Hyaluronan-grafted particle clusters loaded with Mitomycin C as selective nanovectors for primary head and neck cancers

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A B S T R A C T
CD44, a well-documented cell surface receptor, is involved in cell proliferation, migration, signaling, adhesion, differentiation and angiogenesis, which are important properties for normal and cancerous cell function. We recently developed particle clusters coated with hyaluronan (termed gagomers; GAG), and showed that they can deliver the insoluble drug paclitaxel directly into CD44-over-expressing tumors in a mouse tumor model. Here, we tested primary head and neck cancers (HNC) and normal cells taken from the same patient, and found that although CD44 expression in both types of cells was high, GAGs bind only to the cancerous cells in a selective manner. We next formulated the anti cancer agent mitomycin C (MMC) in the GAGs. MMC-based chemoradiation is a potential treatment for HNC, however, due to patient’s toxicity, MMC is not part of the standard treatment of HNC. MMC encapsulation efficiency was about 70% with a half-life drug efflux of 1.2 ± 0.3 days. The Ex vivo study of the targeted MMC-GAG showed significant increase in the therapeutic effect on HNC cells (compared to free MMC), while it had no effect on normal cells taken from the same patient. These results demonstrate the specificity of the nanovectors towards head and neck cancers, which might be applicable as future therapy to many CD44-expressing tumors.

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1. Introduction

The transformation of normal epithelial cells to malignant tumor is a complicated process that involves multiple alterations in the expression of genes that are responsible for cell proliferation, migration, adhesion, invasion and metastatic spread [1]. One of the most important and well-documented genes associated with tumor progression is CD44. Several studies described aberrant expression of CD44 in many types of tumors among them HNC [2].

CD44 is a cell surface glycoprotein receptor for hyaluronan (HA) but interacts with other ligands such as cytokines, matrix metalloproteinases (MMPs) and their growth factors [3]. CD44 is involved in cell proliferation, migration [4], signaling [5], adhesion, differentiation [6], and angiogenesis [7]. These properties are important for normal cell function but they are also essential factors in tumorigenesis. Other then the standard CD44 isoform (CD44s), several isoforms (CD44v1-10) are known as a result of alternative splicing or post translational modifications. Most of the modifications are attributed to the changes at the proximal position of the extracellular membrane site [8,9].

The affinity of HA to the CD44 depends on the receptor type (standard vs. variants), the expression of the different CD44 isoforms on different cells [10] and structural changes in CD44 glycosylation [11]. As a results, HA can bind to several cells carrying CD44 while other will have only limited HA-CD44 interaction or none at all. In Head and Neck Squamous cell carcinoma (HNSCC) cell lines, binding of HA to CD44 was found to promote phospholipase C-mediated Ca2+ signaling and cisplatin resistance, topoisomerase II phosphorylation, and epidermal growth factor- (EGFR)-mediated signaling [12,13]. Other than docking at the cell membrane, HA can block the tumor receptors and delay cancer progression [14].

Papillary thyroid carcinoma is currently the sixth most common malignancy diagnosed in women with increasing incidence that was tripled in the last 5 decades [15]. The majority (80%) of thyroid cancer is papillary carcinoma [16]. Recurrence is documented in 30% of patients and may occur several decades after diagnosis [17].
Medullary thyroid carcinoma derives from the neuroendocrine parafollicular C cells of the thyroid [18]. The disease may have an aggressive course with regional and distant metastasis that might result in decrease survival. The incidence of Head and Neck squamous cell carcinoma (SCC) is approximately 3% of all new cancer cases in the United States. An estimated 11,480 deaths from HNSCC will occur in 2010 [19]. Treating patients with head and neck cancer is complex. The tumor site, extent of disease and pathological findings will guide the appropriate surgical approach, radiation protocol and indication for chemotherapy. Most chemotherapy protocols are based on cisplatinum and 5FU with limited role of ethylazirinpyrroloindoleinedione (MMC).

