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Antibody Conjugated, DNA-Based Nanocarriers Intercalated with Doxorubicin Eliminate Myofibroblasts in Explants of Human Lens Tissue

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Running title: Immunodepletion of Lens Myofibroblasts with Cytotoxic 3DNA

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Non-standard Abbreviations

3DNA: three-dimensional DNA

BMP: bone morphogenetic protein

G8:3DNA:Dox: G8 monoclonal antibody conjugated to three-dimensional DNA intercalated with Doxorubicin

mAb: monoclonal antibody

PBS: phosphate buffered saline

PCO: posterior capsule opacification

TUNEL: deoxynucleotidyl transferase dUTP nick end labeling

α-SMA: alpha smooth muscle actin

-: negative

+: positive

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ABSTRACT

Posterior capsule opacification (PCO) occurs in some adults and most children following cataract surgery. The fibrotic form of PCO arises, in part, from migratory, contractile myofibroblasts that deform the lens capsule and impair vision. In short-term cultures of human anterior lens tissue, myofibroblasts emerge from Myo/Nog cells that are identified with the G8 monoclonal antibody and by their expression of the MyoD transcription factor and bone morphogenetic protein inhibitor noggin. In this study, we tested the hypothesis that targeted depletion of Myo/Nog cells with the G8 mAb conjugated to three-dimensional DNA nanocarriers intercalated with Doxorubicin (G8:3DNA:Dox) would prevent the accumulation of myofibroblasts in long-term, serum and growth factor free cultures of human lens tissue obtained by capsulorhexis. The mAb:nanocarrier complex was internalized into acidic compartments of the cell. G8:3DNA:Dox killed nearly all Myo/Nog cells without affecting the lens epithelial cells. In 30-day cultures, all G8-positive cells expressed noggin and subpopulations had synthesized MyoD, sarcomeric myosin and alpha smooth muscle actin (α-SMA). Myo/Nog cells responded to scratching of the lens epithelium by accumulating around the edges of the wound. Treatment with two doses of G8:3DNA:Dox completely eliminated G8+/α-SMA+ cells throughout the explant. These experiments demonstrate that Myo/Nog cells are the source of myofibroblasts in long-term cultures of anterior human lens tissue and mAb:3DNA nanocarriers specifically and effectively deliver cytotoxic cargo to a subpopulation of cells

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without off target effects. G8:3DNA:Dox has the potential to reduce PCO following cataract surgery.

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INTRODUCTION

A cataract is the clouding of the lens that affects visual acuity, and if left untreated, may lead to blindness. The World Health Organization estimates that by 2020, 32 million cataract operations will be performed each year. While surgery restores vision in the majority of cases, up to 40% of adults and most children develop a secondary cataract, a condition called posterior capsule opacification (PCO) (Awasthi et al., 2009; Wormstone et al., 2009). PCO results from the proliferation and differentiation of lens epithelial cells that form Elschnig pearls (regenerative PCO), or more commonly, myofibroblasts that migrate onto the capsule, a thick basement membrane that surrounds the lens (fibrotic PCO) (Awasthi et al., 2009; Wormstone et al., 2009). A primary cause of loss of visual acuity from PCO is deformation of the lens capsule from myofibroblast contractions (McDonnell et al., 1983; Awasthi et al., 2009; Wormstone et al., 2009).

The source of myofibroblasts in cultures of lens tissue are Myo/Nog cells that were originally identified in the chick embryo blastocyst by their expression of the skeletal muscle specific transcription factor MyoD (Davis, 1987), production of noggin that inhibits bone morphogenetic proteins (BMPs) (Zimmerman et al., 1996) and the G8 monoclonal antibody (mAb) (Gerhart et al., 2001; Gerhart et al., 2006; Gerhart et al., 2009; Walker et al., 2010; Gerhart et al., 2011; Gerhart et al., 2014). Early in development, Myo/Nog cells originating in the epiblast layer are integrated into the eyes and other organs (Gerhart et al.,

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2006; Gerhart et al., 2007; Gerhart et al., 2009). Elimination of Myo/Nog cells in the epiblast results in hyperactive BMP signaling, malformations of the eyes and central nervous system, externalization of organs through the ventral body wall and an absence of skeletal muscle (Gerhart et al., 2006; Gerhart et al., 2009; Gerhart et al., 2011).

Myo/Nog cells are also present in adult tissues, including the human eye (Gerhart, 2012; Gerhart et al., 2014; Bravo-Nuevo, 2015; Brandli, in press). In anterior human lens tissue removed during cataract surgery, Myo/Nog cells synthesize skeletal muscle sarcomeric proteins and surround wrinkles in the capsule denuded of epithelial cells (Gerhart et al., 2014). Depletion of Myo/Nog cells in short-term cultures of chick embryo and human lens tissue prevented the accumulation of myofibroblasts (Walker et al., 2010; Gerhart et al., 2014). Our method of depleting Myo/Nog cells within the embryo and in lens cultures involved incubation with the G8 mAb and lysing with complement (Gerhart et al., 2006; Gerhart et al., 2007; Gerhart et al., 2009; Walker et al., 2010; Gerhart et al., 2011; Gerhart et al., 2014; Bravo-Nuevo et al., 2016).

