Biotinylation Turnover Assay of the A33 Antigen

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Abstract

Colon cancer is a leading cause of cancer death in the United States. Current therapies, such as chemotherapy, will target and destroy both tumor cells and healthy cells. Radioimmunotherapeutic treatment using the A33 antigen was found to have potential in specifically targeting tumor cells. The A33 antigen would be used in a two-step radioimmunotherapeutic treatment involving administration of the A33 antibody, which would bind to the A33 antigen expressed on the surface of tumor cells, and administration of a toxin that will bind only to the A33 antibody and thus selectively destroy tumor cells. In order to determine appropriateness of this antigen as a two step radioimmunotherapy target, its turnover time in cultured colon epithelial cells was determined. This was done by pulsed biotinylation of cell surface proteins followed by extraction of membrane proteins at different time points. Western blots were then conducted and the bands of A33 were isolated. The band density of the antigen at each time point was plotted, yielding a graph of exponential decay from which the turnover time of the antigen was found to be significantly longer than the turnover time of bulk plasma membrane and other membrane proteins. This indicates that the antigen is quite stable and thus has potential in the two step treatment against colon cancer.
1 Introduction

Colon cancer is the third most commonly diagnosed type of cancer and the second most common cause of cancer death in the United States [3]. Before the tumor stage of colon cancer, an adenomatous polyp forms in the colon which can be surgically removed, preemptively preventing the spread of colorectal cancer [ibid]. However, once tumor cells begin to spread throughout the colon, a different approach, such as radioimmunotherapy, is required.

A potentially useful target for the immunotherapeutic treatment of colon cancer is the A33 antigen, a glycosylated membrane protein in the immunoglobulin superfamily [1]. The antigen is expressed on the cell surface and is recognized by the A33 antibody. Previous clinical tests show that the antigen is expressed in 95% of tumors in the colon, rectum, and small intestine [4]. However, the A33 antigen is not cancer specific, and is expressed on all colorectal cells.

Figure 1: A diagram of the A33 antigen attached to the cell membrane of a colorectal cell. The A33 antibody binds to a site on the extracellular part of the antigen [7].
Although the A33 antigen is not cancer specific, it is still useful for cancer immunotherapy. Studies show that although the A33 antibody initially binds indiscriminately to all colorectal cells, after one week, the A33 antibody becomes selective and only remains on tumor cells [9]. One possible hypothesis for such behavior is that noncancerous colorectal cells are shed (the colon epithelium is shed every 3-4 days), while tumor cells are not [2]. Thus, it follows that the A33 antibody bound to the cell linings of healthy colorectal cells is eliminated, while the antibody bound to tumor cells remain.

Figure 2: The diagram on the left shows the A33 antibody (darkened in the figure) in circulation and bound to colorectal cells; the diagram on the right shows that after 7 days, the A33 antibody is only bound on tumor cells. Data courtesy of Chaitanya Divgi, LICR-SKMCC

It can thus be concluded that the A33 antibody will selectively bind to tumor cells in the colon. Since the antigen acts as a target for the A33 antibody, the antigen could be used as part of a two step radioimmunotherapy (RIT) treatment. The first step would involve administration of the A33 antibody, which will bind to the A33 antigen located on tumor cells. The second step would consist of administering a toxin, such as a radionuclide or an enzyme that secretes toxic products, that will bind only to the A33 antibody and selectively destroy tumor cells [ibid].
Currently existing cancer therapies have obvious disadvantages. Chemotherapy, for example, works by killing rapidly dividing cells. These cells include both cancer cells and healthy cells that naturally divide quickly, such as bone marrow cells, gastrointestinal cells, and reproductive cells. The destruction of these healthy cells leads to uncomfortable symptoms, such as nausea, diarrhea, fatigue, susceptibility to infection, infertility, and cognitive impairment [6]. Instead of being systematic in targeting rapidly dividing cells, A33 RIT would be specific, targeting the harmful tumor colorectal cells.

The goal of this project is to find out whether the A33 antigen has a lifetime long enough to remain on tumor cells while normal healthy colorectal cells are shed, so that the antibody-bound antigen marks only harmful cancer cells. The turnover time of the antigen was determined to be 56 hours, indicating that it is a good target for the A33 antibody in two-step radioimmunotherapy against colon cancer.

## 2 Materials and Methods

### 2.1 Sample Generation

#### 2.1.1 Cell plating

A culture containing ls174t human colon cancer cells was trypsinized. The cell culture was diluted to $2 \times 10^6$ cells/mL and was transferred into a 12 well plate. The cells were then incubated at $37^\circ$C for twenty four hours to ensure that the cells adhered in a tight monolayer so that they did not come off the plate during the biotinylation steps.

