattices, which impose maximal load on the fibroblasts as they attempt to contract the gel. In this setting, collagenase activity was higher in freely contracting gels, which was interpreted as evidence for load-dependent regulation of ECM enzymes.

In the heart, ECM and its accompanying regulatory enzymes are secreted by cardiac fibroblast cells. To our knowledge there have been no rigorous studies on the dose-response relationship of MMPs as a function of load imposed on cardiac fibroblasts embedded in collagen lattices.

The hypothesis of this study was that increased levels of load applied *in vitro* to cardiac fibroblast-populated collagen gels would lead to increased levels of MMP expression. To test this hypothesis we set out to: 1) apply a range of biaxial loads to cell-seeded gels, 2) quantify the zymographic activity levels of secreted MMP-2 and MMP-9 as a function of load, and 3) follow the level of MMP activity over a time course of several days.

MATERIALS AND METHODS

Fibroblasts were isolated from adult male rat hearts (ARCF) and cultured in polystyrene flasks for 10 days under standard tissue culture conditions (37^o C, 5% CO_2) in cell culture medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% neonatal calf serum, 5% fetal bovine serum, 1% penicillin-streptomycin, and 0.2% amphotericin-B). To maintain consistency in all experiments, cells were used at passage 2.

An ice-cold collagen mixture was prepared by mixing HEPES, 10x MEM, and bovine type I collagen (Vitrogen 100, Cohesion Technologies) in a ratio of 1:1:8. Cells were trypsin-harvested, suspended in media, and added to the collagen mixture in a ratio of 1:4 to achieve a final concentration of $2.0x10^5$ cells/ml and 2.0 mg of collagen/ml in a total gel volume of 10 ml.

Gels were created by pouring the prepared cell/collagen solution into a custom-made 4x4 cm square casting mold which was housed in a 100x15 mm petri dish. The dish had been pre-coated with 2% bovine serum albumin to prevent cell adhesion, and then rinsed with sterile phosphate buffered saline. On each edge of the mold was a 20-mm long bar of porous polyethylene threaded with 5-0 nylon monofilament suture. The gels were allowed to polymerize for 2 hours in a 37^o C incubator, after which the casting mold was taken off, and a loading rack was placed on the petri dish. Sutures were routed to the outside of the dish and weights were attached so they were suspended freely in the air. The gels were loaded biaxially. There were five loading conditions: free (no bars in the gel), 0 mg, 50 mg, 100 mg, and 200 mg. 20ml of media (DMEM, 10% FBS) was added to the petri dish to float the gel. Gels were placed back in the incubator and left loaded for 5 days.

Cell-seeded collagen gels contract spontaneously, and, in this study, the extent of contraction was inversely proportional to the load applied to the gel. While cell density was initially the same in all gels, by the end of the loading period there were more than 2-fold differences present (Figure 1, the left set of columns). It has been well established that the degree of cell-cell contact acts as an important agent in cell functions. In order to control for the possibility that cell density affects enzymatic activity, a control was created by making a "normalized" set of gels for each cell isolation. These gels were seeded with varying numbers of cells initially, which resulted in more uniform cell densities in final contracted gels (Figure 1, the right set of columns).

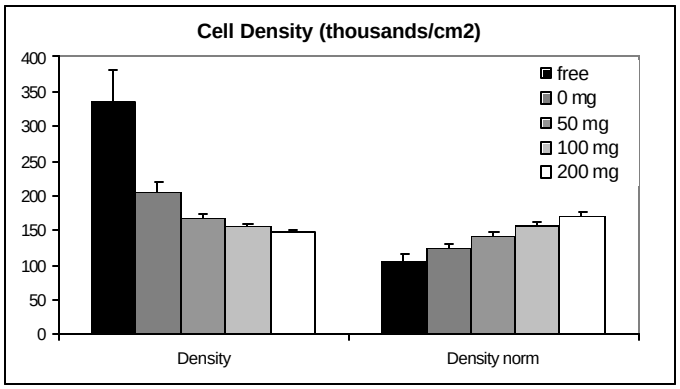

Figure 1 – Cell densities at 3 days for the experimental and control sets [N=8]. The experimental set (left panel) shows significant decrease in cell density with increase load. The "normalized" control set (right panel) shows significant increase in cell density with load.

