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Detection of apoptosis in cancer cell lines using Surface Plasmon Resonance imaging

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ABSTRACT

Induction of apoptosis in cancer cells by therapeutic agents is an important event to detect the potential effectiveness of therapies. Here we explore the potential of Surface Plasmon Resonance imaging (SPRi) to assess apoptosis in cancer cells exposed to therapeutic agents by measuring the cytochrome C release of apoptotic cells. Spots on the SPR sensor were coated with anti-cytochrome C, anti-EpCAM, anti-CD49e monoclonal antibodies and combinations thereof. Cells from the breast cancer cell line MCF7 were introduced into a flow cell, captured on a sensor surface and exposed to culture medium with and without paclitaxel. The cells were followed for 72 h. Clear SPRi responses were observed on the anti-EpCAM coated spots, indicating binding of the MCF7 cells with strong time and drug presence dependent increases in SPRi responses on the spots coated with both anti-EpCAM as well as anti-cytochrome C. This suggests a release of cytochrome C by the MCF7 cells in these specific locations. In addition offline experiments were performed where cultured MCF7 cells were exposed to complete culture medium with paclitaxel, Trastuzumab antibody and Trastuzumab T-DM1 (an antibody drug conjugate). The supernatant of these cells was analyzed and also their drug concentration dependent cytochrome C presence was detected. These preliminary results suggest SPRi to be a unique tool to measure real time response of cancer cells exposed to drugs or drug combinations.

1. Introduction

Surface Plasmon Resonance imaging (SPRi) can be used to measure the expression of antigens on the cell surface and antigens or proteins secreted by cells [12,13]. In recent years a larger number of potential therapeutic agents were identified of which only few entered into the clinic and even fewer showed therapeutic efficacy with a tolerable toxicity profile. All drugs have in common that they ultimately have to kill the cancer cells. These cells will undergo a process baptized apoptosis and during this process specific alterations in the cells take place. Measurement of apoptosis in cancer cells can therefore be used to screen the effectiveness of potential therapeutic agents on cancer cell lines with properties that can be related to the cancers to be treated [4] or to measure the response to therapy in patients [16]. A number of different compounds are excreted during apoptosis and one such compound is cytochrome C. Cytochrome C is a so called hemoprotein that is associated with the inner membrane of the mitochondrion where it is part of the electron transport chain [11]. Under normal conditions cytochrome C is bound to cardiolipin in the inner mitochondrial membrane. This prevents the initiation of apoptosis. Cytochrome C can become detached from cardiolipin in early apoptosis by its oxidization due to the production of mitochondrial reactive oxygen species. Cytochrome C plays an intermediary role in early apoptosis and upon release into the cytoplasm it binds apoptotic protease activating factor-1 (Apaf-1) and activates caspase 9 [6], which in term starts a cascade of events with apoptosis as the end result. As cytochrome C is highly water soluble it can easily dissolve into the cytoplasm and disperse throughout the cell. As apoptosis progresses cytochrome C will inevitably also leak from the cell. This makes the detection by SPR possible.

A variety of methods are described and are commercially available. Characteristics of apoptosis such as cell loss, nuclear morphology, DNA content, cell membrane permeability, mitochondrial membrane potential changes and cytochrome C localization and release are measured. However in order to detect all of these characteristics a number of components are mixed together in a complex and laborious protocol. Measurements of apoptosis are usually end point measurements and cells cannot be followed in real time. The most commonly used techniques to measure apoptosis are microscopy and flow cytometry [1–3,7,9,14,15,17,18]. Though robust and specific, these methods have their own unique challenges and downsides.

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SPR can be a potential alternative technique to study apoptosis. Previously it was shown that SPR can be used to detect apoptosis-associated genes [5], but this was not done using a cell sample. In a different study cells were brought into apoptosis and their morphological changes were monitored using SPR [8].

Here we explored if SPR can be used to detect early apoptosis by measurement of the apoptosis marker cytochrome C excreted in cell supernatants either by collecting the supernatant after exposure to different drugs added at different concentrations or by measuring the response of viable cells that were captured and exposed to drugs in real time in the SPRi instrument. In our experiments we used a cytostatic compound (Paclitaxel) a therapeutic antibody (Trastuzumab) and an antibody drug conjugate (ADC) (Trastuzumab T-DM1), available commercially as Herceptin® and Kadcyla® respectively from Genentech inc., South San Francisco, California, USA. Detecting early apoptosis using SPR would enable rapid therapy screening using live cells which would further contribute towards the development of personalized cancer therapy.