#### 2.1.2 Biotinylation

The media above the ls174t cells (which adhered to the plate bottom) was aerated from the wells and the cell monolayer was washed twice with 1 mL of PBS (phosphate buffered
saline) solution. This ensured that the serum proteins were removed. Cells were treated with a 1.5mg/mL NHS-SS-biotin solution in 4°C PBS at pH 8.0 and were incubated on ice for one hour covered in aluminum foil to keep out light. During this time, the biotinylation reaction took place in which biotin was attached to cell surface proteins. Following the incubation period, 500µL of 100 mM Tris-HCl was added to each well to block the remaining NHS-biotin reagent and thus stop the biotinylation reaction. Cells were incubated on ice again for ten minutes. The labeling solution in the wells was aerated off and the cells were washed twice and incubated at 37°C for chasing.

2.1.3 Lysis

Cells were lysed in lysis buffer (50 mM β-glycerophosphate at pH 7.3, 10 mM NaPP, 30 mM NaF, 50 mM Tris at pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM benzamidine, 2 mM EGTA, 100 µM sodium orthovandate, 1 mM DTT, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 µg/mL pepstatin, 1 µg/mL Microcystin, 1mM PMSF). Time points were taken between 0 hours and 72 hours for each set of samples created. At each desired time point, 500 µL of the lysis buffer was added to the appropriate well. The cells in that well were incubated on ice for ten minutes, and the cell-lysis buffer solution was mixed by pipetting several times to fully dislodge the cells. The solution was vortexed for ten seconds and incubated on ice for ten more minutes. The cells were then centrifuged at 12000 rpm for 15 minutes to clear the lysate. Finally, the supernatants were extracted and stored at -20°C. This procedure was repeated for each time point.

2.1.4 Pulldown

A pulldown was performed where the biotinylated sample was allowed to bind to streptavadin beads. To accomplish this, 100 µL of ultralink streptavadin resin was added to each of the lysate tubes containing the supernatants from the lysis step. The tubes were incubated at
4°C for 12 hours on a rocker and then centrifuged for a minute at 12000 rpm. For each of the tubes, the supernatant was pipetted off, and the remaining resin was put through a series of washes to remove non-specifically bound proteins. The resin was washed twice with 500µL of PBS, 0.5% Triton X-100, 0.1% SDS, and 0.1% BSA; 500µL of PBS, 0.5% Triton X-100, and 0.1% SDS; and 500µL of PBS and 2 mM EDTA for 5 minutes per wash.

Following washing, the supernatant of each tube was carefully pipetted off while leaving the resin bed untouched. The resin was then resuspended in 75µL of 100 mM DTT and 2 mM EDTA in PBS to isolate the biotinylated protein from the attached biotin and streptavidin by reduction of the disulfide bond between the biotin and the protein, thus releasing the proteins into solution. This suspension was incubated at 37°C for 60 minutes and vortexed briefly every 15 minutes. Suspensions were cleared by centrifugation at 12000 rpm for 15 minutes and the supernatants were pipetted into clean tubes.

Figure 3: A diagrammatic representation of the biotinylation reaction. In figure I, which takes place at time t=0 hours, the biotinylation reaction takes place on the cells, where biotin molecules are conjugated to cell surface proteins via disulfide bonds. Every surface protein gets at least one biotin group covalently attached. The cells are left to grow by being incubated at 37°C. Over time, the cells turn over their surface proteins, resulting in a decrease in biotinylated surface proteins. Time points are taken from 0 to 72 hours.
Figure 4: A representation of the streptavadin pulldown step. In figure I, the extract containing the protein is above the streptavadin beads. In figure II, the streptavadin beads are bound tightly to the biotin, which is attached to the protein of interest (the A33 antigen) as well as other proteins via a disulfide bond. After the set of washes following the pulldown, a chemical reaction takes place which breaks the proteins free from the biotin by reduction of the disulfide bond. The end result is soluble protein that was formerly biotinylated.

2.2 Data Gathering and Analysis

2.2.1 Western Blotting

Western blotting was used to determine the amount of A33 antigen in each of the collected samples. 10 $\mu$L of each of the supernatants was mixed with 2 $\mu$L of sample buffer, and the samples were boiled at 100°C for 10 min to denature proteins present. The samples were then resolved onto an Invitrogen NuPAGE 4-12% Bis-Tris Gel at 200V for 50 min using MOPS running buffer. Proteins were transferred onto a nitrocellulose membrane at 30 V for 75 min. The nitrocellulose membrane was then blocked for an hour with 5% nonfat dry milk in PBS and then washed three times with PBS-T (PBS + Tween-20 solution) for 5 minutes per wash. The membrane was then blotted with a primary rabbit polyclonal antibody for 1 hour. After another series of three five-minute washes with PBS-T, the nitrocellulose was blotted again with a secondary goat anti rabbit-HRP antibody for 1 hour. Finally, a third
set of washes with PBS-T was conducted and the nitrocellulose membrane was developed using Pierce SuperSignal ELISA Femto Maximum Sensitivity Substrate. Pictures were taken with a Fluor-S MultiImager, and the image of the membrane was printed for turnover time analysis.

### 2.2.2 Turnover Time Determination

The printed photos of the membrane were scanned onto a computer and the image was analyzed using ImageJ software. A band pattern was found that was brighter (contained more A33) in lanes containing samples at earlier time points and dimmer (contained less A33) in lanes with samples taken at later time points. The band densities at each time point were found and plotted using ImageJ features, yielding a graph portraying the exponential decay of the A33 antigen, and the turnover time (half life) of the antigen was determined by graphical analysis.

