OPTIMIZING NON-VIRAL MEDIATED TRANSFECTION OF HUMAN INTERVERTEBRAL DISC CHONDROCYTES

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Introduction:

Low back pain is the second most common complaint bringing a patient to a physician, and the most common cause of prolonged disability (National Institute of Neurological Disorders and Stroke-National Institutes of Health, 2005). The cost related to its management has been estimated to be between 90 and 100 billion dollars annually (Luo, 2004; Waddel, 1996). Many cases are thought to be the result of degenerative processes of the intervertebral discs (Luoma 2000;Paajanen, 1997; Salminen, 1999). This process is associated with biochemical and biomechanical changes which can eventually result in morphologic findings and symptoms (1,8,18,19,21,28,30). Novel therapeutic regimens that can focus on this degenerative process are the subject of tremendous research interest (Kang, sobijama).

Gene therapy for degenerative disc disease (DDD) is a novel approach which is aimed at correcting cartilage catabolic and anabolic imbalances. The transfection of potentially therapeutic genes both *in vitro* and *in vivo* has been shown to increase proteoglycan production and increase water content of discs after degeneration. Several vectors have been reported, but the most commonly researched has been adenovirus (sobijama, wallach). However, *in vivo* transfection of adenoviral vectors are limited by complications and potential toxicities such as direct cytotoxicity leading to apoptosis, immunologic response following transfection, and insertional mutagenesis and cell transformation. The use viral based gene therapy on human subjects has been the source of recent controversy as a result of several isolated deaths (NY times, zhi 2005). As a result, there is clinical need for a non-viral vector, if gene therapy is to be considered a viable option in the future.

Thus far, there is little data in the literature regarding the use of non-viral vectors in human disc chondrocytes. Early work utilizing non-viral vectors for in vivo transfections was disappointing due to low transfection rates and short durations of gene expression in transfected cells (Felgner 1987; Felgner, 1989; Flechtenmacher, 1996). However, Madry and Trippel (2000) showed that articular cartilage chondrocytes could be successfully transfected in vitro using lipid based reagents. Other methods have been the gene gun and transfer of naked DNA into living cells (Sobijama, 2004). Despite this success, non-viral transfection of the intervertebral chondrocyte has remained elusive. Suitable vectors must be both non-toxic and efficient in order to deliver genes in sufficient quantity to be therapeutic. The purposes of this study were to determine the efficiency, toxicity and optimal conditions for gene delivery into human degenerative intervertebral disk chondrocytes via non-viral vectors *in vitro*. Non-viral reagents were also compared to adenovirus and a similar non-viral vector already known to be effective in transfecting articular chondrocytes. The study was conducted in two phases. Phase 1 screened seventeen lipid based reagents to determine the most efficient vectors as determined by luciferase assay. Phase 2 involved several experiments to determine optimal conditions for *in vitro* transfection.

Methods:

***Isolation and Culture of Human Intervertebral Disc Cells*:**

The use of human disc tissue was approved by the Institutional Review Board of the University of Wisconsin. Consent was obtained from 21 patients undergoing discectomy for trauma, disc herniation, and fusion for scoliosis or degenerative low back pain. The human disc tissue containing both annulus fibrosus and nucleus pulposus was rinsed a minimum of two times with PBS buffer to remove blood and residual tissue. The inner nucleus pulposus was isolated and shaved into pieces of approximately 2 mm with a sterile scalpel. These pieces were rewashed in PBS containing penicillin/streptomyosin and then transferred to a solution of PBS and

collagenase (0.1 mg/mL in PBS) and incubated in a 25 cm2 flask at 37° C with 5% CO2 for approx 16 hours. The flask was then agitated to help separate cells from the digest and the suspension strained using a sterile cell strainer with a 40 um nylon mesh. Centrifugation of the resultant cell suspension at 1100 RPM for 10' sedimented the chondrocytic disc cells. The supernatant was then removed and the cells resuspended in a chondrocyte maintenance medium consisting of 500 ml Dulbecco's Modified Eagle's Medium (DMEM), 5 ml HEPES buffer, 5 ml nonessential amino acids, 5 ml essential amino acids, 5 ml BES and 5 ml TES. The pH was adjusted to pH 7.2 with NaOH and the solution sterilized by filtration. FBS (50 ml) and pen/strep (1.5 ml) were finally added. The resuspended material was then placed in a 25 cc flask and incubated at 37° C in an atmosphere of 5% CO2. Flasks were observed using light microscopy for evidence of chondrocytes and the medium was exchanged every 3-4 days. The cells were allowed to grow under these conditions until confluent at which time they were removed to two 75 cc flasks. When cells had reached 80% to 90% confluency, they were dislodged from the flask with trypsin and transferred to either 75 cm2 cell culture flasks for future use or 24-well plates at a cell density of 5 x 104 cells/ well for transfection experiments.