2. Materials and methods

2.1. SPRi

For SPRi analysis an IBIS MX96 SPR imager was used (IBIS Technologies BV, Enschede, The Netherlands). The height of the flow cell was 300 μm enabling a homogeneous injection of cells and cell medium. The obtained homogenous cell sample injection and larger volume of culture medium improve the cell viability over time.

2.2. CFM spotter

For ligand immobilization on SPR sensor surfaces the Continuous Flow Microfluidic (CFM) spotter was used (Wasatch Microfluidics LLC, Salt Lake City, Utah, USA) [10]. Ligand immobilization buffer was used to prime the CFM system and to dilute the desired ligands. The immobilization protocol lasted 30 min. The CFM spotter with a 6×8 print head configuration is capable of spotting up to 48 different ligands onto the sensor in a single run simultaneously under back and forth confined flow. The confined back and forth flow increases the efficiency of the spotting and avoids the risk of evaporation for contact and non-contact droplet based spotting methods.

2.3. SPR sensors

Easy2Spot® pre-activated G-type Senseye® sensors (Ssens BV, Enschede, The Netherlands) were used as SPR sensor surfaces. The sensors are delivered with a 100 nm hydrogel-like layer. This enables higher capacity coupling of ligands in the evanescent field and gives the ligands a level of mobility. The sensors are pre-activated for easy immobilization without using an additional EDC–NHS activation protocol.

2.4. Antibodies

Anti-cytochrome C (Biolegend inc., San Diego, California, United States of America) was used to capture cytochrome C, which is excreted by the cells when they are undergoing apoptosis. Anti-Epithelial Cell Adhesion Molecule antibody (anti-EpCAM, VU1D9) and Human epidermal growth factor receptor 2 antibody (anti-Her2) (both kindly provided by Immunicon, Huntingdon Valley, Philadelphia, USA) were used to capture cells based on their cell surface molecule expression. Anti-CD49e (BioLegend, San Diego, California, USA) was used as a negative control as MCF7 cells do not express this marker on their cell surface. Antibody Immunoglobulin G fragment crystallizable region (Anti-mouse IgG Fc) (BioLegend, San Diego, California, USA) was used to capture all antibodies present in the sample.

2.5. Apoptosis inducing agents

To induce apoptosis, paclitaxel (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), the therapeutic antibody Trastuzumab and the ADC Trastuzumab T-DM1 (kindly provided by Dennis Waalboer at the VU Amsterdam, The Netherlands) were used.

2.6. Ligand immobilization buffer

A 10 mM solution of sodium acetate (NaAc) immobilization buffer with pH 4.5 was made using anhydrous sodium acetate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and acetic acid (Merck Schuchardt OHG, Hohenbrunn, Germany). First a 0.2 M stock solution was made of both components. Then from these stock solutions 1.93 parts of sodium acetate and 3.07 parts of acetic acid were mixed and finally 95 parts of ultrapure demineralized water were added. The pH was checked and if needed adjusted to pH 4.5.

2.7. System buffer

As system buffer the complete cell culture medium of the MCF7 cell line which was being analyzed was used, unless otherwise noted in the experiment description.

2.8. Cells

The cell line that was used for the experiments was the breast cancer cell line MCF7. MCF7 is an adherent cell line. To harvest MCF7 cells a trypsin protocol was used and the cells were resuspended in complete culture medium (RPMI 1640 + 10% fetal calf serum + 1% penicillin streptomycin + 1% L-Glutamine). RPMI 1640 was purchased from Lonza Group Ltd., Basel, Switzerland. Fetal calf serum and penicillin streptomycin were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

2.9. Deactivation agent

A 1% Bovine Serum Albumin solution (BSA) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in sodium acetate immobilization buffer was used as a deactivation agent. A stock solution of 2-aminoethanol (MP Biomedicals LLC, Illkrich, France) was used to create a 100 mM 2-aminoethanol solution with a pH of 8 and used as an extra sensor deactivation step after the initial BSA deactivation.

2.10. Sensor regeneration agent

As sensor regeneration agent a 200 mM solution of H_3PO_4 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used. Sensor regeneration was performed for 1 min.