MMC is an anti neoplastic antibiotic isolated from the bacterium Streptomyces species. Bioreduced mitomycin C generates oxygen radicals, alkylates DNA, and produces interstrand DNA cross-links, thereby inhibiting DNA synthesis. At high concentrations MMC inhibits RNA and protein synthesis especially in hypoxic cells (NCI). MMC is used as an active component in the treatment of localized bladder cancer and is documented as part of the chemotherapy regimen of breast, prostate, pancreatic and non-small cell lung cancers [20–22]. To date, due to patient’s toxicity, MMC is not part of the standard treatment of head and neck cancer.

In order to take advantage of the MMC effect on cancer cells while avoiding the significant toxicity associated with systemic administration of MMC, we conducted a study where MMC chemotherapy was entrapped in nanoparticulate carriers coated with HA termed gagomers (glycosaminoglycan cluster of particles; GAG) [23] that acted as selective nanovectors to CD44-expressing head and neck primary cells. The study included preparation of single cells from fresh normal and head and neck cancerous human tissue samples transferred directly from the operation room. The efficacy of this delivering platform was tested with MMC-loaded GAGs. CD44 expression, GAGs-cell binding properties and cell viability were investigated.

2. Materials and methods

2.1. Materials

2.1.1. Patient samples

The study cohort consisted of 5 patients with thyroid and mobile tongue cancer. There were 3 males and 2 females. The mean age at diagnosis was 55 years with a range from 33 to 74 years. The most common presenting symptom from the group of patients with thyroid cancer was a painless neck mass, the patient with tongue tumor had presented with an unresolved ulcer of the tongue. All thyroid tumor patients had gone a neck ultrasound (US) and medullary thyroid carcinoma in one patient. This study protocol was procedures reported previously [22,25].

2.1.2. Chemicals

1,2-Dilauroyl-sn-Glycerol-3-Phosphocholine (DLPC) and 1,2-Dilauroyl-sn-Glycerol-3-Glycerol (DLPG) was purchased from Avanti Polar Lipids Inc.(Alabaster, AL, USA). Hyaluronan (HA), Sodium Salt, with an average molecular weight of 7.5 × 10^5 was obtained from Genzyme (Cambridge, MA, USA). FITC—labeled HA was from Calbiochem* (Nottingham, UK). Mitomycin C, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC); Boric acid and Borax (sodium tetraborate (molecular weight cutoff of 12,000) was from Spectrum Medical Industries, Beit Haemek, Israel); 10 mM β-mercaptoethanol (Sigma—Aldrich Co). A suspension of gagomers (0.5 mg/ml) was prepared by rehydration of the dried GAGs powder with an aqeous (pure water) solution of MMC [22]. Rehydration was to the original pre—hylophilization gagomer concentration, to retain original buffering and salinity status. The second formulation, referred to as MMC-GAG2, was prepared as followed: MMC and lipids (DLPE:DLPG mole ratio of 9:1, respectively, DLPG added to assist particle homogeneity) were dissolved in ethane separately, then mixed together. Drug concentration was at 1:4 drug—lipid (w/w). The solution was evaporated to dryness under reduced pressure in a Buchi Rotary Evaporator Vacuum System (Flawil, Switzerland), and hydrated by the swelling solution that consisted of borate buffer. Prior to the addition of activated HA (at weight ratio of total lipid:HA 10:1 (w/w)), the suspension was heated at 45 °C for 2 h and sonicated for 10 min in a bath sonicator. HA was dissolved in acetate buffer (0.1x, pH 4.5) to a final concentration of 2 mg/ml and pre-activated by incubation with EDC at a 40 mg/ml for 2 h at 37 °C. The activated HA was added to the extruded lipid suspension (at weight ratio of total lipid:HA 10:1 (w/w)) and adjusted to pH 9.0 by NaOH followed by incubation over night, at 37 °C. When FITC labeled GAGs were used, the same procedure was performed with the inclusion of 10% HA-FITC (Calbiochem) in the formulation. Excess reactive agents and by-products were removed by dialysis against 20 mM Hepes Buffer Saline (HBS) pH8.2. GAGs were lyophilized and kept at −20 °C until further use. Prior to an experiment GAGs were resuspended in ddH2O to the same pre—hylophilization volume and sonicated for 10 min using a bath sonicator.