Cell lines, transferred to 24 well plates, were allowed to grow for a period of approximately 48 hours and were then transfected with varying ratios of DNA plasmid to reagent, and harvested 48 hours after transfection for analyzation by luciferase assay (phase 1) or flow cytometry (phases 2-3).

Phase 1 Screening of non-viral reagents

Seventeen lipid based reagents, donated by Mirus Corporation®, were evaluated. The reagents are classified into four groups: Histone based vectors, polycation associated, polyethyleneimine associated, and lipid only formulations.

The volume of reagent was varied 5 to 10 fold while holding DNA dose constant at 0.5 ug. Reagents were placed dropwise into serum free media and thoroughly vortexed. The solution

was then incubated for 20 minutes at room temperature. The DNA encoding plasmid pCl-Luc+ (luciferase), .5 ug, was then added and the mixture incubated at room temperature for 20 minutes. Among the vectors used was "LTI", a proprietary (Mirus Corporation) histone based vector which is biochemically similar to the vector previously found to be an effective non-viral vector by Madry et al. (2000). Additionally several "boosters" were evaluated that were thought to enhance transfection efficency.

Histological analysis: After 48 hours cells were graded on a four point scale for toxicity: +1 indicated no signs of atypia; +2 indicated no more than one cell per high powered field (HPF) showing slight cellular changes including irregular borders and sizes; +3 indicated more than one cell with signs of toxicity per HPF; +4 indicated significant cellular toxicity including signs of cell death.

Luciferase assay: After microscopic examination, cells were lysed and combined with a standard reaction mix and then measured with a luminometer. Results were normalized to the activity of the standard LTI (3ul).

Phase 2 Transfection efficiency

After the histological and luciferase activity studies of phase 1 the three best reagents were assessed to determine transfection efficiency and cell viability using flow cytometry. The three lipid-based non-viral vectors, T-Jurkat, TKO, and LT1, were coupled to DNA plasmids coding for green fluorescent protein (GFP) by the following procedure. Reagents were added by micropipette into a sterile test tube containing OptiMem-II (a serum free, antibiotic free basic minimal medium used in transfection) and mixed so that 50 uL of the DNA containing vector could be added dropwise to each well of a 24 well culture plate in the concentrations listed below. First the vehicle was added by micropipette to a solution of Optimem, inverted to mix, and

allowed to stand for ten minutes. DNA was introduced and the solution mixed by inversion and allowed to stand ten minutes. The DNA plasmid to reagent ratios given in table 2 were tested.

Vector	Vector/DNA (ug/uL)
LT1	2
	3
	4
T-Jurkat	3
	5
	7
TKO	4

The reagent-DNA mixture was then added to each well containing chondrocytes and allowed to incubate at 37° C for 48 hours in an atmosphere of 5% CO2. The medium was removed and cells washed with PBS. Trypsin was then added for 1-3 minutes and followed by addition of minimal medium to a volume of 500 uL were then dislodged from the wells with trypsin and transferred to individual vials for flow cytometry analysis.

Flow cytometry: Prior to flow cytometry 3-5 drops of a mixture of PBS and propidium iodide (excitation 560 nm and emission 640 nm) was added to each vial and each vial agitated by vortex. Propidium iodide is selectively taken up by cells that are dying and is read by the flow cytometer at a wavelength of 640 nm to yield survival percentages. A second laser with

wavelength of 535nm was used to detect whether or not GFP (excitation 488 nm, emission 535 nm) was present in the cell. The data was uploaded to Flowjo 5.9®, a flow cytometry analysis program, and analyzed for light scatter patterns which elucidate both surviving and transfected cells. The surviving cells were gated (a selected population of living cells) and the percentage of surviving cells expressing GFP from this group was determined.

Phase 3: Medium Optimization:

The most efficient reagent, LT1 was selected and tested in the normal chondrocyte maintenance medium described above, and a minimal medium mixture devoid of antibiotics, buffers, and amino acids. The same procedure for reagent dosing was used as described above. Additionally the optimum media was tested with pretreatment of hyaluronidase in doses of 0, 4, or 40 units of enzyme activity. The hyaluronidase was added as follows: 24 hours prior to transfection and left in wells throughout the experiment; 24 hours prior to transfection and washed out at the time of transfection; at the time of transfection and left in the wells for the remainder of the experiment.