The following study describes a novel method of eliminating Myo/Nog cells and testing its effects on the emergence of myofibroblasts in long-term cultures of human lens tissue. This refined method of immunodepletion involves conjugating the G8 mAb to three-dimensional DNA-based nanoparticles® (3DNA) intercalated with the cytotoxin Doxorubicin (G8:3DNA:Dox). 3DNA is a branched (dendrimeric) structure built entirely from interconnected monomeric subunits of 5-cytosine-phosphate-guanine-3-free DNA to minimize immune responses

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(Nilsen et al., 1997; Krieg, 2002). Each monomer exists as a pair of hybridized DNA strands complementary only in the central region, creating a double stranded waist and four single-stranded “arms” that are available to hybridize to similar monomers. Multiple “layers” of monomers are crosslinked to form a 3DNA superstructure. The arms of peripheral DNA can be conjugated with different molecules for the purposes of targeting specific cell types, high sensitivity detection of gene expression and delivery of therapeutic payloads (Gerhart et al., 2000; Gerhart et al., 2001; Strony et al., 2005; Gerhart et al., 2006; Mora, 2007; Huang et al., 2016). The 3DNA nanocarriers themselves are biodegradable within the cell, nontoxic when endocytosed in cultured cells or injected into mice, multivalent and efficient at releasing cargo from the endosome during the acidification process (Muro, 2014; Huang et al., 2016).

In this study, we tested the hypothesis that the G8 mAb conjugated to 3DNA nanocarriers intercalated with Doxorubicin (G8:3DNA:Dox) would specifically target and deplete Myo/Nog cells and prevent the accumulation of myofibroblasts in long-term, serum and growth factor free cultures of human lens tissue obtained by capsulorhexis.

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MATERIALS and METHODS

Subjects

This study was carried out in accordance with the Declaration of Helsinki and approved by the Main Line Health Hospitals Institutional Review Board. Informed consent was obtained from subjects undergoing elective cataract surgery. Tissue was obtained from 15 males and 28 females. The age of the subjects ranged from 62-91 with a mean of 78.

Explant Cultures of Human Lens Tissue

Anterior lens tissue was removed by capsulorhexis during cataract surgery. The tissue was divided into two pieces and submerged in 300 µl of DMEM/F12 containing 3 Units of penicillin and 30 µg streptomycin (GIBCO/Life Technologies, Grand Island, NY), in eight well chamber slides (Nunc Lab-Tek, ThermoFisher Scientific, Waltham, MA). Explants suspended in medium were cultured for up to 30 days at 37ºC in 5% CO_2 in air. Fresh medium (100-200 µl) was added to the wells each week.

In some experiments, lens explants were wounded on day 29 in culture. Wounding was carried out by removing the lens tissue from the culture well, placing it in a drop of medium on a glass slide and scratching the epithelium with a dissecting needle while leaving the underlying capsule intact. The tissue was transferred back to its original well and cultured for an additional 24 hours.

Synthesis of Targeting, Cytotoxic DNA-Based Nanocarriers

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Two-layered 3DNA nanocarriers (Genisphere, LLC) consisting of approximately 3,000 DNA bases and 36 single-stranded peripheral DNA regions (Figure 1A) were synthesized as described previously (Nilsen et al., 1997; Vogelbacker, 1997; Wang, 1998). Seven unique sequences of single stranded DNA were designed to hybridize to one another to create building block monomers each with a central double-stranded region and four terminal single-stranded regions. By design, five unique monomers themselves hybridize to each other in specific orientations due to base pairing between complementary single-stranded regions. For manufacturing, monomers are hybridized and crosslinked to each other in a step-wise fashion of forming layers. The two-layered 3DNA utilized in this study had a diameter of approximately 60 nm a zeta potential of approximately -28 meV, as determined by dynamic light scattering (Malvern Instruments Zetasizer). The molecular weight 10^6 Daltons was confirmed by agarose gel analysis, isotope quantitation (Beckman Coulter scintillation counter) and spectrophotometry (Thermo Fisher Scientific Nanodrop). Doxorubicin (Sigma-Aldrich, St. Louis, MO) was intercalated into the central double-stranded regions of 3DNA by incubating at room temperature. Free Doxorubicin was combined with 3DNA at a mole ratio that resulted in greater than 99% of the Doxorubicin being bound by the 3DNA scaffold. The molar ratio of Doxorubicin to 3DNA was approximately 500:1, as confirmed by spectrofluorometry. The G8 mAb was conjugated to a single stranded DNA oligonucleotide, complementary to 3DNA arms, via amine-to-sulfhydryl attachment using a heterobifunctional crosslinker (Pierce Crosslinking Kit,

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Thermo Fisher Scientific). The presence and sequence of the oligonucleotide portion of the conjugate was confirmed using a native polyacrylamide gel. The antibody portion of the conjugate maintained functional binding activity demonstrated by fluorescence localization assays utilizing human lens explant tissue. The antibody-oligonucleotide conjugate was hybridized to 3DNA containing Doxorubicin, yielding approximately four G8 mAbs per 3DNA (Figure 1A). The diameter of the final construct was approximately 120 nm, as determined by dynamic light scattering.

Assay for Internalization of G8:3DNA

Anterior lens tissue was incubated in 250 µl of culture medium containing a 1:500 dilution of LysoSensor green dye (ThermoFisher Scientific) and 200 ηg Cy3 labeled 3DNA nanocarriers conjugated with the G8 mAb for 90 minutes at 37° within four hours of plating. Cy3 labeled 3DNA nanocarriers lacking the G8 mAb were used as a control for the experiment. Tissue was rinsed in phosphate buffered saline (PBS) and fixed in 2% formaldehyde for 10 minutes. Nuclei were labeled with Hoechst dye 33258 (Sigma-Aldrich, St. Louis, MO).

