Viability analysis and apoptosis induction of breast cancer cells in a microfluidic device: effect of cytostatic drugs

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Abstract Breast cancer is the leading cause of cancer deaths among non-smoking women worldwide. At the moment the treatment regime is such that patients receive different chemotherapeutic and/or hormonal treatments dependent on the hormone receptor status, the menopausal status and age. However, *in vitro* sensitivity testing of tumor biopsies could rationalize and improve the choice of chemo- and hormone therapy. Lab-on-a-Chip devices, using microfluidic techniques, make detailed cellular analysis possible using fewer cells, enabling working with a patients' own cells and performing chemo- and hormone sensitivity testing in an *ex vivo* setting. This article describes the development of two microfluidic devices made in poly(dimethylsiloxane) (PDMS) to validate the cell culture properties and analyze the chemosensitivity of MCF-7 cells (estrogen receptor positive human breast cancer cells) in response to the drug staurosporine (SSP). In both cases, cell viability was assessed using the life-stain Calcein-AM (CAAM) and the death dye propidium iodide (PI). MCF-7 cells could be statically cultured for up to 7 days in the microfluidic chip. A 30 min flow with SSP and a subsequent 24 h static incubation in the incubator induced apoptosis in MCF-7 cells, as shown by a disappearance of the aggregate-like morphology, a decrease in CAAM staining and an increase in PI staining. This work provides valuable leads to develop a microfluidic chip to test the chemosensitivity of tumor cells in response to therapeutics and in this way improve cancer treatment towards personalized medicine.

Keywords Breast cancer · Apoptosis · Viability · Cytostatic drugs · Lab-on-a-Chip

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1 Introduction

Breast cancer is the leading cause of cancer among non-smoking women in the world, although variation in incidence, prevalence and mortality exists between countries (Bray et al. 2004).

Treatment modalities used in breast cancer include surgical removal of the tumor, followed by radiotherapy, chemotherapy and/or hormone therapy. The various protocols that exist for chemotherapy and hormone therapy have different and limited rates of success (EBCTG 2005). Currently, selection of chemotherapy and/or hormone therapy is based on a broad scale of factors, including a patients' age and tumor characteristics, such as nodal status, the presence of estrogen receptors and the Her-2/neu status

(Carney et al. 2007). One potential approach to improve the therapeutic efficacy is to use *ex vivo* approaches to evaluate the sensitivity to specific hormone therapies and chemotherapeutic agents using patients' tumor cells. This requires the harvesting of tumor cells via biopsy, conducting chemo- and hormone sensitivity tests and extrapolating the results to the clinical setting.

Microfluidic devices are very helpful in the design of such chemo- and hormone sensitivity tests for multiple reasons. Microfluidic devices offer the possibility of using lower quantities of cells, parallel processing (high-throughput analysis) and detailed cellular analysis (Andersson and van den Berg 2003, 2004). Because of the lower quantities of cells (typically <100), cells can even be obtained via fine-needle aspiration. Furthermore, the obtained cells can be subdivided into numerous samples to test various concentrations and combinations of different chemotherapeutics and hormones (high-throughput drug screening). Furthermore, individually tested samples in parallel promotes statistical analysis.

The balance between programmed cell death (apoptosis) and cell proliferation determines the tumor growth rate, and any alteration between these two factors may be the key element for the uncontrolled expansion of malignant tumors. Chemotherapeutics and/or hormones are prescribed to tackle this homeostatic imbalance. Conventional apoptosis and proliferation assays were performed to evaluate the hormone sensitivity of different breast cancer cell lines *in vitro* (Franke and Vermes 2002, 2003; Franke et al. 2003; Werner et al. 2005). Attempts of testing chemosensitivity of tumor cells *ex vivo* were already made in the eighties, but have not developed into a standard chemosensitivity test so far (Selby and Raghaven 1981). Recently, research groups have established a link between clinical responses to chemotherapeutics and *ex vivo* chemosensitivity analysis (Nakada et al. 2005; Yamashita et al. 2005; Trojan et al. 2005). Moreover DNA microarrays (Gianni et al. 2005) and multiparametric microsensor chips (Otto et al. 2003) have been developed for chemosensitivity testing. The assays described above provide valuable leads for novel therapeutic modalities and improve cancer treatment, however there are limitations. For example, the *in vitro* hormone sensitivity assays need cell culture to obtain millions of cells for analysis and the techniques are very time-consuming and extremely laborious. Cell handling is easier with DNA microarrays, however, the link between genetics and the ultimate goal, cell death in response to chemotherapeutics, yields uncertainty and adds to statistical difficulties.

The focus of this article is to develop a microfluidic chip which enables the analysis of the chemosensitivity of first breast cancer cell lines, and in a later stage a patients' own breast cancer cells in the presence of various chemotherapeutics. As the goal of chemotherapy is to induce cell death, the degree of apoptosis was assessed using fluorescent dyes. It is not until recently that research groups have become interested in developing chips convenient for detecting apoptosis (Wolbers et al. 2004a, b; Qin et al. 2005). So far, microfluidic chips have been developed to characterize the various hallmarks in the apoptotic cascade in real-time at a single-cell level using fluorescent dyes (Valero et al. 2005; Munoz-Pinedo et al. 2005; Chan et al. 2003), measuring the release of biological substances (Tamaki et al. 2002; Kurita et al. 2003), and the fragmentation of the DNA (Kleparnik and Horky 2003). Apoptosis assays place greater demands on cell handling, especially when cell culture of adherent cells is required. Therefore it has to be ensured that the microfluidic device and experimental set-up do not negatively influence the cellular behavior prior to adding chemotherapeutics (Wolbers et al. 2006). Besides the fact that the microfluidic device and experimental set-up must be compatible with cell survival, a flow system is needed to deliver medium and chemotherapeutics. The applied flow rate and the accompanied shear stress have to ensure cell survival. For the chip material, the silicone elastomer poly(dimethylsiloxane) (PDMS) together with glass, for sealing, is chosen. PDMS is biocompatible, gas permeable, and a cheap material in which structures on a micrometer scale can be easily made (McDonald et al. 2000; Sia and Whitesides 2003), and Pyrex glass is a good supporting material for cell adhesion and promotes optical analysis. Overall, the microfluidic device and flow system consist of disposables, which is a great advantage when performing biological experiments.