Statistical Methods:

Phase 1: Data was analyzed after selecting only reagents and doses yielding no toxicity (+1 histological grade). The ratio of luciferase activity values to standard (LT1- 3 ml) were compared between 121 different reagent/dose combinations using an analysis of covariance (ANCOVA). The ranks of the mean log- transformed luminosities, adjusted for presence of booster, harvest time, passage number, diagnosis, location, and confluency were compared for each reagent to select the three reagents that performed well at multiple doses.

Phase 2-3: The most effective vectors and relative toxicity as measured by the percentage of live and transfected cells were analyzed using repeated measures ANOVA using Sass® version 6.12 (Cary, NC) statistical program.

Results:

Phase 1 Screening of non-viral reagents

In the initial screening, T-Jurkat was found to have the highest marker gene activity without incurring critical levels of toxicity as determined by the histological examination. Gene expression following transfection varied by dose in a roughly normal distribution. The toxicity was consistently found to be dose dependent although thresholds varied among the reagents. Many of the reagents were found to have minimal toxicity at relatively effective doses.

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Table 1- Reagent Testing

Vector	Dose	Relative Lumen Ratio to LT1
LT1	**3**	**1**
T-neural	**12**	**3.69**
TKO	**4**	**3.44**
T-Jurkat	**2.5**	**5.05**

Dose response curves:Data Needed

Reagent testing: Following the histological examination of the cells it was decided to repeat experiments using GFP and optimal doses of reagents. In these subsequent experiments LT1 was less toxic than other reagents and similar to control. LT1 had a mean percent survival of 78.1% as compared to 26.8 % for TKO, 16.8% for T-Jurkat, and 70.8% in controls. However, transfection was only 1.5%.

Table 2- Reagent Testing With GFP at Optimal Dose

VECTOR	Dose	%LIVE	%Transfected	N	standard error
Control	**0**	**70.8**	**0.0**	**3**	**7.28605982**
A60	**0**	**70.4**	**94.5**	**3**	**7.28 605982**
A150	**0**	**64.2**	**80.7**	**3**	**7.28605982**
A300	**0**	**68.4**	**92.8**	**3**	**7.28605982**
LT1	**3**	**78.1**	**1.5**	**3**	**7.28605982**
TKO	**5**	**26.8**	**16.6**	**3**	**7.28605982**
TJ	**8**	**16.8**	**2.1**	**3**	**7.28605982**

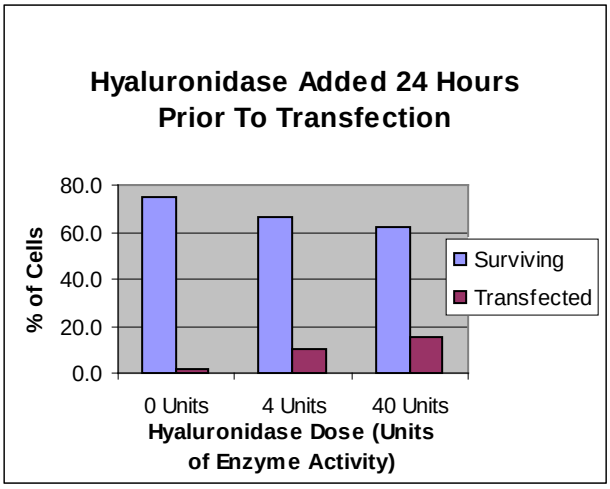
Medium Optimization: LT1 in minimal medium was significantly better than all other reagents in terms of survivability ($p<.005$) and transfection ($p<.05$) percentages. Minimal medium also increased transfection with other reagents, however survivability with TKO and T-Jurkat was still poor.