2.11. Detection of cytochrome C in supernatant samples of cultures treated with paclitaxel

To investigate if SPR is capable of detecting cytochrome C in culture supernatant, several flasks of MCF7 were cultured with various amounts of apoptosis inducing paclitaxel. One flask was without any addition of paclitaxel, the second had 1.5 μl (100 nM) of paclitaxel, the third had 3.0 μl (200 nM) and the last one had 6.0 μl (400 nM) added to 15 ml of complete culture medium respectively. The flasks with added paclitaxel were first cultured to 80–100% confluence before the compound was added by a medium refresh. After the addition of the drugs, the flasks were cultured for another 48 h at 37 °C and 5% CO_2. After these 2 days supernatants were removed from the flasks. An SPR sensor was prepared with varying spots containing: anti-cytochrome C (using a spotting solution of 20 μg/ml antibody in ligand immobilization buffer), anti-EpCAM as a negative control (using a spotting solution of 10 μg/ml

antibody in ligand immobilization buffer) and a spot only exposed to ligand immobilization buffer. After sensor spotting, the surface was loaded into the IBIS MX96 and deactivated with BSA and ethanolamine (see deactivation agent). The program to run the IBIS MX96 used association times of 45 min per sample and after each sample the surface was regenerated with phosphoric acid.

2.12. Detection of cytochrome C in supernatant samples of cultures treated with paclitaxel, Trastuzumab and Trastuzumab T-DM1

In these experiments we wanted to verify if apoptosis markers can be detected in the supernatant of cell samples that were treated with Trastuzumab T-DM1 (an anti-Her2 based ADC with an apoptosis inducing chemical, called emtansin, attached to it), a therapeutic antibody developed for treatment of cancers that express Her2. The chemical compound T-DM1 is delivered to the Her2 expressing tumor cells by binding of the Her2 antibody to the cell surface after which the attached chemical molecule is introduced to the cell. For this experiment MCF7 cells were similarly prepared as for the paclitaxel experiments, however in addition to paclitaxel (3.0 and 6.0 μl, which is 200 and 400 nM respectively, in 15 ml of complete medium) Trastuzumab with and without T-DM1 was used as an apoptosis inducing compound. The recommended concentration for inducing apoptosis in a Her2 expressing cell line using the T-DM1 ADC was 100 nM, as such this concentration was used for both the T-DM1 ADC and plain variant of Trastuzumab (lacking the T-DM1). All samples were cultured for 48 h after the addition of the apoptotic agents. An SPR sensor was prepared with anti-cytochrome C immobilized on its surface, with a concentration of 10 μg/ml. In addition a negative control surface that was only exposed to the immobilization buffer was used. The IBIS MX96 was programmed with an association time of 45 min and dissociation time of 10 min each. After each of the samples the surface was regenerated.

2.13. Real-time detection of induction of apoptosis in cell cultures exposed to paclitaxel

To evaluate if SPRi can be used to detect apoptosis in real-time a sensor was prepared with anti-cytochrome C spots (10 μg/ml), anti-EpCAM spots (10 μg/ml), anti-CD49e spots (10 μg/ml) and an anti-EpCAM/anti-Cytochrome C spot (10 μg/ml in a 50/50 ratio). The anti-CD49e spot is used as a negative control as MCF7 cells express very low levels (if any at all) of CD49e. The mix spot intended to capture the MCF7 cells based on their EpCAM expression. Subsequently the mix spot was intended to follow the anticipated apoptosis process after complete medium containing paclitaxel was introduced to the captured cells. MCF7 cells were harvested from their culture flask and prepared to reach a final cell concentration of 1.8×10^6/ml. The cells were allowed to sediment and bind to the sensor surface for 30 min. After this initial cell binding the unbound cells were washed off and complete culture medium containing paclitaxel was flushed over the sensor surface. The flow cell temperature was kept at 37 °C throughout the experiment and the process was followed for 72 h. The same was done for an identical separate sensor, however on this sensor the cells were not exposed to paclitaxel, but merely to complete culture medium.

3. Results

3.1. Detection of cytochrome C in supernatant samples of cultures treated with paclitaxel

The sensorgram of this experiment is shown in Fig. 1. The anti-cytochrome C spot (green line) shows an increasingly higher interaction response with increasing doses of paclitaxel used in the cell medium. The anti-EpCAM spot shows consistent responses, with a slight decrease in intensity for each subsequent interaction. The blank sensor surface spot shows no interaction whatsoever with any sample, indicating that the sensor surface by itself does not exhibit non-specific background interactions with any of the compounds in the samples.