This paper describes the cellular studies performed on the estrogen receptor positive human invasive lobular carcinoma cell line MCF-7 in two different microfluidic PDMS chips. Firstly, a low volume microfluidic device with cell trap was used to evaluate cell culture. Secondly, a chip with a higher volume without cell trap was used to apply a controlled flow of medium and a chemotherapeutic to study the effect of this drug on the apoptotic pathway. In the experiments presented here, apoptosis is induced by the protein kinase C (PKC) inhibitor staurosporine (SSP; Koivunen et al. 2006) to optimize the microfluidic device towards a potential clinically relevant apoptosis assay. Staurosporine is a frequently used agent for the induction of apoptosis, and known to induce apoptosis in MCF-7 cells (Mooney et al. 2002; Xue et al. 2003; Sayeed et al. 2007). In a later stage, specific chemotherapeutic agents for breast cancer, such as cyclophosphamide or doxorubicin (anthracyclin) will be used. Cell viability was assessed using two fluorescent dyes, i.e., the life-stain Calcein-AM (CAAM; Bratosin et al. 2005) and the death dye propidium iodide (PI; Zamai et al. 2001). This work provides us with valuable leads to develop a microfluidic chip to test the chemosensitivity of tumor cells and improve cancer treatment.

2 Material and methods

2.1 Chip design and fabrication

2.1.1 Chip 1

Chip 1 consists of a single channel with a length of 1 cm, a width of 200 μm and a depth of 50 μm. The round inlet and outlet have a diameter of 1.5 mm. The total channel volume is ~0.1 μl. The middle of the channel consists of vertical columns (17 μm wide, 10 μm length, 40 μm depth) separated by 5 μm spaces, acting as a trapping structure (Fig. 1). The structures were made in PDMS (Sylgard 184, Dow Corning). Curing and base agent were mixed at a ratio of 1:10 for 15 min and degassed. PDMS was poured on the FDTS-coated patterned silicon wafer, degassed, and cured at 60° for 24 h.

After curing, holes were punctured in the PDMS, creating an in- and outlet. The PDMS chip was sealed onto a slide of Pyrex glass (1×2 cm) using oxygen plasma. Therefore, the PDMS and Pyrex glass were cleaned with acetone and ethanol. The surfaces were placed in the plasma oxygenator (Harrick PDC001) for 4 min at an RF of 29.6 W and a pressure of 400 mTorr (high). After sealing, phosphate buffer saline (PBS) was introduced into the chip to preserve the hydrophilicity of the PDMS and facilitate cell loading.

2.1.2 Chip 2

Chip 2 consists of a main channel, which broadens into a chamber for cell culture. The inlet and outlet channel have a width of 1.5 mm and a depth of 44 μm. The cell culture chamber has a length of 10 mm, a width of 5 mm and a depth of 44 μm. The cell culture chamber includes an array of pillars to increase the stability of the chamber. Furthermore, chip 2 consists of two secondary inlets for, for example, the optional addition of versene or fluorescent dyes. The total volume is 4.4 μl (Fig. 2(a)). The structures were made in PDMS and sealed onto a microscope slide as described above (Fig. 2(b)). After sealing, phosphate buffer saline (PBS) was introduced into the chip to preserve the hydrophilicity of the PDMS and facilitate cell loading.

2.2 Cell culture

The estrogen receptor positive human invasive lobular carcinoma cell line MCF-7 was purchased from DSMZ (Braunschweig, Germany). Cells were grown in Roswell Park Memorial Institute (RPMI)-1640 medium, supplemented with 10% (v/v) fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.4 μg/ml fungizone (RPMI$^+$ medium). Media and supplements were all obtained from Invitrogen (Grand Island, NY, USA). Cell cultures were sustained in a 5% CO_2 humidified atmosphere at 37°C. The medium was refreshed every 3–4 days and cultures were split weekly at a ratio of 1:3–1:6 after treatment with versene in distilled water. Versene consists of distilled water with 137 mM NaCl, 1.47 mM KH_2PO_4, 2.68 mM KCl, 7.37 mM $Na_2HPO_4·2H_2O$ and 0.54 mM NA_2EDTA dissolved. In experiments, MCF-7 cells which had satiated the conventional culture flasks (>80% confluence) were used for cell loading.