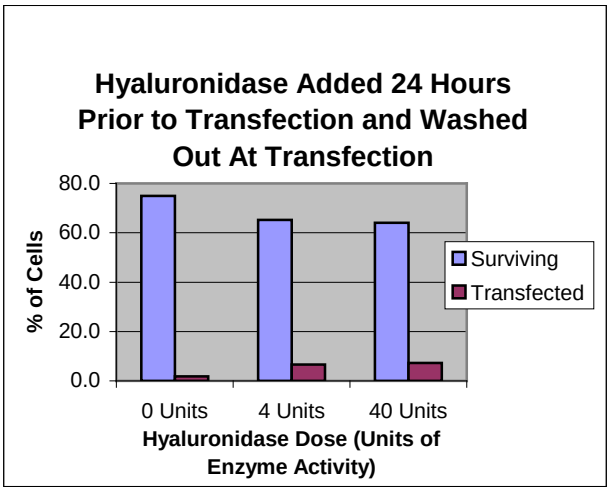
Table 3- Medium Optimization

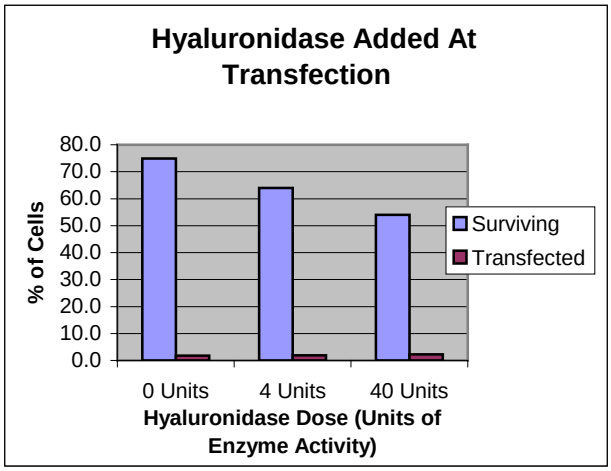
Vector	Medium	% Live	% Transfected
Control	Normal	55.7	0.0
	Minimal	64.0	0.0
LT1	Normal	58.6	3.1
	Minimal	59.5	5.8
TKO	Normal	33.1	2.8
	Minimal	47.2	1.4
T-Jurkat	Normal	23.0	2.9
	Minimal	28.0	2.7

Hyaluronidase Treatments: Hyaluronidase had no effect on the survivability of controls. Hyaluronidase did result in decreased survivability from 74.9% to an overall mean of 62.6% for all treatments compared to controls ($p<.001$). However, transfection percentages increased from 1.8% without treatment to 15.2% with 40 units and 10.4% with 4 units of hyaluronidase given 24 hours prior to transfection and left in throughout the experiment (both values$<.0001$). When treated at the time of transfection, efficiency was not significantly different to samples without hyaluronidase added. Additionally, hyaluronidase added 24 hours prior to transfection and washed out at the time of transfection significantly increased transfection percentages ($p<.001$).

Hyaluronidase Optimization and Cell Transfection







Discussion:

After the initial Phase 1 screening of the vectors it appeared T-jurkat was the most efficient vector in terms of its ability to transfect cells with minimal toxicity. However, in Phase 2 when more subjective measurements using light microscopy were replaced with the highly accurate cell counting of flow cytometry, it was found that LT1 was the most efficient reagent in terms of transfection ability and cell toxicity compared with other reagents. This result was surprising in light of the initial experiments, and illustrates the need for accurate assessments of not only transfection, but of cell survivability in gene transfection experiments.

Treatments in minimal medium yielded significant increases in transfection and no significant difference in toxicity compared to controls. It is hypothesized that the additives in the media designed to protect cells in culture may actually impede the ability of the vector to access the cell membrane to deliver its contents.

The Hyaluronidase treatments seem to further increase the ability of the vector to gain access to the cells and significantly improved transfection. However, there appears to be a fine balance between the degradation of the extracellular matrix allowing access to the cells and the cells ability to survive such an insult. It is well known that cells that have been disrupted from cell to cell adhesion molecules induce cell apoptotic signals. It is therefore possible that the matrix potentiates cell survivability and disruption induces an apoptotic signal and cell death. This finding is important as potentially therapeutic genes delivered with matrix degradation enzymes may be able to transfect some chondrocytes while at the expense of other healthy cells. Thus, the net therapeutic effect may be minimal and at worst the gene therapy may be damaging to the cells. Further study will need to be done to characterize the importance of this finding.

Finally it is not known which therapeutic genes should be delivered to cells. An ideal gene would be one with the ability to not only up-regulate the matrix producing proteins of the cell it transfects, but also have the ability to up-regulate signalling proteins which would allow

senescent cells to reactivate, divide and produce healthy matrix. This would decrease the necessity for a large bolus of cells to be transfected, and hopefully decrease toxicity. The absolute number of cells that must be transfected to produce a therapeutic benefit should be the focus of future research. Transfection of chondrocytes *in vitro* with controller genes coupled to non-viral vectors should help answer some of these questions. Although further research must be done to examine the questions posited above, our results suggest that the non-viral vector LT1 may be used to safely transfect IVD chondrocytes in vitro and may help facilitate gene transfection of IVD chondrocytes with therapeutic genes.

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