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A novel pretargeting method for measuring antibody internalization in tumor cells

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Abstract

A novel pretargeting method has been developed to quantitate antibody cellular internalization. In this study, the antibody was conjugated with a phosphorodiamidate morpholino oligomer (MORF) specific for the complementary MORF (cMORF) as effector. Half the tumor cells were incubated with the MORF-antibody (pretargeting group) and the other half with the same MORF-antibody at the same concentration but radiolabeled (direct targeting group). After incubation, the same dosage of radiolabeled cMORF is added to the wells of pretargeting group. The radioactivity of the direct targeting cells represents the sum of both internalized and cell surface bound antibodies while the radioactivity of the pretargeting cells is due only to surface bound antibodies since the radiolabeled cMORF does not penetrate the cell surface. Therefore the difference in radioactivity accumulation between pretargeting and direct targeting provides the internalized fraction. In this example, the internalization of a MORF conjugated anti-PMSA antibody 3C6 in LNCaP cells was examined and the average cell surface residence time was determined as 2 h. This method of measuring antibody internalization is directly applicable to pretargeting applications but can be a universal alternative to the conventional acid-wash method with the advantage of leaving the cell membrane undamaged.

Keywords

Internalization; Pretargeting; Tumor; Antibody; Immunodetection

INTRODUCTION

Tumor pretargeting requires that the pretargeting antibody in the first administration remains on the cell surface and accessible to the radiolabeled effector in the second administration^{1, 2}. Accordingly, the internalization properties of any antibody under consideration for tumor pretargeting should be established. Although several methods of measuring internalization are in practice, only the acid wash method introduced in early 1980s has the advantage of straightforward discrimination and quantification of the cell surface bound and the internalized antibodies^{3, 4}. After incubation with a radiolabeled antibody, an acid wash removes the surface bound antibody along with its radioactivity. The remaining cell-bound radioactivity is assumed to be due to internalized antibody.

We report herein on a novel pretargeting approach to evaluate internalization as an alternative to the acid wash method. In this approach, an antibody is conjugated with a group specific for a radiolabeled effector. After incubation with cells, the internalized antibodies are sequestered

and not “visible” to the radiolabeled effector, thus providing a simple method to discriminate between internalized and surface bound antibodies. This method of quantitating antibody internalization has the advantage of leaving the cell membrane undamaged and, in particular, has the potential of evaluating internalization in the in vivo situation.

MATERIALS AND METHODS

Principles

Figure 1 schematically illustrates the principles involved. As shown in the top panel, the cells are divided equally and incubated under identical conditions for the same period of time with either an oligomer conjugated antibody, in this case the 3C6 antibody conjugated with a phosphorodiamidate morpholino oligomer (MORF-3C6) (pretargeting), or the identical conjugated antibody at the identical concentration but radiolabeled, in this case a complex of MORF-3C6 and the radiolabeled complementary oligomer (^{99m}Tc -cMORF) (direct targeting). During the incubation period, some antibody may internalize as shown in the middle row of the figure. At the end of the incubation, the same amount of radiolabeled cMORF is added to the pretargeting group as that added earlier to the direct targeting group. Media are removed and all cells are then immediately washed for counting as shown in the bottom panel. Radioactivity of the direct targeted cells after removal of the medium represents the total cell accumulation of antibody and corresponds to the sum of both internalized and surface bound antibodies. Radioactivity of the pretargeting cells represents only the surface bound antibodies since the labeled effector does not penetrate the cell membrane⁵. The accumulation difference between the pretargeting and direct targeting cells accounts for the antibodies that become internalized over the incubation period.