2.3 Experimental set-up

2.3.1 Chip 1

Cell loading was accomplished by pipetting cells in the inlet. Hydrostatic forces moved the cells into the channels and cells were trapped at the trapping site. The chip was put in a Petri dish and covered with RPMI$^+$ medium to prevent further flow. The chip was placed back in the incubator to analyze long-term cell culture. For this, cells were cultured for 1, 2, 3, 5 and 7 days, without changing the medium. For every time point a separate chip was used. Viability was assessed by staining the cells in the chip with 2 μg/ml

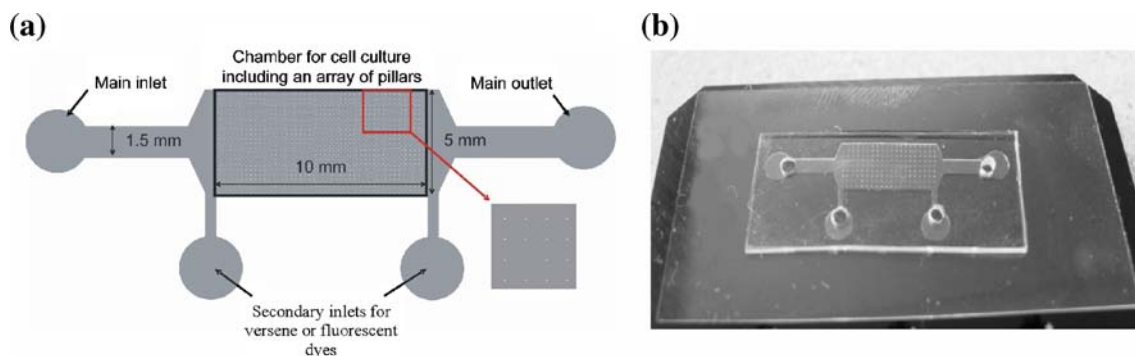**Fig. 1** Schematic drawing of PDMS chip 1
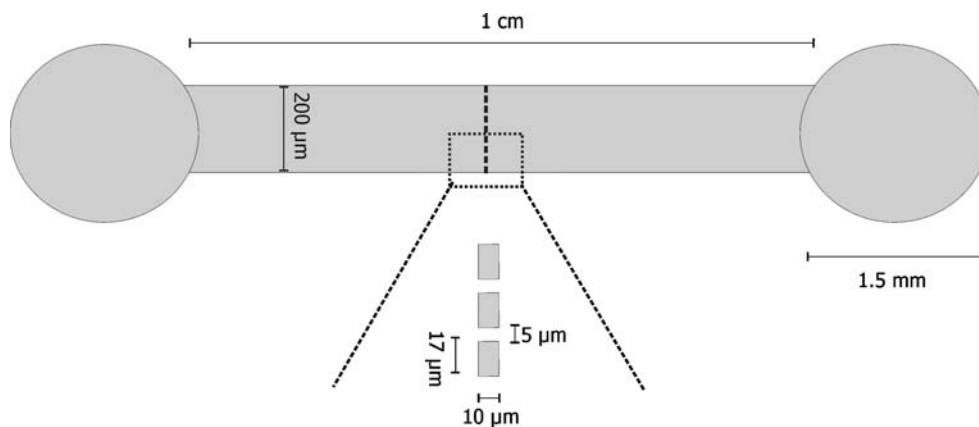
Fig. 2 Schematic drawing of PDMS chip 2 (**a**) and picture of the final chip design in PDMS sealed onto a microscope slide (**b**). Courtesy S. Le Gac 2007

Calcein-AM (CAAM; Molecular Probes Invitrogen) and 10 µg/ml Propidium Iodide (PI; Sigma, St. Louis, MO, USA). Cell viability was obtained optically, checking cell morphology with light microscopy and fluorescence with a mercury lamp.

2.3.2 Chip 2

Cells were introduced by pipetting 20 µl of 4×10^6 cells/ml in the inlet. Cells dispersed evenly throughout the chip by hydrostatic forces. The chip was put in a Petri dish, covered with RPMI$^+$ medium and placed in the incubator for 2 days. The medium in the chip and in the Petri dish were refreshed after 1 day. After 2 days, flow was applied. In the main inlet, a pipette tip (Finntip 200Ext 5–200 µl; Thermo Electron Corporation, Vantaa, Finland) was punctured, in which a PA-6 (inner Ø 0.25 mm and outer Ø 0.75 mm; Liquid scan) tube was put to connect it to a BD plastic syringe (Becton Dickinson, Franklin Lanes, NJ, USA) via a BD Microlance needle (30 G × 1/2 in.; 0.3×13 mm, yellow). Flow was applied by a KdScientific 101 syringe pump at a flow rate of 0.5 µl/min. The outlet and secondary outlets were left open. Flow was applied for 30 min. Apoptosis was induced by 50 µM staurosporine (SSP; Sigma). RPMI$^+$ medium served as a control. After 30 min of flow, the chip was disconnected from the flow system and put in a Petri dish fully covered with RPMI+ medium and placed in the incubator for 24 h.

After 24 h, the chip was connected to the flow system to flow a solution of 2 µg/ml CAAM and 10 µg/ml PI through the chip at a flow rate of 0.5 µl/min for 30 min. Cell viability was obtained optically, checking cell morphology with light microscopy and fluorescence with a mercury lamp.

2.4 Microscope system

The microfluidic chip was mounted on to an *X–Y–Z* translation stage of an inverted wide fluorescence microscope (Leica DM IRM, Leica Microsystems, Wetzlar,

GmbH, Germany). The microscope is equipped with a mercury lamp as an excitation source for fluorescence measurements, 5×, 10×, 20×, 50×, 63× objectives, and different fluorescent cubes, of which the BGR filter cube was used in these experiments (excitation BP 420/30 and emission BP 465/20 [blue]; excitation BP 495/15 and emission BP 530/30 [green]; excitation BP 570/20 and emission BP 640/40 [red]). In addition, a computer-controlled CCD camera (Leica DFC300 FX) is mounted on to the microscope for image recording, using the accompanied Leica Application Suite Software (version 2.3.4 R2).

3 Results

3.1 Chip 1

For the culture of adherent cells in a microfluidic device, cells must be able to adhere to the bottom of the chip. PDMS is non-toxic and MCF-7 cells adhered optically. However when the PDMS was rinsed the cells flushed away. Coating with gelatin 1% or BSA 3% did not improve this. Possibly other coating agents would improve adherence. Nonetheless Pyrex glass was used for the cells to adhere (bottom of the chip), since MCF-7 cells adhere to Pyrex glass without the need of coating. Structures (upper part of the chip) were made in PDMS because of its fabrication properties and oxygen permeability.