Depletion of Myo/Nog cells in Explants of Human Lens Tissue

The culture medium was replaced with 250 µl medium containing G8:3DNA:Dox, G8:3DNA or 3DNA:Dox 18 hours after plating. The final concentration of 3DNA and Doxorubicin was 0.64 ng of 3DNA and 0.29 µM Doxorubicin. Cells were fixed 24 hours later and labeled with the G8 mAb,

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affinity purified, F(ab´)2 goat anti-mouse IgM μ chain conjugated with DyLight or Alexa 488 (Jackson ImmunoResearch, West Grove, PA) and fluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reagents (Roche Diagnostics, Mannheim, Germany) as described previously (Gerhart et al., 2011; Gerhart et al., 2014). In another set of cultures, the drug-containing medium was replaced with fresh medium. A subset of cultures received a second dose of G8:3DNA:Dox or 3DNA:Dox 10 days later, incubated was added to the cultures for 24 hours and then replaced with fresh medium for the remainder of the culture period, for a total of 30 days.

Immunofluorescence Localization

Anterior lens tissue was labeled with antibodies following fixation in 2% formaldehyde and permeabilization in 0.5% Triton X-100 as described previously (Gerhart et al., 2000; Gerhart et al., 2001; Gerhart et al., 2006). Primary antibodies to the following molecules were used in this study: G8 (Gerhart et al., 2001), alpha smooth muscle actin (α-SMA) (Sigma-Aldrich), noggin (AF719; R&D Systems, Minneapolis, MN), MyoD1 (NCL-MyoD1; Novocastra Labs Ltd, UK), slow sarcomeric myosin (A4.951) (Webster et al., 1988) and αB-crystallin (CPTC-CRYAB-1). The mAbs to sarcomeric myosin and αB-crystallin were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Dept. of Biology, Iowa City, IA. The antibody to α-SMA was directly conjugated with fluorescein. Other primary antibodies were visualized with affinity purified,

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F(ab´)2 goat anti-mouse IgM µ chain, goat anti-mouse IgG or donkey anti-goat IgG secondary antibodies conjugated with DyLight or Alexa 488, Dylight 549 or rhodamine (Jackson ImmunoResearch). Controls for background fluorescence included anterior lens tissues incubated with secondary antibodies alone. Nuclei were stained with Hoechst dye.

Staining was analyzed with the Nikon Eclipse E800 epifluorescence microscope equipped with the Evolution QE Optronics video camera and Image Pro Plus image analysis software program (Media Cybernetics, Rockville, MD) and the Nikon Eclipse 90i, Roper camera and Nikon's Advanced Research software. Figures were annotated and adjusted for brightness and contrast with Adobe Photoshop CC.

Statistical Analyses

The percentage of the total cells that was labeled with one antibody was determined by counting the numbers of fluorescently labeled and unlabeled cells in 20 consecutive fields as described previously (Gerhart et al., 2014). For double labeled tissues, the percentage of cells labeled with one antibody that was also stained with the second primary antibody was calculated by counting the number of single and double labeled cells in each field. The means and standard deviations of control groups were compared to each other and to cultures treated with G8:3DNA:Dox by the t Test using GraphPad software.

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RESULTS

G8:3DNA is Internalized into Acidic Compartments of the Cell

The G8:3DNA conjugate was used to specifically deliver the cytotoxin Doxorubicin to Myo/Nog cells. In order to kill Myo/Nog cells, the complex must be internalized into the cell, as illustrated in Figure 1B. Release of Doxorubicin from DNA nanoparticles is enhanced at acid pH (Kim, 2016). Demonstration of internalization into acidic compartments within Myo/Nog cells was carried out by simultaneous incubation of explants of human anterior lens tissue with 3DNA nanocarriers conjugated with the G8 mAb and the fluorochrome Cy3 (G8:3DNA:Cy3) and the LysoSensor green dye that only fluoresces at acidic pH (≤ pH 5).

The LysoSensor dye that is freely permeable through membranes fluoresced within both lens epithelial and Myo/Nog cells with various intensities (Figure 1C and D). Consistent with previous results (Gerhart et al., 2014), the G8 mAb labeled a small subpopulation of cells within the lens explant (Figure 1D). Overlap of the two fluorochromes in merged images indicates that G8:3DNA:Cy3 was internalized into acidic compartments of Myo/Nog cells (Figure 1C-E). Only trace amounts of 3DNA:Cy3 lacking the G8 mAb were internalized into a small number of cells within the explant (Figure 1F-H).