Cell, antibody, and radiolabeled effector

The androgen dependent, PMSA positive cell line LNCaP (American Type Culture Collection, Manassas, VA) and the PMSA specific antibody 3C6 (Northwest Biotherapeutics, Bothell, WA) were used in this study. The cell culture medium was RPMI containing 1% glucose, 1% sodium pyruvate, and 15 % FBS. The oligomer used was the synthetic DNA analogue phosphorodiamidate morpholino oligomer (MORF) (Gene-Tools, Philomath, OR) with a base sequence TCTTCTACTTCACAACTA. Conjugation of the NH_2 -MORF to the 3C6 was achieved with the commercial Hydralink kit (Solulink, San Diego, CA) following the manufacturer's recommended procedure⁶. The 3C6 antibody was conjugated with succinimidyl 4-hydrozino nicotinate acetone hydrazone while MORF was conjugated with succinimidyl 4-formylbenzoate. After purification, combination of hydrazine modified 3C6 and benzaldehyde modified MORF resulted in a hydrazone bond linking both. The average MORF groups per molecule of MORF-3C6 used in this study was 1.15 as measured by HPLC as previously described⁷.

The cMORF effector was complementary to the MORF on the antibody and was conjugated with NHS-MAG₃ and radiolabeled with ^{99m}Tc as described previously⁸. The labeling efficiency was always greater than 95%. Formation of the MORF-3C6/ ^{99m}Tc -cMORF complex was achieved simply by mixing MORF-3C6 with labeled cMORF at a molar ratio of MORF on 3C6 to ^{99m}Tc -cMORF of typically about 3–5.

Flow cytometry analysis

The immunoreactivity of 3C6 after conjugation with MORF was confirmed by FACS analysis as recommended by the antibody manufacturer⁹, with native 3C6 as positive control and the incubation medium as negative control. Specifically, nine vials each containing 1.5 million LNCaP cells were divided into three groups. After pelleting, cells in the study group were suspended in 150 μL cold PBS containing 0.5% BSA and 3.0 μg MORF-3C6. In the positive

control group, native 3C6 was used in place of the MORF-3C6 while in the negative group and the MORF-3C6 was eliminated. After 30 min incubation on ice, the cells were washed twice with 1 mL 0.1% BSA in PBS and then stained with 1.5 µg FITC labeled secondary AffiniPure Rabbit Anti-Mouse antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) in 150 µL 0.5% BSA in PBS. After another 30 min on ice, the cells were washed again and suspended in 750 µL 0.1% BSA in PBS for flow cytometry analysis on a FACS Calibur cytometer (Becton-Dickinson, San Jose, CA). Data analysis was achieved with Flowjo software (Tree Star, Inc. Ashland, OR).

Internalization measurements

About 0.40 million LNCaP cells were seeded in each well of a 12-well plates coated with polylysine P1274 using the manufacturer's recommended procedure (Sigma, Saint Louis, MI). Three days later when the confluency was greater than 75%, the internalization study was performed at 37 °C. At that time, the cell culture medium was replaced with 1 mL internalization buffer (RPMI medium containing 1% glucose, 1% sodium pyruvate, and 1% FBS). To cells in one half of the wells (direct targeting) was added 150 µL of internalization buffer containing 0.35 µg MORF-3C6 and 5.3 ng cMORF as the MORF-3C6/^{99m}Tc-cMORF complex, while to the cells in the other half (pretargeting) was added 150 µL of internalization buffer containing only the 0.35 µg of MORF-3C6.

The initiation of incubation was staggered so that all cells regardless of incubation time were washed and counted together. Five minutes prior to washing, a 10 µL ^{99m}Tc-cMORF aliquot containing 5.3 ng ^{99m}Tc-cMORF was added to each well of the pretargeting cells, followed by immediate agitation. The cell medium was then removed, the cell washed and lysed with 1% SDS in 0.2 N NaOH, and radioactivity in the medium and cell was measured in an auto gamma counter.

Comparison with internalization measurements by acid wash

The comparison was accomplished under three different cell incubation conditions expected to provide increasing degree of internalization: incubation at 4 °C for 30 min, 37 °C for 30 min, and 37 °C for 3 h. The 12 wells of a polylysine-coated plate with LNCaP cell at greater than 75% confluency were divided into three groups: direct targeting, pretargeting, and acid wash. For each of the three incubation conditions, the first two groups were treated as described above. Cells in the acid wash group were incubated with the identical dosage of MORF-3C6/^{99m}Tc-cMORF. At the designated time of incubation, the cell media in the acid wash group were removed, the cells were washed, and 1 mL of acid buffer (0.2 M acetic acid in 0.5 M NaCl, pH 2.5) was added to each well as described^{3, 4}. Five minutes later, the acidic media was removed and the cells in each well were washed and prepared for radioactivity measurements together with the samples from the first two groups.