Chip 1 consists of a cell trap, which promotes the entrapment of MCF-7 cells in the middle of the channel (Fig. 1). In this way, the amount of cells which could adhere to the Pyrex glass, increased. Thus, the immediate flow of cells from the inlet directly to the outlet was prevented. By loading cells into the vertical inlet, it was expected, due to the hydrophilicity of the chip, that medium including cells would easily flow through the channel because of hydrostatic forces. However, only part of the loaded cells flew into the channel, others sank to the bottom

of the inlet. This could be caused by the relatively small size of the channel compared to the size of the inlet, the used cell concentration or the stickiness of the cells. The used cell concentration is convenient for the chip dimensions of chip 1. MCF-7 cells are adherent epithelial-like cells which grow in colonies, forming aggregates, as it is the nature of these tumor cells. However, MCF-7 cells need to be in suspension to transfer these cells into the chip. This might account for the stickiness, and together with the narrow channel, complicate the entrance into the channel. In this way, a natural selection could occur due to differences in stickiness. If stickiness is related to cell viability, this could confound the viability analysis. In our case, MCF-7 cells, cultured in a conventional culture flask, were detached with versene, and immediately loaded in the chip, to minimize the effect on the cell viability. Resuspending did not yield extra cells in the channel and possibly damages the cells mechanically.

The MCF-7 cells (size≈10 µm) that entered the channel were mechanically trapped (Fig. 3(a)). Although, the space between the pillars is only 5 µm, cells were still able to pass the cell trap. Moreover, the cells could flow underneath the cell trap because the pillars have a height of 40 µm, in comparison to the channel which has a depth of 50 µm. Advantageously, the flow of medium was not obstructed by the trapped MCF-7 cells, however, when the MCF-7 cells formed a few rows in the cell trap the flow diminished. At the cell trap, the round cells were compressed at a high density, but during cell culture, the cells attached to the Pyrex glass, retaining their epithelial-like morphology and migrating away from the trap (Fig. 3(b) and (c)). Cell culture was performed under static conditions on the microscope stage at 37°C (thermoplate), using specific cell culture medium, i.e., CO_2 independent medium (Invitrogen), which is a non-HEPES proprietary medium suitable for supporting cell growth under atmospheric conditions.

Furthermore, the viability of long-term cell culture of MCF-7 cells was analyzed. In order to bring cells in their exponentially growing phase (doubling time±50 h), cells need to be cultured for 2–3 days before starting apoptosis experiments. MCF-7 cells were trapped in chip 1 and cultured in a conventional incubator. Cell viability was checked after 1, 2, 3, 5 and 7 days using the life-stain Calcein-AM (CAAM) and the death dye propidium iodide (PI; Fig. 4). Viable cells stained green and dead cells red. Medium in the channel was not changed, and for every time point a separate chip was used, as fluorescent dyes are toxic for cells. As demonstrated in Fig. 4, the majority of the MCF-7 cells were viable, showing green CAAM staining and negligible red PI fluorescence. It was observed that MCF-7 cells migrated away from the trap in time, forming aggregates in the channel, and because of this clustered cell growth, quantifying the viability of the cells was difficult. Nevertheless, plotting the course of the overall green (CAAM), red (PI) and total fluorescent intensity in time provides a good indication of the viability. The plot of Fig. 4 shows an increase in the green fluorescence, defining that the cell number has increased (proliferation). The red fluorescence increases only slightly, indicating that some cells have died, though the number is similar to what apply for conventional cell culture. However, these viability results maybe somewhat positively skewed because the introduction of the fluorescent dyes in the chip induced a flow, resulting in lost of detached (including apoptotic bodies) or loose cells that flew towards the outlet. Moreover, due to the use of fluorescent dyes it was necessary to use a different chip for every individual time point. Nevertheless, the same pool and concentration of MCF-7 cells was used to perform the 7-days viability experiments. Hence, it can be assumed that cells survived and proliferated, since they were CAAM positive (increase in fluorescent intensity in time) and confluence increased, as shown in Fig. 4.

The viability experiments as described above were performed without any medium change. For growth and viability maintenance, MCF-7 cells were dependent on the nutrients present in the medium in the channel and the diffusion of nutrients through the inlet in the channel and the diffusion of gases through the PDMS. The thickness of
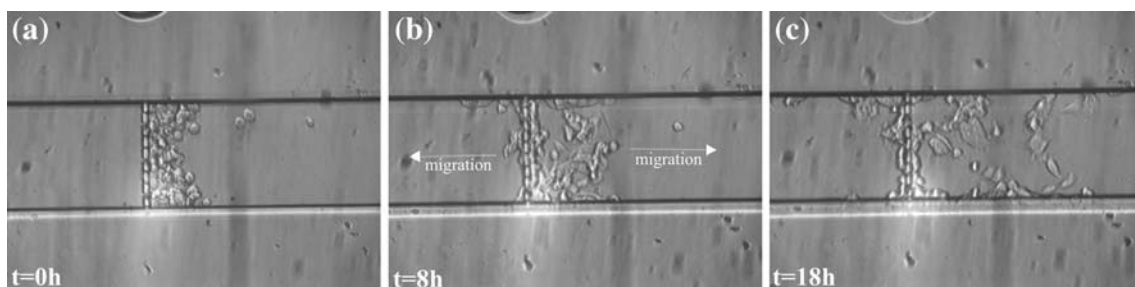