3.2. Detection of cytochrome C in supernatant samples of cultures treated with paclitaxel, Trastuzumab and Trastuzumab T-DM1

Fig. 2 shows the sensorgrams of supernatants of cell suspensions exposed to paclitaxel, Trastuzumab or Trastuzumab T-DM1. An anti-cytochrome C spot of 10 μg/ml (green line) was used in this experiment. In order to be able to study the apoptotic effect of the used compounds

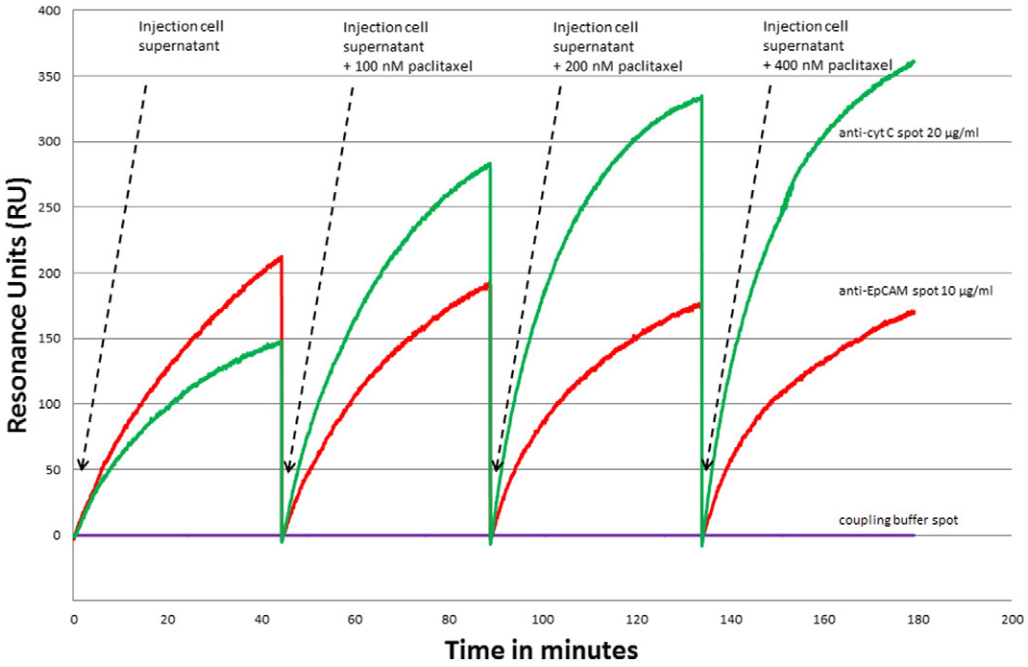

Fig. 1. Sensorgram showing interactions of cell culture supernatant samples treated with various concentrations of paclitaxel. The response of the anti-cytochrome C spot increases as the concentration of paclitaxel that was used in the samples is increased.