### 3 Results

#### 3.1 Turnover Time of the A33 Antigen

Turnover time of the antigen was analyzed by time-course pulldowns of biotinylated A33.

![Western blot](image.png)

Figure 5: Western blot of samples containing the A33 antigen; lane A contains unbiotinylated sample and lane X contains the MagicMark standard.
The band densities for each time point were then calculated using ImageJ. Each band density is relative to a standard unbiotinylated sample, whose density was measured in lane A of Figure 5. The bands are found between 40 kDa and 50 kDa on the image.

<table>
<thead>
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<th>Time Point (hours)</th>
<th>Actual Band Density</th>
<th>Model Band Density</th>
<th>Square Error</th>
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<tr>
<td>43</td>
<td>17.77</td>
<td>(REMOVED)</td>
<td>(REMOVED)</td>
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</table>

Table 1: Data analysis of the Western blot via ImageJ software

The data from the sample collected at the 43 hour time point was not included, as the band density deviated sharply from the graph. This discrepancy most likely occurred due to an error during sample generation. The following figure contains the plotted band densities versus time and the best fit graph of exponential decay:

Figure 6: Band densities of each of the samples versus time.
As can be seen, the data from samples at 0 hours, 4 hours, 9 hours, 25 hours, and 33 hours fell extremely close to the modeled curve of exponential decay. Once the graph was generated, the half life was calculated and was found to be 56 hours.

4 Discussion

Since samples collected at earlier times have higher band densities and are thus darker, these band densities could be calculated and plotted to determine turnover time of the A33 antigen. Each sample at a different time point showed a different amount of biotinylated A33 antigen expression, since over time, the cultured cells turned over their surface proteins, resulting in a decrease in biotinylated surface proteins.

The turnover time for the A33 antigen was thus found to be 56 hours in the blot shown. In additional trials, similar results were found, such as a turnover time of 53 hours in another blot of a second sample set.

This experimentally derived half life of around 2 days is likely to be enough time for the healthy colon cells to be significantly shed, rendering specificity to the antibody-bound A33 antigen such that it will remain solely on tumor cells and can be targeted by a radionuclide or another substance toxic to tumor cells. Hence, A33 is indeed a viable option that can be utilized in a two step radioimmunotherapy treatment.

In each blot that was run with each set of samples, there was always at least one data point that had to be ignored because it deviated sharply from the general curve. One explanation is that the particular sample with the incorrect band density was extracted or pulled down incorrectly. Another possible explanation is that the sample was loaded improperly or did not migrate properly during the transfer.

Usually, to assure that the curve generated by the band densities from the blot were due solely to the antigen turning over rather than different concentrations of protein being
loaded into different lanes of the gel, a loading control would be performed that would yield the graph of a straight line. However, such a loading control could not be performed in this protocol, since live mammalian cells were being cultured and thus any attempt at a loading control would have resulted in a skewed graph due to their exponential cell growth.

While the half life of the antigen was found to be 56 hours, this figure is not a cutoff time before which the primary antibody agent and the secondary toxic moiety must be administered. The reason for this is that the A33 antibody, which is the primary agent that binds to the A33 antigen, has been found to fall off of the antigen with a half life of 6 hours [2]. However, it has been found that upon dissociating, the antibody can rebind to any free antigen. Thus, in 56 hours, while half of the A33 antigen would have turned over, there will always be more naked antigens for the antibody to target and bind to. The long turnover time of the antigen is a guideline that shows and affirms that the antigen does indeed have potential in cancer therapy.

Although the colon epithelial turnover time is longer than the turnover time of the A33 antigen, the antigen can still be used in radioimmunotherapy treatment. The reason for this is that after 2 days (the half life of the A33 antigen), if the toxic moiety that is supposed to target solely the A33 antibody bound to the antigen is administered, the healthy colon cells that will be affected by the moiety will already be in the process of being shed; the moiety thus will have minimal adverse affect upon the healthy colorectal system. This has been affirmed by clinical trials, where instead of a two step radioimmunotherapy treatment, a one step treatment was used in which the toxic moiety (a radionuclide) was already bound to the A33 antibody. In this case, the entire colon was exposed to radiation; despite this, the dose limiting toxicity was not gastric [9].

The A33 antigen thus also has an advantage in cancer therapy over other targets, because it remains on the cell surface for an extended period of time. Any antigen targeted by an antibody that is internalized becomes inaccessible to the toxic molecule in a two step ra-
dioimmunotherapeutic treatment, while if the antigen remains on the surface, such treatment becomes possible.

5 Conclusion

The turnover time of the A33 antigen was found to be 56 hours. This data suggests that the antigen will remain on the surface of tumor cells for about 2 days before being degraded, which indicates that the antigen is stable for this period of time and can thus be targeted via a two-step radioimmunotherapeutic treatment against colorectal cancer.

6 Acknowledgments

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References


