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Carbohydrate Dependent Targeting of Cancer Cells by Bleomycin-Microbubble Conjugates

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Graphical Abstract

Biotinylated bleomycin A₅ was attached to streptavidin-derivatized microbubbles and a solution containing the conjugate was passed over a monolayer of cultured MCF-7 cells. The bleomycin-derivatized microbubbles adhered to the MCF-7 cells, and the association could be monitored by the use of a microscope. Three other cancer cell lines gave similar results. The bleomycin-microbubble conjugate did not bind to a normal breast cell line (MCF-10A), nor to the matched non-cancer cell lines corresponding to the other cancer cell lines targeted by bleomycin. No binding to any tested cell line was observed when the microbubbles lacked conjugated bleomycin A₅, or when the microbubble contained a bleomycin A₅ analogue lacking the carbohydrate moiety.

The bleomycins (BLM A₅, **1**) are clinically used antitumor agents efficacious in the treatment of squamous cell carcinomas and malignant lymphomas.¹ Their anti-tumor activity is believed to result from their ability to mediate the selective oxidative cleavage of DNA,² and possibly also RNA.³

One property of bleomycin that has been recognized for decades is its ability to target tumors; this has been documented in numerous tumor imaging studies that employed BLMs bound to radionuclides.^{4,5} The importance of tumor targeting to the therapeutic efficacy of BLM is underscored by the therapeutic dose of BLM (~5 μ moles), which is quite low

relative to the doses of many clinically used agents. Understanding the molecular nature of tumor targeting by BLM would facilitate the synthesis of analogues with improved properties, and might also enable the selective delivery of other probes and drugs to tumor cells.

Microbubbles, consisting of a shell enclosing a gas core, are used in ultrasonography as contrast agents and intravascular blood flow tracers. The shell is usually composed of albumin, carbohydrates or lipids. The gas core is usually air, nitrogen or a perfluorocarbon.⁶ While individual microbubbles may exist in varying sizes, they are usually smaller than red blood cells and their mean diameter is typically within the range 1–4 μm . This small size permits free flow of the microbubbles through the (micro)circulation. In recent years, microbubbles have been modified with ligands that bind specific receptors expressed by cell types of interest, including inflamed and cancer cells.⁷ The majority of ligands to date have been monoclonal antibodies. Presently we describe the conjugation of BLM A₅ to microbubbles, demonstrate that the conjugate adheres selectively to cancer cells, and report that the carbohydrate moiety of BLM A₅ is required for tumor cell targeting.

The C-terminus of BLM A₅ (**1**) was acylated with biotin, as shown in Figure 1, to afford BLM A₅-biotin conjugate **2a**.⁸ The preparation of BLM A₅-conjugated microbubbles was then accomplished by admixture of BLM-biotin **2a** to commercially available microbubbles derivatized with streptavidin.⁹ A schematic representation of the resulting targeted microbubble is shown in Figure 2.

MCF-7 human breast carcinoma cells were grown on sterile glass cover slips (40 mm) and incubated at 37 °C until they reached 40–60% confluency in a 5% fetal bovine serum/RPMI 1640 medium. They were then assembled into a parallel plate flow chamber with a constant temperature maintained at 37 °C. The suspension containing the BLM-microbubble conjugate was introduced into the flow chamber at a controlled rate of 0.01 mL/min, and possible attachment of the microbubbles to the cultured MCF-7 cells was imaged using an inverted microscope fitted with a camera. As shown in Figure 3, the BLM microbubble conjugate adhered to the cultured MCF-7 cells, but microbubbles lacking attached BLM **2a** failed to adhere to the cultured MCF-7 cells.

To judge the ability of the BLM A₅-microbubble conjugate to bind selectively to tumor cells, the experiment was repeated using the “normal” breast cell line MCF-10A. As shown in Figure 4, there was no adhesion of the BLM A₅ microbubble-conjugate to the cultured MCF-10A cells. Thus the BLM A₅-microbubble conjugate adhered selectively to the cancer cell line.¹⁰

The realization of a system for monitoring the selective interaction of BLM A₅ with tumor cells provides a vehicle for evaluating those structural elements of BLM that contribute to tumor cell targeting. Accordingly, biotinylated deglyco BLM A₅ (**2b**) was prepared to permit a direct assessment of the role of the carbohydrate moiety of BLM in tumor cell targeting. BLM **2b** was conjugated to the same microbubbles and employed in experiments designed to test adhesion to cultured MCF-7 and MCF-10A cells. As shown in Figure 4 for the

cultured MCF-7 cells, no adhesion of the deglyco BLM A₅ microbubble conjugate to either cell line was observed.

The foregoing experiments enable direct, visual observation of the binding of a BLM conjugate to cultured tumor cells and demonstrate that BLM adheres selectively to tumor, as compared with the same “normal” cells. Also established is the requirement for the carbohydrate moiety of BLM to support tumor cell targeting. Not yet resolved by these experiments is the related question of the sufficiency of the BLM carbohydrate moiety to support tumor cell targeting, a finding that could enable novel strategies for selective drug delivery and antitumor therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- (8). Cu(II)•BLM A₅ was treated with the *N*-hydroxysuccinimide ester of biotin, as outlined in Figure 1, in analogy with attachment of BLM A₅ to a solid support (Abraham, A. T.; Zhou, X.; Hecht, S. M. *J. Am. Chem. Soc.* **2001**, 123, 5167). Following the removal of Cu²⁺ by treatment with EDTA, the BLM A₅-biotin conjugate (**2a**) was purified by C₁₈ reversed phase HPLC.

- (9). Targestar^B Ultrasound Contrast Agent, containing streptavidin attached through a PEG spacer, was used for attachment of BLM **2a** and deglyco BLM **2b**. These microbubbles have a consistent mean diameter of $\sim 2.5 \mu\text{m}$ with $\sim 1.5 \times 10^6$ streptavidin molecules/microbubbles. A 1-mL aqueous suspension of 1.5×10^8 streptavidin-derivatized microbubbles was treated with 50 μL of aq. 500 μM BLM **2a** or **2b**. The combined solution was agitated gently for 20 min, then free BLM was removed by repeated centrifugation ($400 \times g$, 3 min, 10 $^{\circ}\text{C}$) and washing with buffer.
- (10). Analogous experiments were carried out using other sets of cultured cancer and normal cells. Selective adhesion of the BLM A₅ microbubble conjugate was observed using SW480 colon carcinoma cells (but not CRL-1541 colon cells), DU145 prostate carcinoma cells (but not CRL-2221 prostate cells) and A549 lung carcinoma cells (but not CCL-75 lung cells).

Figure 1.
Synthesis of biotinylated derivatives of bleomycin A₅ and deglycobleomycin A₅.

Figure 2.
Constitution of the bleomycin A₅-microbubble conjugate.

Figure 3. Monolayers of cultured MCF-7 breast cancer cells treated with the BLM A₅-microbubble conjugate (top) or with underivatized microbubbles (bottom).

Figure 4. Monolayers of cultured MCF-10A breast cells treated with the BLM A₅ microbubble conjugate (top) and of cultured MCF-7 cells treated with the deglyco BLM A₅ microbubble conjugate (bottom).