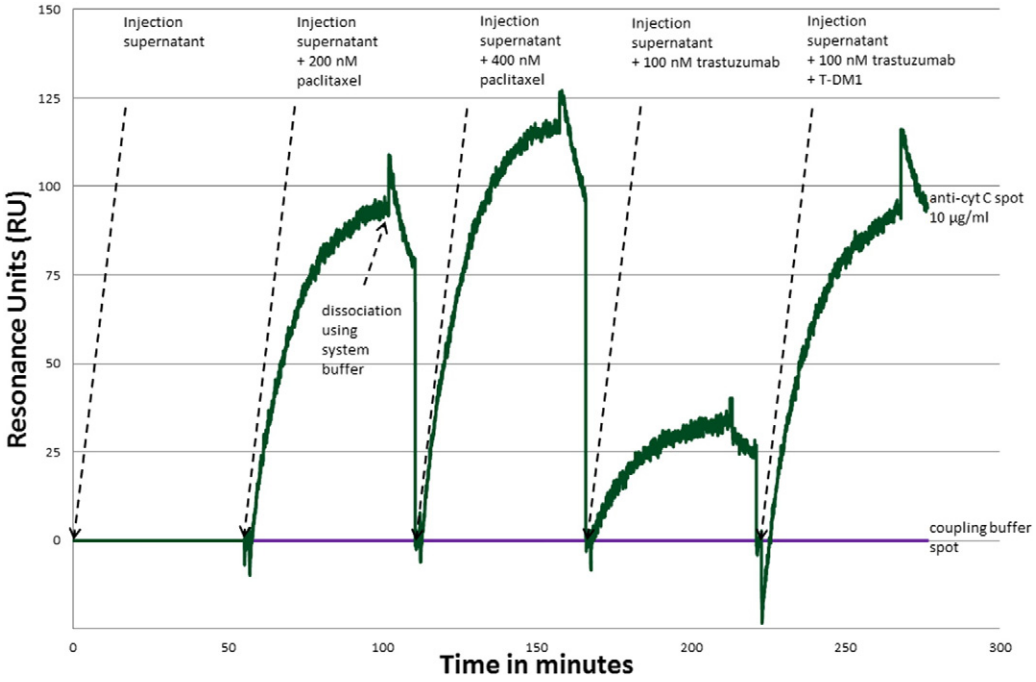

Fig. 2. Double referenced sensorgram showing the effect of paclitaxel and Trastuzumab with and without T-DM1 on the excretion of cytochrome C by MCF7 cells. The first negative control sample with no apoptosis inducing agents was used for blank subtraction. The differential responses increase when paclitaxel, Trastuzumab and the T-DM1 ADC were used. The responses of the T-DM1 ADC and paclitaxel are larger, implying an increase in apoptosis in these cell samples.

better a data processing method "blank subtraction" was used, which is sometimes referred to as double referencing in the SPR realm. In essence, this means that the RU values of a negative sample are subtracted from each of the subsequent (presumably positive) samples, which were already referenced using a negative control surface. This way any background or non-specific signals are removed from the resulting output and only the cleaned up specific differential signal remains. The first sample which had no apoptosis inducing compounds added to the

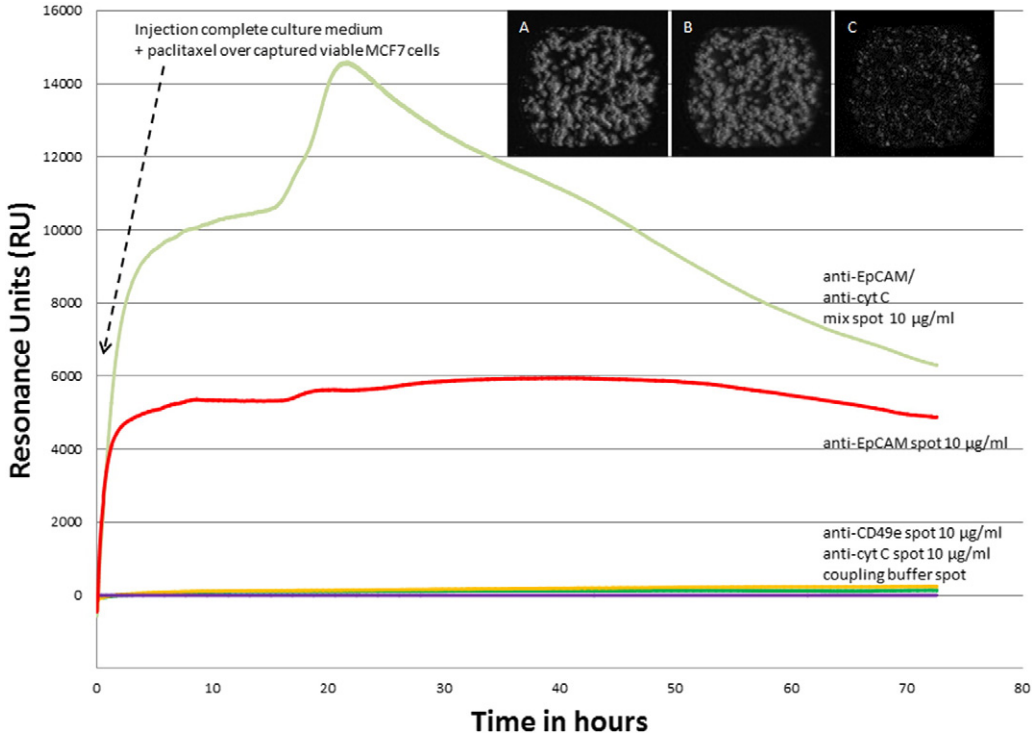

Fig. 3. Sensorgram showing a real-time detection of apoptosis in cells exposed to paclitaxel. At t = 0 bound cells are exposed to complete culture medium containing paclitaxel. The anti-EpCAM/anti-cytochrome C mix spot shows the highest response indicating that cytochrome C is actively excreted by the cells and is being bound on the surface. Insert A shows the SPR reflectivity image of MCF7 cells at the beginning of their exposure to paclitaxel, insert B shows the cells at the end of the experiment and insert C shows the differential image of inserts A and B.