2.1.3. Preparation of MMC-loaded GAGs

Two formulations of MMC-loaded GAGs were prepared. The first formulation, referred to as MMC-GAG1, was prepared by rehydration of the dried GAGs powder with an aqueous (pure water) solution of MMC [22]. Rehydration was to the original pre—hylophilization gagomer concentration, to retain original buffering and salinity status. The second formulation, referred to as MMC-GAG2, was prepared as followed: MMC and lipids (DLPE:DLPG mole ratio of 9:1, respectively, DLPG added to assist particle homogeneity) were dissolved in ethane separately, then mixed together. Drug concentration was at 1:4 drug—lipid (w/w). The solution was evaporated to dryness under reduced pressure in a Buchi Rotary Evaporator Vacuum System (Flawil, Switzerland), and hydrated by the swelling solution that consisted of borate buffer. Prior to the addition of activated HA (at weight ratio of total lipid:HA 10:1 (w/w)), the suspension was heated at 45 °C for 2 h and sonicated for 10 min in a bath sonicator. HA was dissolved in acetate buffer (0.1x, pH 4.5) to a final concentration of 2 mg/ml and pre-activated by incubation with EDC at the concentration of 40 mg/ml for 2 h at 37 °C. The activated HA was added to the lipid—drug suspension and adjusted to pH 9.0 by NaOH followed by incubation over night, at 37 °C. Excess reactive agents and by-products were removed by extensive dialysis against HBS, pH8.2. GAGs were lyophilized and kept at −20 °C until further use. Prior to an experiment GAGs were resuspended in ddH2O to the same pre—hylophilization volume and sonicated for 10 min using a bath sonicator. The MMC concentrations in the swelling and in the rehydration solutions were in the range of 10 mg/ml—500 mg/ ml. Dialyzed and encapsulated MMC in phosphate buffer saline (PBS) was determined by incubation in the test media at 37 °C, pulling out samples over a time span of 0—35 h, and determining the concentration of GAGs-encapsulated drug.

2.2. Particle size distribution and zeta potential measurements

Particle size distribution and zeta potential measurements were determined by light scattering using Malvern nano Zetasizer (Worcestershire, UK). Each experiment result is an average of at least three independent measurements.

2.2.1. Preparation of single cells from normal and cancerous human tissue samples

The preparation of single cells from normal and cancerous human tissues was adapted from [24]. Short time after removal, the tissues were minced with scissors in
where $f(t)$ is the cumulative drug that diffuses from the sac into the reservoir at time $t$, normalized to the total drug in the system at time 0, $f_j$ is the fraction of the total drug in the system occupying the $j$th pool at time 0, and $k_j$ is the rate constant for drug diffusion from the $j$th pool.

2.2.6. Encapsulation efficiency defined as the ratio of entrapped drug to the total drug in the system, encapsulation efficiency can be determined by 2 independent methods. Method 1 is by centrifugation. Samples of complete gagomers preparation (i.e., containing both encapsulated and unencapsulated drug) are centrifuged as described above. The supernatant, containing the unencapsulated drug, is removed and the pellet, containing the encapsulated drug, is resuspended in drug-free buffer. Drug is assayed in the supernatant and in the pellet, as well as in the complete preparation. The results of these assays serve to calculate encapsulation efficiency as well as to verify conservation of matter. Method 2 uses data analysis of efflux kinetics. As discussed above, magnitudes of the parameter $f_j$ are obtained through data analysis. When the efflux experiment is carried out on samples from the complete gagomer preparation, the sum of $f_j(s)$ for the pool(s) of encapsulated drug is also the efficiency of encapsulation as previously reported by us for liposomal systems [25,26].

2.2.7. CD44 expression levels in tissue samples
Cancerous and Normal cells from each patient were washed with PBS and incubated with Alexa Fluor 488 anti CD44 Rat anti-human (clone 1M7) from Biolegend (San Diego, CA, USA) on ice for 30 min followed by washing with PBS and subjected to flow cytometry using a FACSscan (Becton Dickinson, San Jose, CA, USA). Analysis was done using a flow cytometry analysis software, flowjo software (Ashland, OR, USA).