Fig. 3 Static culture of MCF-7 cells in chip 1. Suspended MCF-7 cells were loaded in the chip and the cells which flew in the channel were trapped at the cell trap (**a**). Cell culture was performed on the microscope stage at 37°C (thermoplate) using specific cell culture medium, i.e. CO_2 independent medium. The round MCF-7 cells attached to the Pyrex glass, retrieving their epithelial-like morphology (**b**), and migrated away from the trap (**b** *white arrows* and **c**). Magnification is ×10
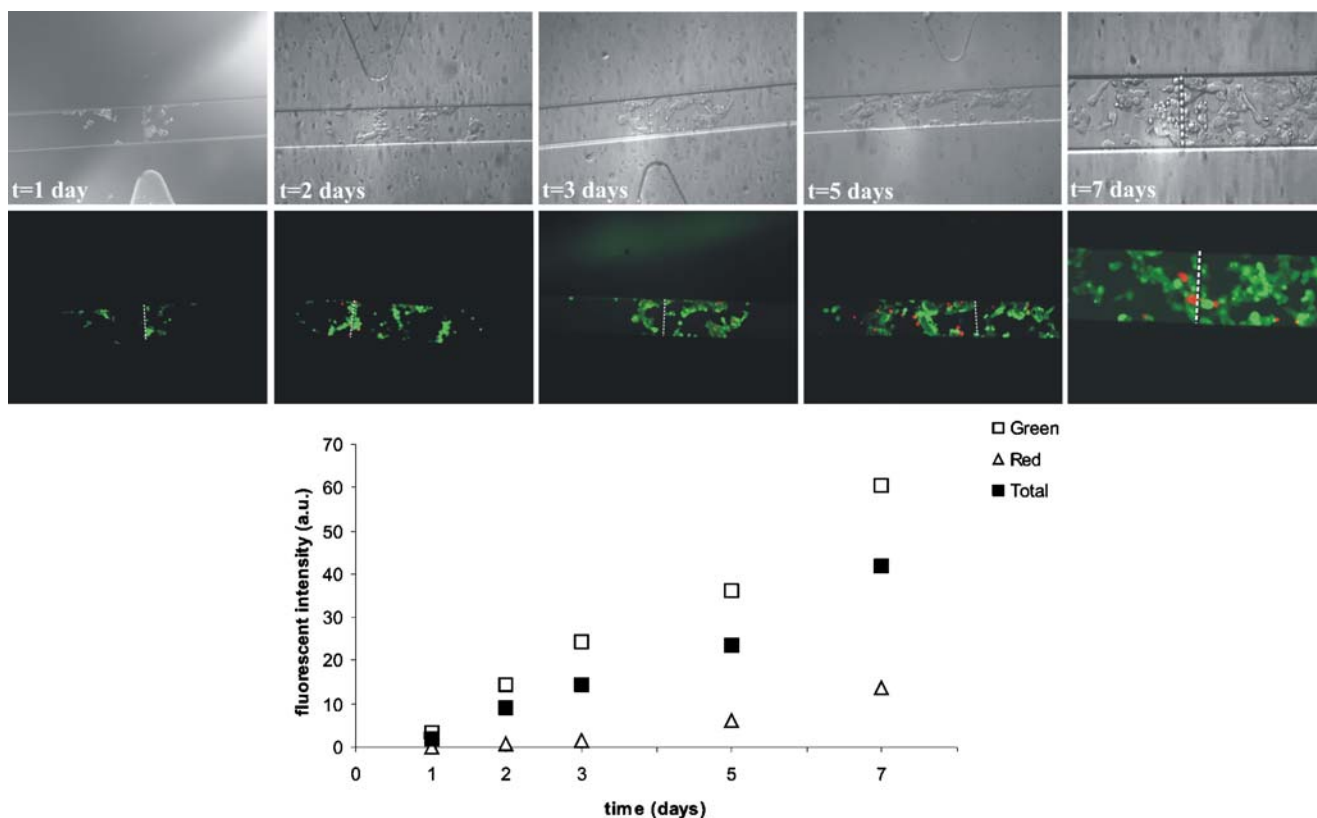
Fig. 4 Long-term static cell culture of MCF-7 cells in chip 1. Viability was assessed using the life-stain Calcein-AM (CAAM; *green*) and the death dye propidium iodide (PI; *red*). LM pictures with underneath the corresponding fluorescent pictures are shown for the different time periods. Magnification of the first four pictures *(t=1 day till t=5 days)* is ×10 and for the last picture *(t=7 days)* is ×20. Plot below the pictures shows the course of the overall *green* (CAAM), *red* (PI) and total fluorescent intensity during the 7 days of culture. Note that the picture of *t=7 days* is taken with a higher magnification than the pictures of *t=1 day* till *t=5 days*. This also accounts for the increase in the fluorescent intensity at *t=7 days*

the PDMS (0.1–1.0 mm) and the distance from the trap to the inlet and outlet did not have any effect on cell viability inside the channel. It could be observed that cell growth was favored at spots where the cells were in direct contact with the medium and had no PDMS on top (Fig. 5).

To conduct apoptosis experiments, a controlled flow was required to (continuously) introduce the apoptotic inducer in the microfluidic chip. Therefore, pipette tips were chosen because of the simplicity and the disposability. Unfortunately, it was not possible to use pipette tips to apply a controlled flow in chip 1, because puncturing a pipette tip in the inlet caused a sudden, strong flow (which detached the cells) as a result of the large volume of the pipette tip in comparison with the channel volume. To reduce this sudden strong flow, a new chip was developed (chip 2), with dimensions which enabled the connection to the flow system with use of pipette tips.