medium during culture was used as the blank subtraction sample, and therefore this differential signal is zero. The samples treated with paclitaxel show more intense interactions on the anti-cytochrome C spot similar to those observed in Fig. 1. The sample with Trastuzumab lacking the T-DM1 only showed a small differential signal as compared to the control sample whereas the differential response of the sample with Trastuzumab T-DMI showed a similar response as the paclitaxel treated samples. These results indicate the presence and release of cytochrome C in the culture medium of MCF7 cells, which increases after the cells are exposed to Trastuzumab, Trastuzumab T-DM1 or paclitaxel suggesting an increase in apoptosis. The blank sensor surface (purple line) shows no differential signal. The cytochrome C spots show a slight bulk shift when association and dissociation is initiated. This is due to the refractive index differences between the sample and the system buffer.

3.3. Real-time detection of induction of apoptosis in cell cultures exposed to paclitaxel

Fig. 3 shows the SPRi response of cells captured on an SPRi sensor and subsequently exposed to paclitaxel. No response is observed on the spots coated with anti-CD49e, anti-cytochrome C or coupling buffer. This indicates that MCF7 cells do not bind to these spots and therefore no cytochrome C release can be detected. The anti-EpCAM spot shows a rapid response that remains relatively constant over a period of 60 h. This demonstrates binding and continuous interaction of the MCF7 cells to the anti-EpCAM present on the spot. The largest response is observed on the anti-EpCAM/anti-cytochrome C mix spot showing an almost immediate drastic increase in RU after the bound cells were exposed to complete culture medium containing paclitaxel. After ~18 h of exposure to paclitaxel a sudden increase can be seen in the sensorgram that reaches a peak at ~21 h after which it slowly decreases to a level close to that observed for the anti-EpCAM spot.

Fig. 4 shows a different passage of MCF7 cells that were followed similarly, however here the cells were only exposed to complete culture medium that did not contain paclitaxel. Similar to the experiment illustrated in Fig. 3 no response is observed on the spots coated with anti-CD49e, anti-cytochrome C or coupling buffer. The SPRi response of the anti-EpCAM/anti-cytochrome C spot showed an immediate increase, the slope of this response decreased after ~42 h and was lower as compared to the anti-EpCAM spot after this time. The SPRi response of the anti-EpCAM spot only started after ~4 h with a similar slope as the anti-EpCAM/anti-cytochrome C spot also here after ~42 h the slope decreased but to a lesser extent as compared to the mixed spot. We have previously described this phenomenon and contributed it to cells that keep interacting with the surface and their adherent nature, which contributes to the continuously increasing signal [12].

4. Discussion

We explored whether SPRi could be used to follow apoptosis of cells by measuring the cytochrome C release as a progression marker of the apoptotic process. Before conducting real time cell measurements we harvested the culture medium of MCF7 cells exposed for two days to different concentrations of paclitaxel and showed an SPRi response on anti-cytochrome C spots. Cytochrome C was detected in cells not exposed to paclitaxel indicating the natural apoptosis of cells in culture and with increasing doses of paclitaxel Cytochrome C release increased (see Fig. 1). Unexpectedly SPRi responses were also observed on anti-EpCAM spots that were intended as negative controls. As MCF7 expresses EpCAM on their cell surface a possible explanation for the interactions could be that during the apoptosis process the MCF7 cells excreted vesicles in the supernatant. These vesicles have the potential to express similar markers as its cell of origin, but also have the capability to be distinct from their origin [19] and therefore can be the reason for the interactions seen on the EpCAM spots. To exclude nonspecific binding a separate experiment was conducted in which no cells were added to the RPMI 1640 complete culture medium with paclitaxel (data not shown). Except for a large bulk refractive index shift in the raw data, no interaction was detected, indicating that the presence of the MCF7 cells during culture was responsible for the SPRi response