G8:3DNA:Dox Specifically Targets Myo/Nog Cells in Lens Explant Cultures

Explants of human lens tissue were incubated in serum free medium containing G8:3DNA:Dox beginning 18 hours after plating. The following day,

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tissue was labeled with the G8 mAb and TUNEL reagents to detect cell death. Incubation of lens tissue with control conjugates consisting of G8:3DNA or 3DNA:Dox did not affect the viability of Myo/Nog or lens epithelial cells (Figure 2A-F; Table 1). Treatment of lens explants with G8:3DNA:Dox induced cell death in the Myo/Nog cell population, as evidenced by the co-localization of G8 and TUNEL reagents (Figure 2G-I). Cells with α-SMA also were targeted by G8:3DNA:Dox Figure 2J-L). The G8-negative (-) lens epithelial cells labeled with an antibody to αB-crystalline were unaffected by G8:3DNA:Dox (Figure 2M-O). Quantitation of the results demonstrated that: 1) no significant differences were found between the percentages of G8-positive (+) cells in untreated explants and those treated with the control conjugates or G8:3DNA:Dox; 2) significant differences were found in the percentages of TUNEL+ cells in all three control cultures compared to G8:3DNA:Dox ($p < 0.0001$), 3) approximately 99% of the G8+ cells were TUNEL+, and vice versa, following incubation in G8:3DNA:Dox, and 4) none of the conjugates induced cell death in the lens epithelial population (Table 1). These experiments demonstrate that the G8 mAb specifically delivers 3DNA:Dox to the Myo/Nog subpopulation and one dose of G8:3DNA:Dox kills nearly all G8+ cells.

Myo/Nog Cells Are Present in 30-Day Lens Explant Cultures

Lens tissue was labeled with antibodies to Myo/Nog cell markers and muscle proteins to determine whether they survived for 30 days in serum free medium and to pave the way for assaying the long-term effects of G8:3DNA:Dox

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on the accumulation of myofibroblasts. While all G8+ cells continued to synthesize noggin, only a subpopulation of these cells contained detectable levels of MyoD (Figure 3A-F; Table 2). The decrease in the percentage of MyoD+ Myo/Nog cells from that previously seen in Myo/Nog cells in short-term cultures (Gerhart et al., 2014) may reflect either a quiescent state in which MyoD mRNA but not protein is detectable in G8+ cells (Gerhart et al., 2000) or a decrease in MyoD expression following differentiation (Bentzinger, 2012). Consistent with either of these interpretations is the finding that only a subpopulation of G8+ cells contained the differentiation marker sarcomeric myosin (Figure 3G-I; Table 2). Most G8+ cells contained α-SMA; however, some α-SMA+ cells lacked G8 (Figure 3J-L; Table 2). These results suggest that Myo/Nog cells present in 30-day human explant cultures are in different stages of maturation.

Two Doses of G8:3DNA:Dox Completely Eliminates Myofibroblasts in 30-Day Human Lens Cultures

The long-term effects of treatment with G8:3DNA:Dox were analyzed one month after plating. In order to activate any remaining Myo/Nog cells, a scratch wound was produced in the epithelium on day 29. G8+/α-SMA+ cells had accumulated around the wound a day later (Figure 4A-C). Low percentages of G8+ and α-SMA+ cells (0.5% and 0.4%, respectively, n = 2)) were present in 30-day cultures following treatment with a single dose of G8:3DNA:Dox on the first day after plating (Figure 4D-F). Therefore, explants were exposed to a second

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dose of conjugates 13 days after plating. G8+/α-SMA+ cells rimmed the wound and extended processes onto the capsule when treated with two doses of the non-targeting 3DNA:Dox (Figure 4G-I). No significant differences were observed in the percentages of G8+ and α-SMA+ cells in untreated and control conjugate treated cultures, and the cells were distributed similarly along the wound and throughout the culture (Table 3; Figure 4). However, the second dose of G8:3DNA:Dox completely eliminated G8+ and α-SMA+ cells in all explants (Figure 4J-L; Table 3).

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DISCUSSION

This study demonstrates an innovative approach to targeted immunotherapy that capitalizes on the properties of a novel DNA-based nanocarrier, 3DNA, to deliver a cytotoxic payload. 3DNA's versatility in design and multivalency allows for rapid modification of the synthesis platform to accommodate a wide range of therapeutic and diagnostic applications. For example, 3DNA can be derivatized with fluorescent oligonucleotides for detection of low abundance mRNA by *in situ* hybridization, siRNA, small molecule cytotoxins and targeting moieties (Gerhart et al., 2000; Gerhart et al., 2001; Strony et al., 2005; Gerhart et al., 2006; Mora, 2007; Muro, 2014; Huang et al., 2016). This study is the first to report functionalization of 3DNA nanocarriers with a cell type specific mAb and the cytotoxin Doxorubicin to target a minor subpopulation of cells. As opposed to other cytotoxins that require conjugation to the nanocarrier, Doxorubicin can be intercalated into various conformations of DNA (Um et al., 2006; Hoffman, 2008; Roh et al., 2010; Nishikawa et al., 2011; Roh et al., 2011; Jiang et al., 2012; Zhao et al., 2012; Kim, 2013). Furthermore, the amount of drug and targeting molecule can be titrated to maximize cell death and minimize off target effects. Doxorubicin was the drug of choice for this study because of its cytotoxic effects after release from DNA, well-characterized pharmacokinetic and pharmacodynamics properties, and history of use as a chemotherapeutic agent for human cancers (Trouet, 1972; Um et al., 2006; Carvalho et al., 2009; Roh et al., 2010; Nishikawa et al., 2011; Roh et al., 2011; Jiang et al., 2012; Zhao et al., 2012; Kim, 2013).

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3DNA nanocarriers intercalated with Doxorubicin were conjugated with the G8 mAb to target Myo/Nog cells. The conjugate was internalized into acidic compartments of Myo/Nog cells. G8:3DNA:Dox specifically killed Myo/Nog cells in human lens explant cultures, indicating that Doxorubicin escaped the endosome. This result is consistent with previous studies demonstrating that: 1) four-layered DNA dendrimers conjugated with an antibody to intercellular adhesion molecule-1 are internalized into endosomes and permit the escape of endosomal contents into the cytoplasm (Muro, 2014), and 2) Doxorubicin is released more rapidly from DNA at an acidic pH (Kim, 2016).