3.2 Chip 2

As shown in Fig. 2, chip 2 consists of a larger surface area to culture cells. Due to widening of the channel, cell

loading was improved. In this case, MCF-7 cells were loaded at a higher concentration (20 μl 4×10^6 cells/ml), since the surface is larger and there is no cell trap. The cells were introduced into the chip with a pipette tip and easily dispersed over the surface. In this chip design, the rapid deceleration and compression of cells, which did occur at the cell trap in chip 1, was prevented. After 2 days in the incubator for cell culture, chip 2 was connected to the flow system. This also caused a displacement of liquid in the chip, however, because of the larger dimensions, the MCF-7 cells did not detach. A flow of 0.5 μl/min for 30 min was applied, either containing RPMI$^+$ medium for the control group, or 50 μM staurosporine (dissolved in RPMI$^+$ medium) for the apoptosis group. To prevent cellular disfunction and preserve the viability of MCF-7 cells under flow conditions, the duration of the flow was kept short and the flow rate as low as possible to have a minimal effect of the shear stress ($\ll 0.5$ dyn/cm^2), but ensure that all cells have been in contact with the apoptotic inducer. Due to this short period, the use of specific CO_2 independent medium was not necessary. After 24 h, the viability of the MCF-7 cells was checked with CAAM and PI. Figure 6 shows that
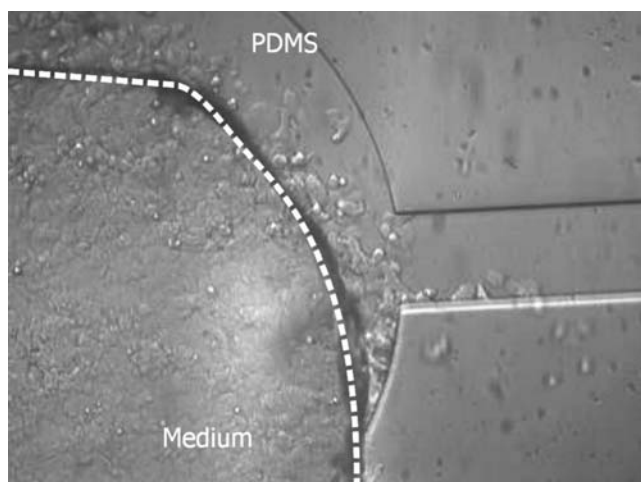
Fig. 5 LM picture of the cell growth of MCF-7 cells at the inlet at day 5. Cell growth was better at the medium side, forming confluent cell cultures, whereas the cell growth under the PDMS was less pronounced. Magnification is ×20. The dotted line shows the dividing line between cell cultured under the PDMS or in direct contact with the medium

the majority of the MCF-7 cells in the control group (Fig. 6(a)) maintained an aggregate-like cell pattern, appearing in colonies. Most of the MCF-7 cells in the control group were viable, as shown by the green CAAM staining. However, some PI positive (red) cells could be observed, present as small red dots surrounding the (green) colony. In conventional cell culture, the amount of apoptosis is normally between 5% and 10%, comparable with the results shown in Fig. 6(a). When MCF-7 cells were subjected to a flow with SSP, the aggregate-like phenotype disappeared (the size of the colonies decreased), and cells were predominantly present in small clusters. Incubation with SSP demonstrated more PI positive cells (Fig. 6(b)) as compared to the control group (Fig. 6(a)). Plotting the green (CAAM) vs. the red (PI) fluorescent intensity (Fig. 7) shows that the addition of SSP to MCF-7 cells decreased this green:red ratio (control 0.74 ± 0.19 vs. SSP 0.47 ± 0.15), which occurred due to a decrease in the fluorescence of the life-stain and an increase in the fluorescence of the death dye. Thus, SSP induced apoptosis in MCF-7 cells. Even more, this effect is probably even larger, because due to the applied flow to add the fluorescent dyes, part of the dead cells washed away.

### 3.3 Discussion

3.4 Goals and obstacles

Matching cancer patients to the appropriate chemotherapeutics would increase the effectiveness of these pharmaceuticals. Microfluidic devices have the potential to aid in this process. For microfluidic devices to fulfill their potential multiple obstacles must be taken. For example, cells obtained via biopsy have to survive in the microfluidic device and the obtained results have to be extrapolated to the clinical situation. This paper described the first steps towards a microfluidic device which enables chemosensitivity testing of breast cancer cells to improve treatment. The requirements for such assays were analyzed in two different PDMS chips. The first design was used to explore the cell culture properties of MCF-7 cells in the microfluidic device and the second design to test the chemosensitivity in response to the apoptotic agent staurosporine. However, it should be stated that the microfluidic device and experimental set-up as described in this paper is not specific for breast cancer, but applicable for other (cancer) cell lines and any other drug of interest to fine-tune multiple therapies and the treatment of diseases, in which specifically the process of apoptosis is suppressed or enhanced.

