STUDY OF THE INTERNALIZATION AND SUBCELLULAR TRAFFICKING OF MODEL ANTISENSE COMPOUNDS IN HEP G2 CELLS.

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Introduction

Antisense therapy has the potential to specifically treat a wide variety of diseases on the cellular level by inhibiting undesired protein synthesis.[1,2] This potential has stimulated a significant amount of research in this area but problems limit antisense in reaching its potential. One of the greatest obstacles of antisense therapy is delivery of the oligonucleotide to its target. The antisense oligonucleotide must reach its complementary mRNA in the cytoplasm or nucleus to be effective. To do this, the oligonucleotides must pass one or more biological membranes, but their charge and/or size should prevent this.

The goal of this research is to develop methods to study the internalization and subcellular trafficking of compounds into cells. From this basis, we can systematically change the delivery system to improve cytoplasmic or nuclear delivery of oligonucleotides or other therapeutic agents.

The oligonucleotides and model compounds are delivered via water soluble polymers[3] (see Figure 1). The polymer contains targeting moieties which results in internalization by receptor mediated endocytosis. The oligonucleotides and target compounds are covalently attached to the polymer backbone by nondegradable or degradable spacers. The degradable spacers are composed of a tetrapeptide sequence, Gly-Phe-Leu-Gly, which is cleaved by enzymes[4] in the lysosomes allowing release of the oligonucleotide or model compound in the lysosome. This will allow us to determine whether the model compound can escape when released in the lysosome.

Internalization and subcellular trafficking are monitored by confocal fluorescent microscopy. The oligonucleotide or other target compound is labeled with a fluorescent tag such as fluorescein to allow detection. Confocal microscopy has the advantage over conventional microscopy in that light from outside the focal plane is excluded from the detector. This allows one to scan the cell looking at thin slices at different depths (in the z-direction). With a bright sample, slices as thin as 1 μm are easily obtained.

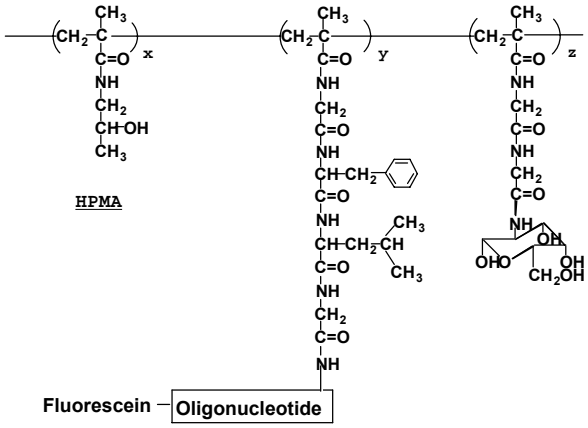

Figure 1. Structure of targetable polymer-oligonucleotide conjugates.

Other polymer spacers (currently being developed) release the oligonucleotide in the endosome and allow endosome escape to be determined.

In the final stage, membrane disrupters, such as fusogenic peptides, can be incorporated into the system to increase the efficiency of delivery.

Experimental Methods

A 21-mer oligodeoxynucleotide phosphorothioate, complementary to nucleotides 1903-1923 of the human hepatitis B virus[5] (ayw subtype) was used. Fluorescein was attached to the oligonucleotide via a fluorescein labeled phosphoramidite. The oligonucleotide was terminated with a primary amine at either the 3' or the 5' end to allow coupling to the polymer delivery system. The delivery system consisted of copolymers of N-(2-hydroxypropyl)methacrylamide (HPMA), and comonomers containing N-acylated galactosamine (for targeting to hepatocytes) and 4-nitrophenylate groups on the termini of the side-chains. The oligonucleotide and other compounds were conjugated to the polymer via aminolysis of 4-nitrophenylate. Conjugation was attempted in solution and in a two-phase reaction with the oligonucleotide remaining attached to the CPG beads on which it was synthesized. Conjugation was detected by PAGE. Hep G2 cells were cultured in

minimum essential media-alpha modification and 5% fetal bovine serum. Fluorescence was quantified on a fluorometer. Protein content was determined by the Lowry assay.