A single dose of G8:3DNA:Dox dramatically reduced the numbers of G8+ and α-SMA+ myofibroblasts in explants of human lens tissue cultured in serum free medium for 30 days. This study supports and advances our previous data in which the G8 mAb and complement were used to lyse Myo/Nog cells in short-term human lens cultures (Gerhart et al., 2014). Two doses of G8:3DNA:Dox completely eliminated G8+ and α-SMA+ cells after 30 days in culture, even when the tissue was challenged by wounding. The conclusion drawn from this study is that all myofibroblasts emerge from Myo/Nog cells in this long-term culture system of human anterior lens tissue. The drug was non-toxic to lens epithelial cells that are important for securing the position of the intraocular lens and maintaining the capsule following cataract surgery (Spalton et al., 2014).

The potential of Myo/Nog cells to react to wounding of the lens epithelium after 29 days in culture has important implications for potential therapies directed at preventing PCO. Sustained delivery of G8:3DNA:Dox injected during cataract

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surgery may be critical for depleting Myo/Nog cells that escape exposure to the drug during the acute phase of injury and for killing Myo/Nog cells that may migrate into the lens in response to prolonged stress.

This relatively simple, serum and growth factor free culture system of human anterior lens tissue supports the viability of Myo/Nog and lens epithelial cells for a period of at least one month and is suitable for screening drugs with disease modifying potential. Our method of cell depletion that utilizes a cell type specific targeting antibody conjugated to 3DNA:Dox may be therapeutic in pathological conditions outside of the lens in which a subpopulation of cells contributes to disease progression and recurrence.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Gerhart, Getts, George-Weinstein

Conducted experiments: Gerhart, Greenbaum, Mathers

Contributed new reagents: Costa, Clemente, Getts

Performed data analysis: Gerhart, George-Weinstein

Wrote or contributed to the writing of the manuscript: Gerhart, Getts, George-Weinstein

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FOOTNOTES

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FIGURE LEGENDS

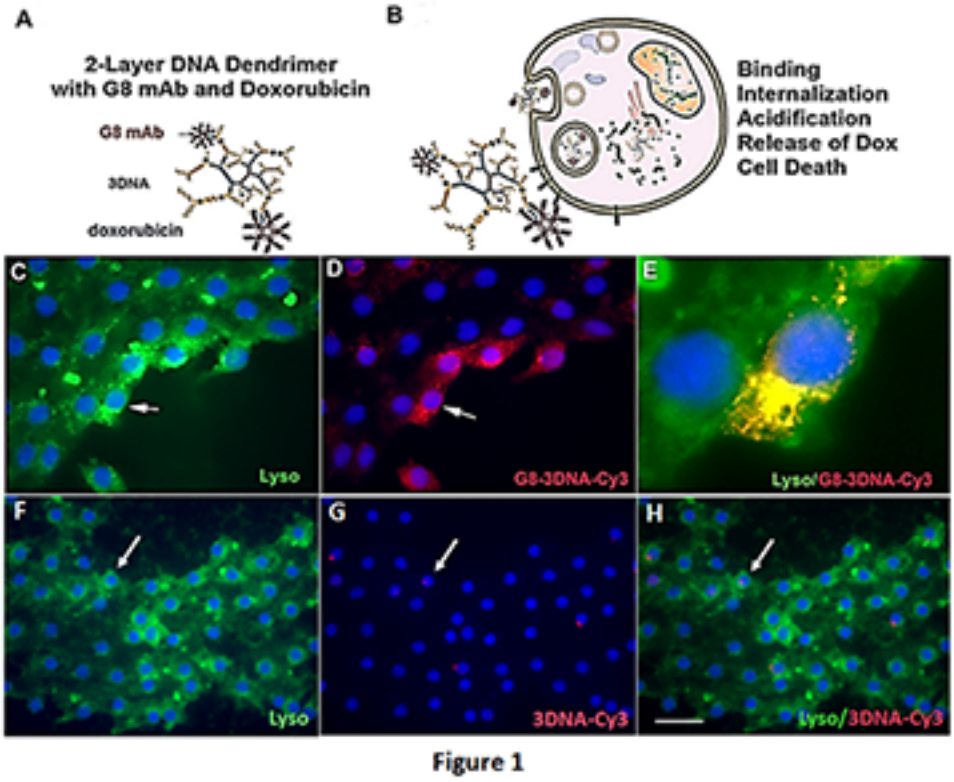

Figure 1

Figure 1. Structure and internalization of G8:3DNA. A. Schematic representation of the G8 IgM mAb coupled to a 2-layer 3DNA nanocarrier intercalated with Doxorubicin. B. Proposed mechanism of targeted cytotoxicity in which Doxorubicin diffuses from 3DNA following antibody binding and internalization of the conjugate into acidic compartments of the cell. C-E: Anterior lens tissue was incubated for two hours with G8-3DNA-Cy3 (red) and LysoSensor dye that fluoresces green at acidic pH (C-E) or 3DNA-Cy3 and LysoSensor dye (F-H). Nuclei were labeled with Hoechst dye (blue). Co-localization of red and green appears yellow in the merged image in E. Minimal incorporation of the non-targeting 3DNA-Cy3 conjugate was visible in the tissue (G). Bar = 9 µM in F-H, 5.67 µM in B and D and 2.25 µM in E.