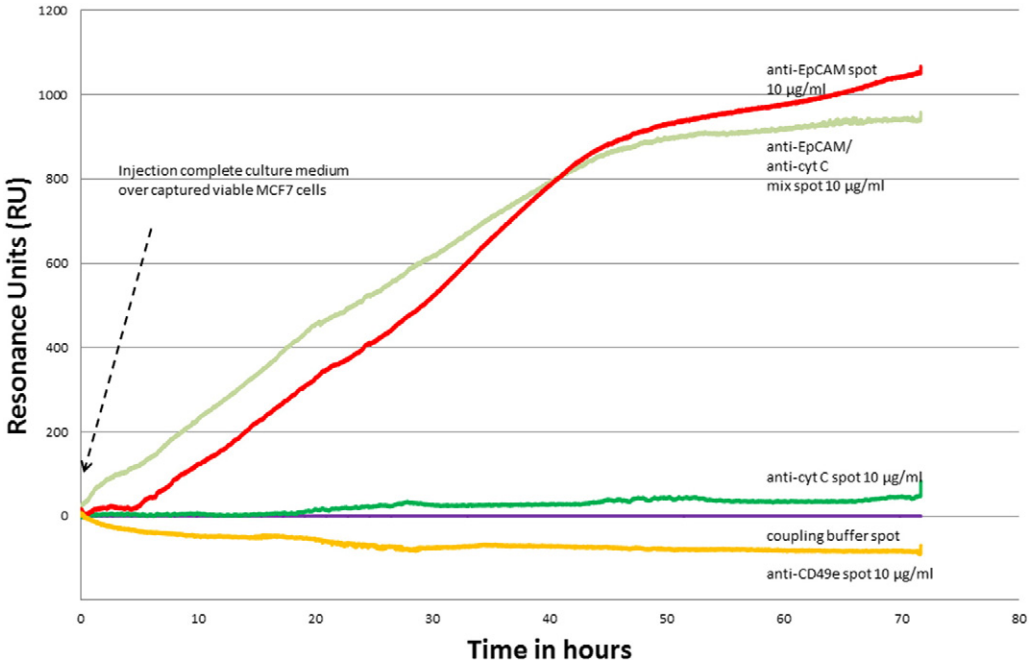

Fig. 4. Sensorgram showing a measurement of MCF7 cells that were exposed to culture medium without paclitaxel on spots coated with coupling buffer (purple line), anti-CD49e (yellow line), anti-cytochrome C (green line), anti-EpCAM (red line) and a mix of anti-EpCAM and anti-cytochrome C (light green line). The cells show similar responses on both the anti-EpCAM and the anti-EpCAM/anti-cytochrome C mix spot, indicating that the interaction seen here is a continuation of their binding interaction with the ligands immobilized on the sensor surface and there is no detection of apoptosis related cytochrome C.

obtained from the supernatant. This further strengthens our conclusion that indeed we are detecting the apoptosis specific cytochrome C high response values up to 14,000 RU as well as EpCAM but the SPRi responses of the latter did not increase with increasing concentrations of paclitaxel.