Nude mice pretargeting study

A pretargeting study in LNCaP xenografted BALB/c nude mice was performed on the approval by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School. Pretargeting conditions were selected based on earlier pretargeting experiences¹⁰. Fifteen mice were inoculated subcutaneously in the back with 5×10^5 LNCaP cells in 0.1 mL of a (v/v=1:1) mixture of PB and Matrigel (BD Biosciences, Bedford, MA). After about one month, tumor started to appear as violet spots under the skin. At about 2 months, ten mice with the largest tumors were selected and divided into two groups. One group of five animals each received 4.0 µg MORF-3C6 (2 MORF groups per molecule) intravenously and three days later all mice were given 0.50 µg (about 80 µCi) ^{99m}Tc-cMORF. The other group of five animals was used as controls, receiving only 0.50 µg ^{99m}Tc-cMORF at the same specific activity and the same time. All mice were sacrificed 3 h postinjection of radioactivity by heart

puncture under anesthesia. Organs and tumor were harvested and counted in the gamma counter for biodistribution.

RESULTS

Flow cytometry analysis

Figure 2 presents histograms from the cytometry analysis of LNCaP cells treated first at 4 °C with either MORF-3C6, native 3C6, or medium only and then with a FITC labeled secondary antibody specific for murine antibodies. As shown, the intensity of the fluorescent signal (F_{L1-H}) from the cells treated with MORF-3C6 appears to be identical to that from cells treated with native 3C6. These results indicate that when conjugated at about one MORF group per antibody, the MORF-3C6 preserves its immunoreactivity for PMSA on LNCaP cells as well as its immunoreactivity to the secondary antibody. If either were compromised, the intensity of the fluorescent signal would have been reduced.

Internalization measurements

Cell accumulation of radioactivity are presented in Figure 3A in percent of the total radioactivity added and the results show that incubation at 37 °C with the MORF-3C6/ ^{99m}Tc -cMORF complex in the direct targeted cells resulted in a steadily increasing cell accumulation over time, while accumulation in the pretargeted cells after 30 min remained constant at about 1.2 %. The difference in the two curves represents the internalized antibody fraction. The percent internalized is presented in Figure 3B, the slope of which provides an estimate of the internalization rate at about 0.6 %/h. The surface bound percentage of about 1.2 % in Figure 3A may be used along with the internalization rate of 0.6 %/h in Figure 3B to further estimate the average surface residence time. $1.2\%/0.6\%/h = 2\text{ h}$. Since the 2 h residence time is short compared to the normal pretargeting interval of 48–72 h, it may be predicted that pretargeting in LNCaP tumored animal will result in a low tumor accumulation of the radiolabeled effector if the rate of internalization is similar in cell culture and in tumor xenograft.

Comparison with internalization measurements by acid wash

Figure 4 presents histograms showing the percent accumulation of radioactivity for the direct targeting, pretargeting, and acid wash groups in cells incubated at 4 °C for 30 min, 37 °C for 30 min, or 37 °C for 3 h. In the case of acid wash, the open bar shows the percentage of antibody that is sensitive to acid and therefore presumably not internalized, while the closed bar above shows the percentage of antibody that is resistant to the acid wash and presumably internalized. The combined height of the open and closed bars for the acid wash cells agrees with the height of the bars for the direct targeting cells under all three incubation conditions, as expected. Furthermore, the difference in the height of the open bars between the direct targeting and pretargeting groups increases in proportion to increasing internalization as shown by the height of the closed bars in the acid wash group in the order: 4 °C for 30 min, 37 °C for 30 min, and 37 °C for 3 h, also as expected. However, the internalized percent measured by acid wash is lower than that determined by pretargeting. This may reflect the loss of internalized antibody itself and/or its cMORF label during acid wash, possibly due to membrane damage. Evidence of such damage has been reported and has led to suggestions that buffers of higher pH (about 5.0) be used for the dissociation of surface bound radioactivity ^{11,12}.