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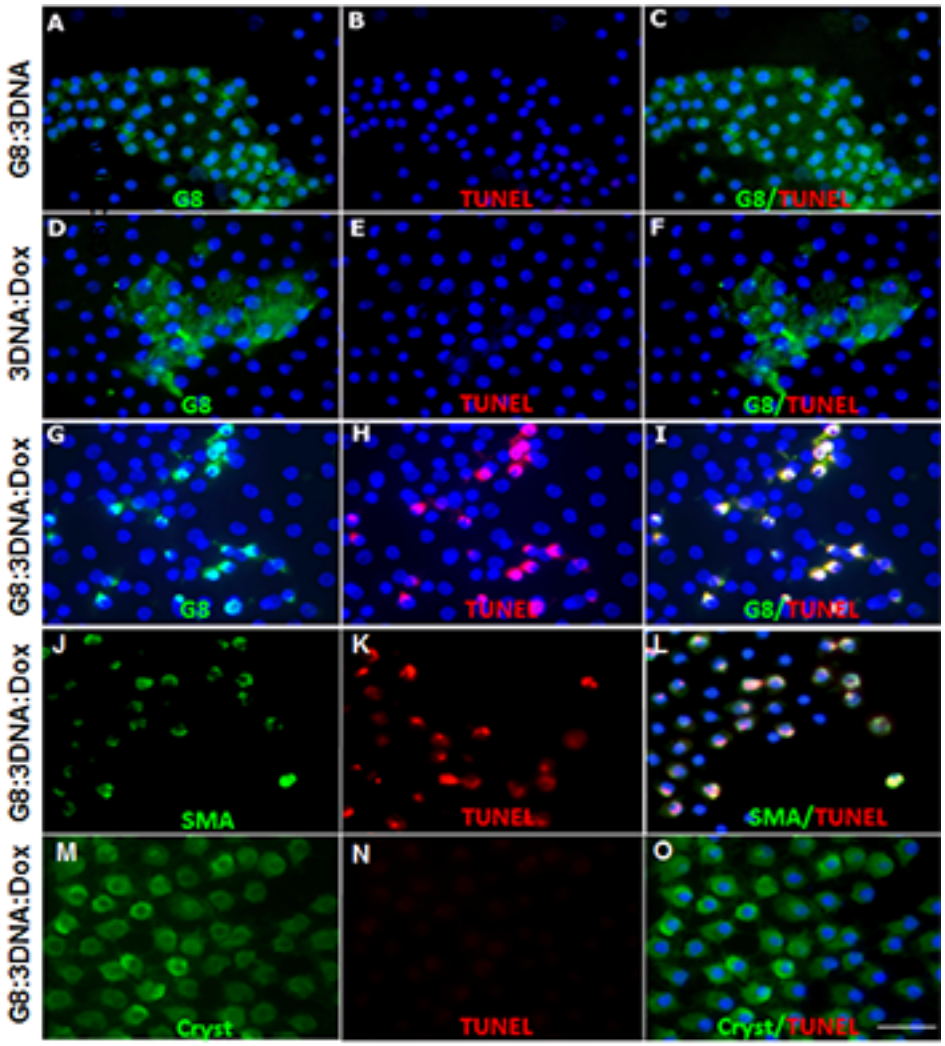

Figure 2

Figure 2. G8:3DNA:Dox kills Myo/Nog cells in human lens tissue. Anterior human lens tissue was incubated with G8:3DNA (A-C), 3DNA:Dox (D-F) or G8:3DNA:Dox (G-)) for 24 hours, beginning on the second day in culture. Lenses were fixed and labeled with antibodies to G8, α-SMA or αB-crystalline and fluorescent secondary antibodies (green) and TUNEL reagents (red). Nuclei were stained with Hoechst dye (blue). Overlap of red and green appears yellow

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in merged images. G8:3DNA:Dox (G-I), but not the control conjugates (A-F), specifically killed Myo/Nog cells in lens explants. Bar = 9 µM.