Results and Discussion

Several polymers with M_w between 30,000 – 43,000 Da were synthesized with and without N-acylated galactosamine. Fluorescein or fluorescein labeled oligonucleotides with a primary amines were coupled to the polymer. Conjugation of the fluorescein to the polymer was more efficient than conjugation of the oligonucleotide which needs to be optimized to produce larger amounts.

Cells were incubated with various amounts of polymer labeled with fluorescein for 24 hours. The amount of internalized polymer was determined by measuring the relative fluorescence of dissolved cells. One polymer contained galactosamine whereas the control polymer did not. Figure 2 shows the relative amount of polymer internalized at various concentrations. More polymer containing the targeting moiety galactose was internalized as compared to the control polymer at similar concentrations. This indicates that the cells had functioning receptors.

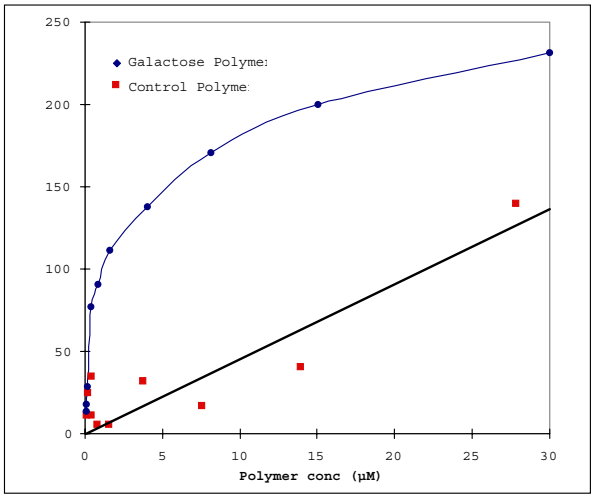

Figure 2. Relative amount of polymer with and without galactosamine internalized by Hep G2 cells over 24 hours.

Confocal microscopy was used to look at cells incubated with the two fluorescein labeled polymers. Cells incubated with polymer containing galactose were brighter than those incubated with control polymer as expected. The confocal microscope allows detection of the fluorescein in several cellular compartments. Soon after internalization, endosomes and lysosomes are clearly visible as small vesicles distributed in the cytoplasm. Incubation with large amounts of polymer resulted in fluorescence being detected in the cytoplasm and/or nucleus. Figure 3 shows nuclear accumulation in the left picture, nuclear exclusion of the labeled polymer in the right picture and many intact endosomes and lysosomes.

Other experiments showed that free fluorescein was able to quickly diffuse through cell membranes as was expected. Thus, if fluorescein was cleaved from the polymer, it would diffuse throughout the entire sample and only increase the background fluorescence.

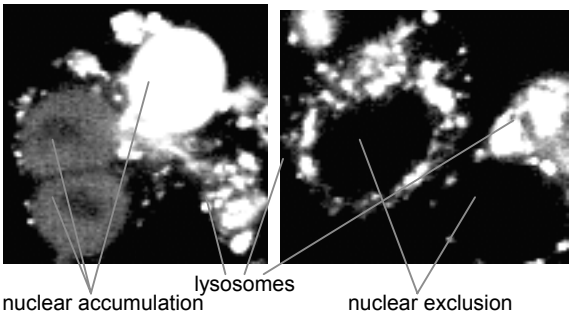

Figure 3. Perinuclear section of cells incubated with fluorescein labeled polymer.

Conclusion

A water soluble polymer delivery system was synthesized using HPMA copolymers. Hep G2 cells were cultured and found to contain active receptors. Confocal microscopy appears to be way to determine whether bulk amounts of fluorescently labeled compounds are restricted to endosomal and lysosomal vesicles or if they escape into the cytoplasm and subsequently enter the nucleus. KDJ was supported by an AFPE fellowship and NIH Grant GM08573.

References

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