3.5 Chip design and cell culture

In apoptosis assays it is essential that the chip design and materials used create a microfluidic environment that meets cellular needs and sustains cellular viability (Wolbers et al. 2006). MCF-7 cells have been cultured in microfluidic devices, either in a 3D-cone structure (Torisawa et al. 2005) or a channel device to which continuous flow was applied (Neville et al. 2007). In our microfluidic PDMS-glass device type 1, cell viability was maintained for up to 7 days in a static cell culture fashion in the incubator. It is described that the thickness of the PDMS has an influence on the permeation of gasses such as O_2 and CO_2 (Leclerc et al. 2003), however, in our experiments PDMS with a thickness range in between 100 μm to several mm showed to have no influence on the cell viability (data not shown). Cell survival is also dependent on the quality of the available medium. A higher concentration of cells will exhaust the medium faster, however, cell–cell contacts are necessary for survival (Yu et al. 2005). During long-term cell culture no flow was applied in the microchannel. This could have had an adverse effect on cell survival and proliferation because of acidification of the medium and depletion of nutrients (Zhu et al. 2004). However, Fig. 4 shows that cells managed to survive and proliferated in the medium present in the chip for up to 7 days. Although observed in a different cell line and in a microchannel with dimensions in the mm range, the relationship between the microchannel geometry and cell proliferation was explored (Yu et al. 2005). It was described that cells cultured in microchannels in the absence of flow proliferated significantly more slowly than cells cultured in conventional culture flasks, and entered a quiescent state. However, this inhibited cell proliferation disappeared when cells returned to a conventional culture flask. A possible explanation for

Fig. 6 MCF-7 cells cultured in chip 2. MCF-7 cells were incubated with 50 μM staurosporine (SSP) under a continuous flow of 30 min (**b**). MCF-7 cells flown with RPMI$^+$ medium served as a control (**a**). Thereafter, MCF-7 cells were put in a Petri dish and placed in the incubator for 24 h. After 24 h, the chip was connected again to the flow system to flow CAAM (*green*) and PI (*red*), to check the viability. This figure shows the LM pictures after fluorescent staining, with underneath the corresponding fluorescent pictures. *White squares* and *circles* highlight the pillars in the chip. Magnification is ×20

(a) Control (RPMI$^+$ medium)

(b) Staurosporine
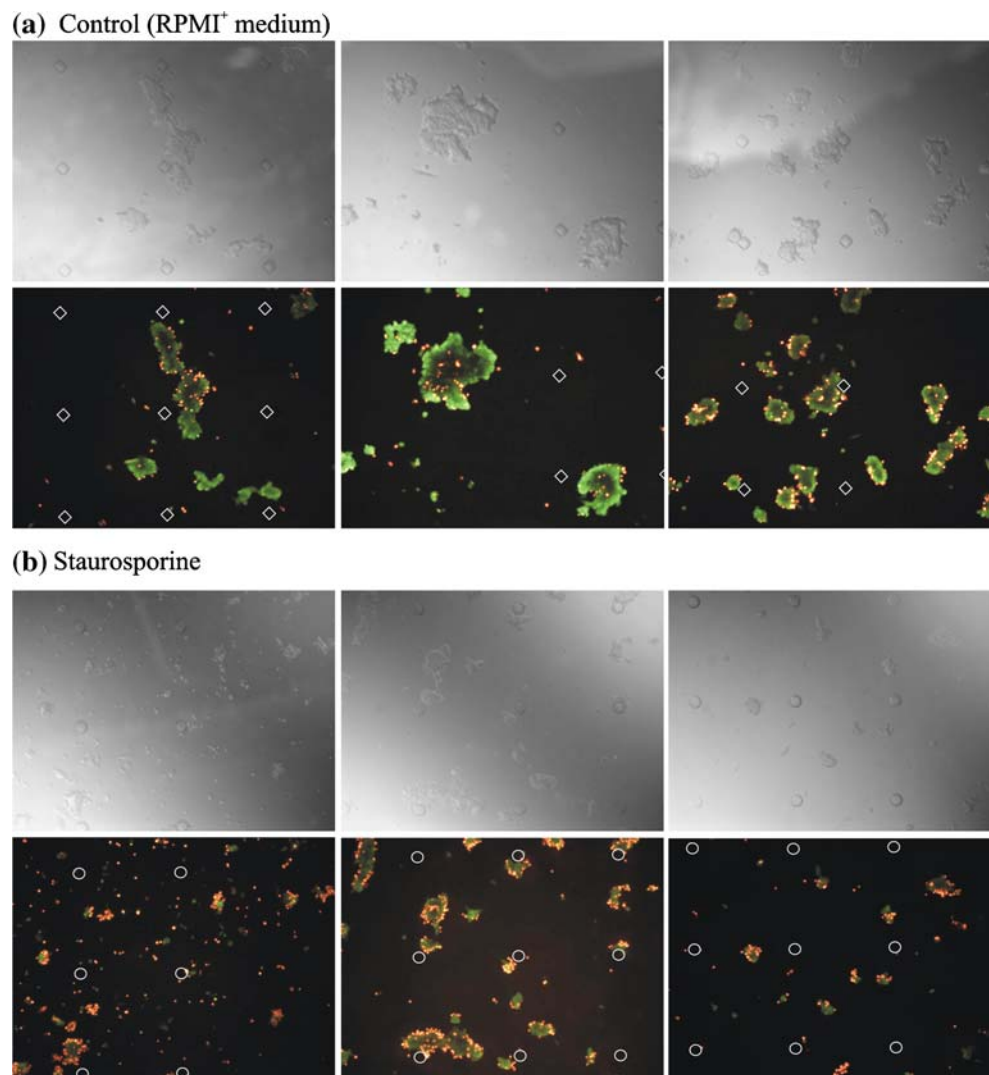
the inhibited proliferation is that (cell-secreted) functional molecules surround the cells, enclosing the cell in a special microenvironment. Figure 5 of this paper also showed that cells grew better under a ceiling of only medium (in the punctured part) than under the ceiling of medium and PDMS. This suggests that the culture conditions change and even possibly deteriorate rapidly when under a PDMS ceiling, because of the lack of diffusion, causing the cells not to die, but entering a quiescent state.