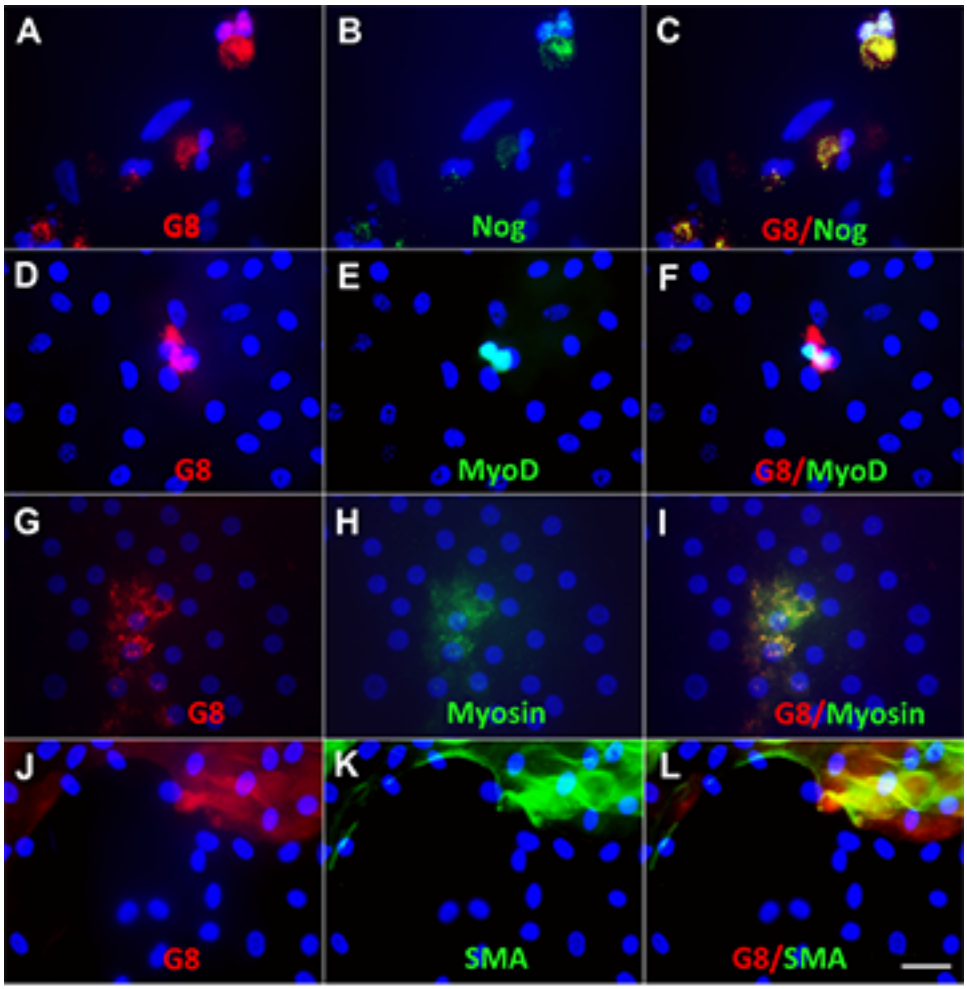

Figure 3

Figure 3. Expression of Myo/Nog and muscle cell markers in long-term cultures of human lens tissue. Anterior lens tissue was cultured for 30 days and labeled with antibodies to G8, noggin, MyoD, sarcomeric myosin, α-SMA and fluorescent secondary antibodies. Nuclei were stained with Hoechst dye (blue). Overlap of red and green appears yellow in merged images. G8+ cells contained

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noggin (A-C), MyoD (D-F), sarcomeric myosin (G-I) and α-SMA (J-L). Bar = 9 μM.

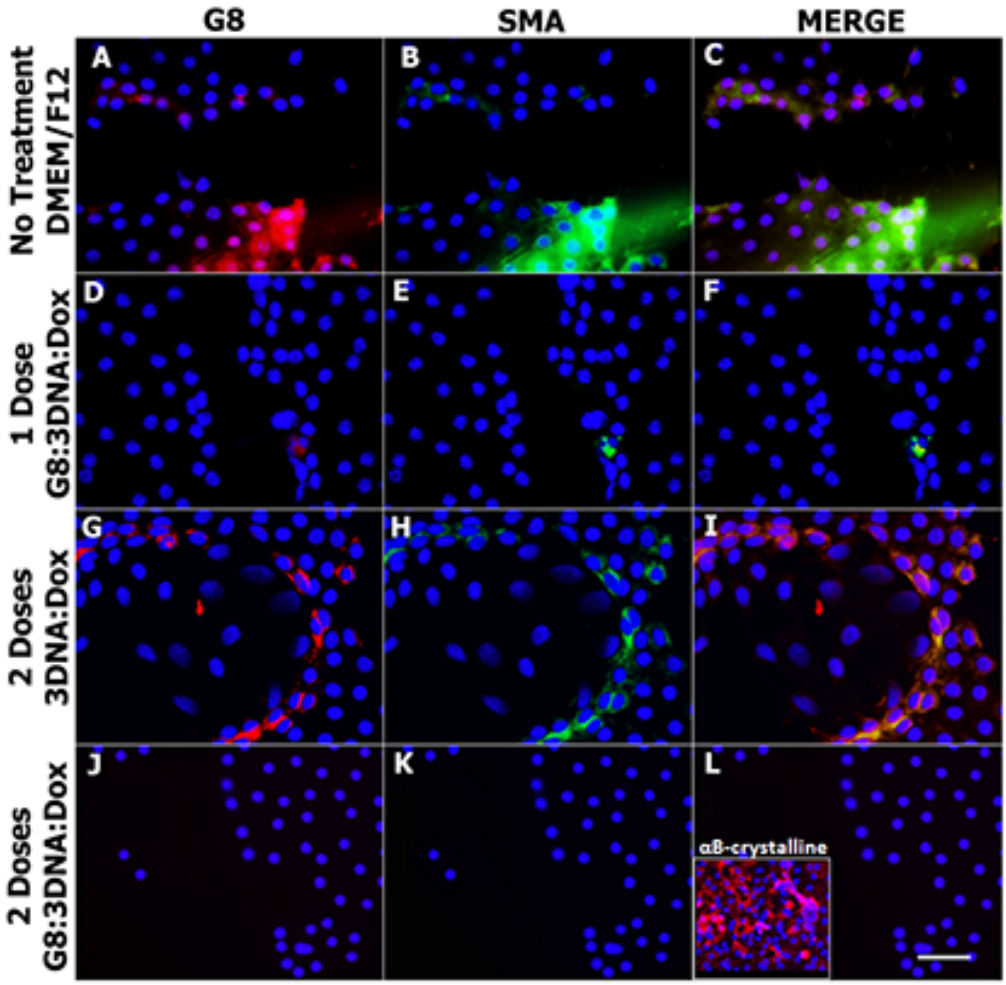

Figure 4

Figure 4. Effect of G8:3DNA:Dox on myofibroblast accumulation in long-term cultures of human lens tissue challenged by wounding. Anterior lens tissue was incubated with DMEM/F12 medium alone or medium containing 3DNA:Dox or G8:3DNA:DOX for 24 hours on day 1 only or days 1 and 13. The tissue was wounded on day 29 and stained for G8, fluorescent secondary