2.2.8. GAG binding assay
Cancerous and Normal cells from each patient were washed with PBS. Cells were incubated with 60 μg FITC (labeled)-GAGs that have undergone sonication for 10 min. Incubation with the cells was on ice for 30 min followed by washing with PBS and subjected to flow cytometry using a FACSscan (Becton Dickinson, San Jose, CA, USA). Analysis was done using the flowjo software (Ashland, OR, USA).

2.2.9. Cell viability assay
Cancerous cells were seeded onto 96 multi-well plates (4 × 10^3 cells/well). 24 h later, the media was replaced by treatment media that contained 1 μM MMC, as free or encapsulated drug, or by treatment media that contained an equal amount of CA, USA. Analysis was done using the flow cytometry analysis software, flowjo software (Ashland, OR, USA).

2.2.10. Statistical analysis
Data were analyzed using Student’s t-test. Differences between treatment groups were evaluated by one-way ANOVA with significance determined by Bonferroni adjusted t-tests.

3. Results
3.1. Structural and physicochemical characterization of MMC-entrapped in GAG
Drug-free GAG exhibit size ranges of 100 nm similar to previously reported structures [23]. GAG-entrapping MMC exhibit size ranges of 350 ± 35 nm in diameter which was in the same range of previously reported gagomers as paclitaxel carriers [23].

Zeta potentials of −60 mV ± 7 were determined for both MMC-GAG1 and MMC-GAG2, respectively. These magnitudes and directions fit with the lipid composition of the two formulated particles with the surface modification by the negatively charged hyaluronan coating.

The kinetics of MMC efflux from the gagomers is shown in Fig. 1. Two different formulations were studied. In formulation 1 the MMC was entrapped by resuspending the lyophilized gagomers powder with MMC in saline (MMC-GAG1). In formulation 2 MMC was entrapped inside the gagomers in the course of the gagomers’ formation (MMC-GAG2). The formulation was processed according to equation (1). The efficiency of MMC entrapment was 68 ± 5%, and 97 ± 9% for MMC-GAG1 and MMC-GAG2, respectively as calculated from equation (1). MMC-GAG1 formulation demonstrated fast efflux of the encapsulated MMC over time when compared with a slower dissipation of MMC from the second formulation (Fig. 1). The half-life of MMC efflux from the GAGs were 1.2 ± 0.3 days and 32.5 ± 1.8 days for MMC-GAG1 and MMC-GAG2, respectively. Since, the release rate of formulation 2 was too slow, we chose to continue our studies with MMC-GAG1 formulation.

3.2. CD44 expression on patients cell samples
Representative histograms of the expression of CD44 on both cancerous and adjacent normal cell samples are presented in Fig. 2. Cells (normal and cancerous tissue samples) were stained with an antibody against pan-CD44 or its isotype control and analyzed using flow cytometry. High CD44 expression was documented in all patients with no significant difference between normal and malignant cells.

3.3. GAGs binding to CD44-expressing head and neck cancer and normal cells
Once we demonstrated the presence of CD44 on the cell surface of the patient samples, we examined the binding of GAGs to both normal and cancerous cells expressing CD44. Drug-free GAGs -labeled with FITC were incubated for both type of samples for 30 min on ice. Representative histograms are shown in Fig. 3. The results demonstrate a significant higher expression of the GAG on the surface of cancer cells as opposed to minimal or low expression on the corresponding normal tissue (Fig. 3).

3.4. No therapeutic benefit of GAGs-MMC on normal tissue samples
Upon demonstrating binding of GAGs to cancerous cells with minimal binding to normal cells (Fig. 3) GAGs were loaded with MMC and cell viability was assayed in normal cells. As shown in Fig. 4, none of the tested formulations decrease cell viability (~95% mean cell survival ± 7) in all tested samples.
Fig. 2. Representative histograms of CD44 expression on normal and cancerous head and neck primary cells. Panels A–E correspond to patients 1–5, respectively. Gray and black lines indicate isotype control for normal and cancerous cells, respectively. Red and blue lines indicate CD44 expression for normal and cancerous cells, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
3.5. **GAG-MMC decrease cell viability of primary head and neck cancer cells**

GAGs can probe for cancerous cells with high affinity (Fig. 3), therefore, applying MMC-GAG on cancerous cells is expected to have an improved therapeutic outcome compared to free MMC. Indeed, when applying MMC in its free form only a minor effect on cancer cell viability (85–90% mean cell survival ± 6) was observed. Drug-free GAG had no effect on cell proliferation, however, MMC-loaded GAGs decreased cell viability significantly (10–20% mean cell survival ± 4) (Fig. 5).