Nude mice pretargeting study

The biodistribution of ^{99m}Tc -cMORF in LNCaP tumored mice at 3 h is shown in Figure 5. The mice have been pretargeted three days earlier with MORF-3C6. The tumor accumulation (about 1% ID/g) is not greatly in excess of the accumulations in normal organs, suggesting a poor MORF-3C6 expression on the tumor cell surface probably due to antibody internalization

over the three days. In addition, compared to control, tumor levels in the study animals are about three times higher, but levels in normal tissues such as liver, heart, lung, spleen, and muscle in the study animals are also higher. The blood shows the largest difference between pretargeted and control animals because of the formation of MORF-3C6/^{99m}Tc-cMORF in the blood of the pretargeted animals. Therefore the higher tumor accumulation could also be attributed mainly to the presence of MORF-3C6/^{99m}Tc-cMORF in capillaries and extra-vascular space instead of on the cell membrane. Regardless, the poor tumor accumulation of MORF-3C6 in pretargeted LNCaP tumored mice was predicted by the pretargeting internalization measurements.

DISCUSSION

The choice of antibody and cell lines for this investigation was largely dictated by our interest in pretargeting prostate cancer by the MORF conjugated antiPMSA antibody 3C6, using radiolabeled cMORF as effector and the PMSA positive LNCaP cells as tumor model. In common with conventional tumor targeting, pretargeting requires high affinity antitumor antibodies but in contrast, pretargeting also requires antibodies with minimal tendency to internalize. For this reason, antitumor antibodies considered for pretargeting should be investigated for their internalization properties.

We have described in this report a novel cell pretargeting approach to measure antibody internalization. The pretargeting approach does require more cell manipulation since two incubations are required. Nevertheless, since the internalization properties of an antibody may vary with chemical modifications, in the measurement of internalization using a construct that is identical to that intended for in vivo use must be regarded as an advantage. Furthermore, in contrast to internalization measurements by acid wash, the cell surface is not damaged when internalization is measured by pretargeting, leaving the cells in a condition suitable for additional cell measurements. As perhaps its greatest advantage, the measurement of internalization by pretargeting may be applied in vivo in contrast to all other methods of internalization measurements.

CONCLUSION

A quantitative method for measuring internalization of antibodies has been described. Besides being relatively simple, this method of measuring antibody internalization is not only directly applicable to pretargeting applications but also can be a universal alternative to the conventional acid-wash method.

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Figure 1. Schematics illustrating the principle of the pretargeting method for measuring antibody internalization in tumor cells.

Figure 2.
Results of flow cytometry analysis of LNCaP cells treated with MORF-3C6, native 3C6 (positive control), or nothing (negative control) followed by staining with a secondary antibody-FITC

Figure 3. Cell accumulation of radioactivity presented separately for direct targeting and pretargeting (panel A) and their difference (panel B) representing the percentage of antibody internalized over time. (Error bars represent one s.d., N=3)

Figure 4.

The percent cell accumulations in the direct targeting, pretargeting and acid wash groups for cells incubated at 37 °C for 3 h, 37 °C for 30 min, or 4 °C for 30 min. In direct targeting, the open bar shows the percentage of antibody both on the cell surface and internalized. In pretargeting, the open bar shows the percentage of antibody on the cell surface. In acid wash, the open bar shows the percentage of acid removable antibody and the closed bar shows the percentage of acid resistant and presumably internalized antibody. (Error bars represent one s.d., N=4) Note the change in scale.

Figure 5. Histograms showing the biodistribution in percent injected dosage per gram at 3 h of labeled cMORF in LNCaP tumored mice pretargeted by MORF-3C6. Control animals did not receive the MORF-3C6. (Error bars represent one s.d., N = 5)