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antibodies (red) and α-SMA (green), or G8 and αB-crystallin the following day. Nuclei were stained with Hoechst dye (blue). Overlap of red and green appears yellow in merged images. G8+/α-SMA+ cells were present around the wound in cultures lacking drug (A-C) and those treated with 3DNA:Dox (G-I). Myo/Nog cells extended processes onto the capsule (I). G8+/α-SMA+ cells were rare in cultures treated with a single dose of G8:3DNA:Dox (n = 4) (D-F). Two doses of G8:3DNA:Dox completely eliminated G8+/α-SMA+ cells (J-L). Lens epithelial cells stained for αB-crystallin were unaffected by two doses of G8:3DNA:Dox (inset in L). Bar = 9 μM.

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Table 1. Depletion of Myo/Nog cells with G8:3DNA:Dox in human lens cultures.

Anterior human lens explants were incubated with medium alone (untreated), G8:3DNA, 3DNA:Dox or G8:3DNA:Dox for 24 hours.

Cells were double labeled with the G8 mAb and fluorescent secondary antibodies, and TUNEL reagents.

The results are the mean ± standard deviation.

Untreated, G8:3DNA and 3DNA:Dox, n = 5

G8:3DNA:Dox: n = 8

Significant differences were found between the percentages of TUNEL+ cells in all three control groups compared to G8:3DNA:Dox ($p < 0.0001$), untreated and 3DNA:Dox ($p = 0.0005$), and G8:3DNA and 3DNA:Dox ($p = 0.02$).

Treatment	% G8+	% TUNEL+	% G8+ with TUNEL	% TUNEL+ with G8
Untreated	4 ± 1	0.7 ± 0.2	0	0
G8:3DNA	4 ± 1	0.5 ± 0.3	0	0
3DNA:Dox	4 ± 2	0.1 ± 0.1	0	0
G8:3DNA:Dox	5 ± 1	5 ± 1	99 ± 2	99 ± 2

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% G8+ or TUNEL+ = (number of fluorescent cells ÷ total number of cells in 20 fields) X 100. % G8+ with TUNEL = (number of G8+ cells co-labeled with the second primary antibody ÷ total G8+ cells) X 100. Reciprocal pairs are also presented. G8:3DNA:Dox, but not control conjugates, specifically killed G8+ cells.

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Table 2. Expression of Myo/Nog and muscle cell markers in 30-day human lens cultures.

Anterior human lens explants were cultured for 30 days and doubled labeled with antibodies to G8 and noggin, MyoD, α-SMA and sarcomeric myosin and fluorescent secondary antibodies.

The results are the mean ± standard deviation.

The number of subject is indicated in parentheses.

Markers	**Percent Positive**	
G8	6 ± 4	(18)
Noggin	5 ± 3	(7)
MyoD	1 ± 2	(4)
Myosin	4 ± 3	(4)
α-SMA	9 ± 5	(9)
G8+ cells with Noggin	100	(4)
Noggin+ cells with G8	99 ± 3	(4)
G8+ cells with MyoD	24 ± 24	(4)
MyoD+ cells with G8	100	(4)
G8+ with Myosin	53 ± 29	(4)

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Myosin+ cells with G8	100	(4)
G8+ cells with α-SMA	82 ± 22	(6)
α-SMA+ cells with G8	67 ± 32	(6)

% positive = (number of fluorescent cells ÷ total number of cells in 20 fields) X 100. % G8+ with second primary antibody = (number of G8+ cells co-labeled with the second primary antibody ÷ total G8+ cells) X 100. Reciprocal pairs are also presented. All G8+ cells contained Noggin. Subpopulations of Myo/Nog cells contain different muscle proteins.

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Table 3. Effects of G8:3DNA:Dox on the accumulation of G8+ and α-SMA+ cells in 30-day, wounded human lens explants.

Anterior human lens explants were incubated with medium alone, 3DNA:Dox or G8:3DNA:Dox for 24 hours on the second and thirteenth day in culture.
Wounds were created in the epithelium on day 29.
Cells were double labeled with antibodies to G8, fluorescent secondary antibodies and α-SMA on day 30.
The results are the mean ± standard deviation.
The number of cultures is indicated in parentheses.
***p < 0.0001.

Marker	% Positive No drug	% Positive G8:3DNA	% Positive 3DNA:Dox	% Positive G8:3DNA:Dox
G8	9 ± 2 (4)	9 ± 1 (4)	8 ± 4 (n = 8)	***0 (n = 7)
α-SMA	9 ± 2 (4)	10 ± 1 (4)	7 ± 3 (n = 6)	***0 (n = 7)

% Positive = (number of fluorescent cells ÷ total number of cells in 20 fields) X 100. The number of cultures scored is indicated in parentheses. Two doses of G8:3DNA:Dox completely eliminated G8+/α-SMA+ cells in long-term lens cultures.

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