Gelatin zymography was used to detect activity of MMPs released into the surrounding media. The media was sampled at 3 and 5 days, the samples were prepared for electrophoresis and run on SDS-PAGE gelatin gels. After electrophoresis, gelatin gels were stained with 0.05% Coomassie blue dye for 30 min, destained and imaged. Three bands were visible on stained gels: MMP-2, activated MMP-2, and MMP-9. NIH Image software was used to perform densitometry of MMP bands and thus determine the levels of zymographic activity.

RESULTS

There were two different groups of cell-seeded gels in this study. In the first group (2M) each gel was cast with 2 million cells, while in the "normalized" group each gel was cast with a different number of cells in order to normalize the cell density in the final contracted state of the gel. Cell density calculated at 3 days showed that for the 2M gels there was a significant ($p<0.0001$) decrease in the cell density with the increase in load. In the "normalized" group of gels the trend was significantly ($p<0.0001$) reversed with the 200mg gels exhibiting the highest cell density (Figure 1).

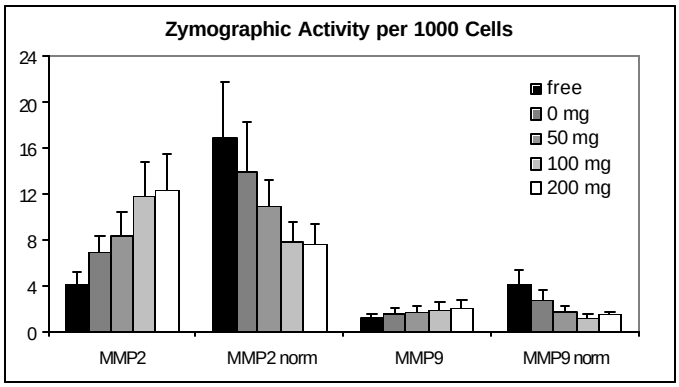

Figure 2 – Densitometry measurements of zymograms for MMP-2 and MMP-9 [N=8].

MMP-2 showed a significant ($p=0.02$) increase in 2M gels with the increase in the biaxial load, while in "normalized" gels there was a significant ($p=0.04$) decrease in MMP-2 expression (Figure 2, panels on the left). The activated form of MMP-2 showed a significant ($p=0.001$) increase with increasing load in 2M gels, while in "normalized" gels there was no statistical variation with load (figure not shown). The other enzyme of interest, MMP-9, showed a significant ($p=0.02$) increase in 2M gels and a significant decrease ($p=0.0004$) in "normalized" gels as a function of increasing load (Figure 2, panel on the right).

Correlating MMP-2, activated MMP-2, and MMP-9 activity data with inverse of the cell density and with the load across all experiments showed that there was some correlation with the cell density (average $R^2=0.499$), but no correlation with the load imposed (average $R^2=0.140$).

DISCUSSION

In order to examine changes in secreted MMP activity of cardiac fibroblasts seeded in collagen lattices as a function of load imposed on the cells, a novel experimental system [2] was used. Carefully calibrated loads were applied to gels and the resulting changes in MMP activity successfully measured.

The hypothesis that the increase in load results in increased production of MMP was, at first glance, validated for all three enzymes measured: MMP-2, MMP-2a, MMP-9. However, further analysis of MMP enzymatic activity in "normalized" gels showed that these trends were either reversed (MMP-2, MMP-9) or disappeared (MMP-2a) when cell densities were reversed. Furthermore, R^2 values obtained through linear regression of the measured MMP activity data yielded a much better fit with cell density than with load imposed. We conclude that production of MMP-2 and MMP-9 enzymes in cardiac fibroblast-seeded gels is likely determined in part by the final cell density in contracted gels but not by the amount of load.

In light of previous studies done on MMP activity, our findings are surprising. However, no previous studies incorporated a control for cell density, making those findings subject to possible reinterpretation. It should be pointed out that the range of cell densities used in this protocol was relatively low (= 2.0×10^5 cells/ml), resulting in collagen gels with a low degree of overlap between cells. It is possible that experiments at much higher or much lower cell densities would yield different findings. In any case, it is clear that cell density must be considered carefully in design of future experiments.

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ACKNOWLEDGMENTS

Funding agencies: JWH was a Kopf Family Fellow of the American Heart Association Heritage Affiliate (#9930279T). Individuals: Ms. Cheryl Jobe for technical assistance, Prof. Thomas K. Borg.