![Image of graphs showing binding of FITC-labeled GAGs to normal and cancerous cells, labeled A to E.](image-url)
4. Discussion

The role of CD44 as a target for HA coated nanoparticles carrying therapeutic payloads is well documented in the literature [22,25,28]. Recently, we showed that a cluster-particle system – coated with HA (GAG) can carry paclitaxel and deliver it directly into mouse CD44-expressing tumors in a safe manner [23]. The application of this platform on fresh head and neck human cancer samples transferred directly from the operating room to the laboratory is presented here. The current report acts as a proof of principle evaluating the role of selective nanovectors containing chemotherapeutic payload as a potential future therapy for head and neck cancer.

To achieve a successful systemic administration of carrier-loaded chemotherapy, the nanoparticle needs to navigate through the circulation, extravasate the blood vessels, recognize and dock specifically onto the tumor cell. Next, the particle should contain enough chemotherapy to act as sustained release drug depot on the cells’ membrane or to penetrate the cell and deliver its payload [29]. The current work described here focuses on the recognition and binding properties of the GAG system in human primary tissue samples. Our results clearly show that hyaluronan can target the drug carrier selectively to the human head and neck cancer cells and deliver its payload in a functional manner (Figs. 3 and 5).

The key benefit of this platform is associated with the selectivity towards cancer cells with limited damage to adjacent normal tissue. Ilia Rivkin [23] described the kinetics of the GAG-CD44 binding properties on mouse cell line and the pharmacokinetics in a tumor-bearing mice model and concluded that GAGs are beginning to be cleared from the target cell receptor’s binding region within 6 h post incubation and complete their clearance within 12 h post incubation. Therefore, the high affinity of GAG to the

Fig. 4. Viability of normal head and neck primary cells after treatment with 1 μM free or MMC-entrapped in GAGs. Data are expressed as the mean ± SD of at least three independent experiments, six repeats per data point in each experiment. N/S – not statistically significant.
CD44-expressing tumors enable efficient long standing delivery of therapeutic payloads to the tumor cells. On the other hand, drug-free GAGs had no effect on cancerous and normal control cells (Fig. 4). This can be attributed to the nanoparticle composition, which include HA and neutral in charge lipids, all of which do not induce CD44 conformational changes or provoke immune activation, as oppose to charged lipids [30].

Although CD44 is present on normal and cancer cells, CD44 conformation varies between different malignancies [6,9]. Here we show that CD44 was highly expressed on both cancer and normal cells (Fig. 2). However, GAGs (composed of HA) showed higher affinity to the cancer cells (Fig. 3). This can be explained by the presence of different splice variants of CD44 expressed on the surface of cancer as opposed to normal cells [9]. For example, CD44 variant but not standard CD44 expressed on lymphoma cells strongly bound HA [31]. Moreover, variant CD44 cells showed in vivo accelerated local tumor formation and aggressive metastatic behavior [31]. Gilles Salles [32] demonstrated the high expression of CD44 variant (V6) on neoplastic cells such as non Hodgkin’s lymphoma. CD44v3 is associated with tumor invasion and decrease apoptosis in colon cancer whereas CD44 v6 expression correlated with tumor metastasis and a decrease in disease-free survival [33,34]. CD44v3 is associated with the tendency of breast cancer to metastasis [35]. Others have found CD44 markers on pancreatic cancer [36], prostate cancer [37]and ovarian cancer [38]. Studies conducted on head and neck cancer samples have demonstrated the presence of v3, v6 and v10 CD44 isoforms that can explain our results [2]. Compared to the primary tumor, a greater proportion of metastatic lymph nodes demonstrated strong expression of CD44 variants. Expression of CD44 variant isoforms were associated with advanced T stage (v3 and v6), regional (v3) and distant (v10) metastasis, perineural invasion (v6), and radiation failure (v10).