To assess whether cytochrome C release could also be detected in the supernatant of cells exposed to different therapeutic agents, the MCF7 cells expressing Her2 were treated with drugs targeting this receptor (Trastuzumab with and without T-DM1). The responses shown in Fig. 2 show similarities with the responses observed in Fig. 1. Again the paclitaxel treated samples show increased interaction levels with the anti-cytochrome C spots. The sample treated with Trastuzumab without the T-DM1 conjugate shows a lower interaction level. This might be explained by the action of Trastuzumab, when it binds to the HER2 expressing cell, it reduces cancer cell proliferation by arresting the cells in the G1 phase of the cell cycle. The T-DM1 ADC showed increased interaction levels just like the paclitaxel sample. This shows that we can indeed observe a difference between the Trastuzumab ADC with added therapeutic/cell toxic component emtansin (T-DM1) and Trastuzumab without such a component. The SPR sensorgrams imply that indeed we can see a difference in the quantitative cytochrome C response and that using this principle it might be possible to study therapeutic alternatives and their effectiveness. However, if we want to use the SPRi platform as a way to analyze effects of therapeutics directly on the cells, the cells and excreted products should be followed in real time during exposure to the drugs. This was performed in the experiments illustrated in Figs. 3 and 4. Here we immobilized anti-CD49e, anti-cytochrome C, anti-EpCAM and anti-EpCAM/cytochrome C on the sensor surface. The mix anti-EpCAM/cytochrome C spots can capture the MCF7 cells on the SPRi sensor as these cells have EpCAM expression as described earlier [12]. The anti-cytochrome C part of the mix spot was intended to detect release of cytochrome C from the cells that would progress into apoptosis. Fig. 3 indeed shows a great difference in responses between the spots. The largest and most obvious response up to levels of 14,000 RU indeed comes from the anti-EpCAM/cytochrome C mix spots after the medium with paclitaxel was added. In addition a peak is seen at around 25 h after paclitaxel addition. One explanation is that the cells around this time point massively release cytochrome C. Afterwards there is a decrease in intensity visible, which may be explained by the start of the disintegration of the cells, which detach from the sensor surface and the cytochrome C that possibly dissociates from the mix spots into the medium. A decrease is also seen on the anti-EpCAM spot but to a lesser extent. The intensities in signal are also remarkably different, the mix spot in Fig. 3 shows a much higher response compared to the anti-EpCAM spot. Already after about 1 h considerable intensity differences of several thousands of RU between the 2 spots can be seen. Insert A of Fig. 3 shows the MCF7 cells at the beginning of their exposure to paclitaxel, insert B shows the cells at the end of the experiment and insert C shows the differential image of inserts A and B. It is clearly visible that in insert C the cells have underwent morphological changes and seemingly binding of some sort has occurred adjacent to them. Based on the sensorgrams we assume the slight halos around the cells to be cytochrome C that was excreted throughout the experiment, we have previously described a similar "halo effect" [13]. In the control experiment seen in Fig. 4 the anti-EpCAM and mix spots remain largely similar throughout the experiment. This real time experiment could in the future be used to screen potential therapeutic agents on live cells and follow the process to see if they can induce apoptosis. The control experiment shown in Fig. 4, was done in order to see what would happen with the same cells (MCF7 of a different passage) when they are not stimulated to go into apoptosis. This shows that cells merely continuously interact with their ligands on the sensor surface but to a lesser extent up to 1000 RU after 40 h, a phenomenon we also described previously [12]. We assume that on top of the interaction with the ligands on the sensor surface the cells also exhibit their adherent properties. As they are being monitored using complete culture medium as a system buffer and at a temperature of 37 °C, the cells are most likely spreading generating higher SPR-signals while they are bound to the sensor surface. Cell-spreading is a behavior they also exhibit in culture flasks. Responses of the anti-EpCAM and the anti-EpCAM/anti-cytochrome C mix spots seem similar and therefore no apoptosis was detected, which agrees with the intended circumstances of the assay. In addition in both Figs. 3 and 4 the negative control spots (anti-CD49e, anti-cytochrome C and coupling buffer) show no interaction whatsoever as opposed to Fig. 1, where the anti-CD49e spot shows a small interaction. Earlier in the discussion we already mentioned that in the supernatant sample vesicles might have been present explaining the interactions seen in Fig. 1. These interactions did not occur in Figs. 3 and 4 as the cells that were bound locally on the mix and anti-EpCAM spots were too few in number to cause the sample to become saturated with vesicles and if any vesicles were formed they would have (re)bound locally after formation on the mix and anti-EpCAM spots.

5. Conclusion

SPRi can be used to detect apoptosis through measurement of cytochrome C excretion. This was achieved through binding of cytochrome C to an SPR sensor coated with antibodies recognizing cytochrome C. Experiments were conducted with supernatants of cells of the MCF7 breast cancer cell line exposed to cell culture medium with or without paclitaxel, Trastuzumab or Trastuzumab T-DM1 that were introduced in the SPRi instrument. The largest SPRi responses were observed on spots coated with anti-cytochrome C indicating that Cytochrome C release is a relevant biomarker of apoptosis progression. In addition, living MCF7 cells were introduced into the SPRi instrument and exposed to cell culture medium with or without paclitaxel and high SPRi responses were observed on the anti-EpCAM and anti-EpCAM/anti-cytochrome C coated spots, demonstrating that the apoptotic process can be monitored in real-time and without the use of traditional fluorescent labels or stains.

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