Fig. 5. Viability of cancerous head and neck primary cells after treatment with 1 μM free or encapsulated MMC. Data are expressed as the mean ± SD of at least three independent experiments, six repeats per data point in each experiment. * denoted p < 0.01; ** denoted p < 0.001.
CD44 v6 and CD44 v10 were also significantly associated with shorter disease-free survival [2].

Another explanation of the high affinity of cancer cells to the GAGs can be retrieved from the studies of Shinji Ogino [8] that described Two-State Conformations in the Hyaluronan-Binding Domain. CD44 hyaluronan-binding domain (HABD) alters its conformation upon HA binding, from the ordered (O) to the partially disordered (PD) conformation. They also demonstrated that the HABD undergoes equilibrium between the O and PD conformations, in either the presence or absence of HA. An HABD that the HABD undergoes equilibrium between the O and PD partially disordered (PD) conformation. They also demonstrated that the HABD undergoes equilibrium between the O and PD conformations, in either the presence or absence of HA. An HABD that

Mitomycin C was found to be an effective treatment in head and neck cancer when given with radiation and other chemotherapy [39]. Combined with radiation, MMC was thought to inhibit hypoxic tumor cells, which are resistant to radiation [40]. The combination of DDP with 5-Fluorouracil and MMC together with radiation improved local tumor control, but was associated with severe mucositis in the majority of patients and hematologic and gastrointestinal toxicity in nearly half of the patients [41]. To date, MMC is not part of the standard treatment of head and neck cancer due to patient’s toxicity. The usage of a targeted MMC delivery system might diminish patient toxicity.

Loading MMC into a liposomal nanoparticle was previously developed and described by our group. Significant progress was made formulating the liposomal-MMC in order to achieve robust in vivo delivery [26,42]. We reported that when MMC was delivered via HA coated liposomes, drug accumulation in the tumor was 30-fold higher than when the drug was administered in its free form [22]. Next, an effort was made to obtain an extended circulation time. This was achieved by coating the liposomes with hyaluronan [43]. Our study confirms earlier results [22]demonstrating in vitro increased MMC potency of 100-folds when delivered via HA coated liposomes to cells expressing HA receptors as opposed to cells with low HA receptors expression.

The mechanism, by which MMC-GAG cause tumor cell death can be speculated. Harboring of the GAGs along the cell membrane and release of the drug content causing increase drug influx that accumulates in cell death. Secondly, particle internalization, or conformational changes in the GAGs that opens the structure and facilitate entry of the drug into the cell cytoplasm is another potential explanation [23]. Similar to our study, Iliia Rivkin [23], showed that incubation with GAG-encapsulated doxorubicin resulted in substantial intracellular drug accumulation.

Although the number of subjects in these study contained only 10 fresh samples (normal and cancerous tissue), the results were consistent in all subjects and our study should be regarded as a proof of concept and a platform for further studies.

Three of our patients were diagnosed with papillary carcinoma of the thyroid, which harbor an excellent prognosis, the drug delivery platform gained the same results in patients with an aggressive and life threatening disease as medullary carcinoma of the thyroid and SCC of the oral tongue. Chemotherapy and other biological agents (such as epidermal growth factor receptor (EGFR) inhibitors) may play an important role in the treatment of advanced disseminated papillary cancer as well as medullary carcinoma and SCC. Further progress in targeted drug delivery system may decrease tumor recurrence and improve survival.

5. Conclusions

Our study demonstrated selective binding of HA coated lipid particle cluster (GAG) to cancer cells with very low binding to adjacent normal cells. In addition, when MMC was entrapped in the GAGs, high therapeutic benefit was observed for head and neck primary tumors. The degree of cell viability post GAG-MMC treatment might predict the potential outcome of the patient exposed to the MMC delivery system. We argue that the present study might be regarded as a potential treatment for head and neck cancer or as an adjuvant treatment for residual microscopic disease following gross surgical resection.

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