Applying a continuous flow is a possible solution to provide the cells with the appropriate amount of nutrients and oxygen, and to remove waste products (Hung et al. 2005). However, the applied flow rate will expose the cells to mechanical forces, such as the shear stress. The maximum amount of shear stress that can be endured by cells depends on the cell type, since certain cells (e.g., endothelial cells) require shear stress for their development, whereas other cell types are negatively affected by shear

stress (such as chondrocytes; Li et al. 2005; Healy et al. 2005; Kim et al. 2007). The optimal flow rate is therefore different for every cell type and chip design, depending on the chip dimensions. MCF-7 cells are from an epithelial origin, hence they do not require a (high) shear stress for development. In this case, the optimal flow rate needs to provide a good balance between on the one hand favorable medium conditions and on the other hand a low as possible shear stress. As stated in the results section, a flow of 0.5 μl/min was applied in chip 2, resulting in a shear stress of 0.17 dyn/cm^2 in the inlet and outlet channel. The shear stress in the cell culture chamber is even lower, due to broadening of the channel and the presence of micropillars. This flow rate did not affect the cellular viability, though it could be observed that after 60 min of flow, the characteristic colony-forming growth pattern diminished and individual cells could be identified (data not shown). Applying a continuous flow could introduce a confounding

Fig. 7 Plot of the overall green (CAAM) vs. the red (PI) fluorescence of the individual pictures shown in Fig. 6: *left*, *middle* and *right* (*small squares*), respectively, for the control (*empty squares*) and staurosporine (*SSP*; *filled squares*) treated MCF-7 cells. Average (*big square*) defines the mean of the measured green:red fluorescent intensities of the three individual pictures (*small squares*), with the accompanied SD
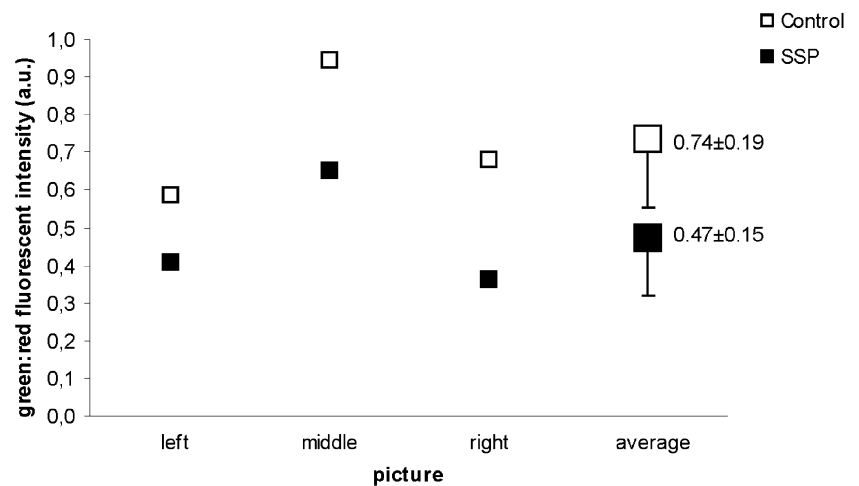
variable in the apoptosis assays. To prevent this, the shear stress needs to be reduced by further decreasing the flow rate, changing the chip dimensions, or applying flow in intervals. In our experiments flow was applied for only 30 min, which preserved the cellular viability (Fig. 6).

3.6 Apoptosis induction

To analyze the chemosensitivity of MCF-cells apoptosis assays were performed. Staurosporine induced apoptosis in MCF-7 cells, exhibiting a diminished aggregate-like phenotype and PI positivity (Fig. 6). Morphology and fluorescence analysis are widely used in apoptosis assays. For an apoptosis assay to be useful the assay has to be controllable and apoptosis rates must be quantifiable and repeatable. Quantification of apoptosis in adherent cells that grow in colonies and maybe even in layers is only indicative when solely relying on optical methods, and therefore it is better to implement the measurement of electrophysiological parameters, such as pH, pO_2 and impedance measurements (Otto et al. 2003). Furthermore, this aggregate-like phenotype complicates the interaction of the drug with all cells separately, hence probably not every cell was exposed to the same drug concentration during the 30 min of flow and the subsequent 24 h incubation. Moreover, the cells that in the final stage of the apoptotic process have detached from the surface and flow towards the outlet have to be counted in. In the experiments presented here, these detached cells were not included, so the amount of apoptosis observed might be an underestimation, for not only the untreated cells, but especially the SSP-treated cells. The chemosensitivity results obtained in the microfluidic chip are difficult to compare with standard conventional assays, such as flow cytometry, due to differences in experimental settings. The chosen staurosporine concentration used in our experiments was

substantial higher than described in literature, in order to assure quick apoptotic effects in our caspase-3 negative MCF-7 cells (Mooney et al. 2002; Xue et al. 2003; Sayeed et al. 2007). The implementation of a dose-gradient generator in future chip designs will enable dose–response analysis (Thompson et al. 2004).

3.7 External validity and future plans

If eventually the chip as described here is optimized to serve as a solid apoptosis assay to analyze drug responses, it will still be doubtful if *in vivo* cancer sensitivity to a certain chemotherapeutic agent can be predicted by *in vitro* testing. It is impossible to copy *in vivo* circumstances to the *in vitro* situation. However the quality of the model depends on predicting chemosensitivity, not on the resemblance between the *in vitro* and the *in vivo* environment. For testing chemosensitivity, apoptosis assays were chosen since apoptosis is the objective and most downstream effect of chemotherapy. DNA microarrays have less requirements regarding cell handling, nevertheless are more upstream. The uncertain link between genetics and apoptosis in response to certain chemotherapeutics introduces more variability. Breast tumors are heterogeneous and the cells of a single tumor could be heterogeneous because of tissue conditions (ischemia and necrosis) (Stingl and Caldas 2007). Therefore, it is essential to use a patients' own (tumor) cells obtained via biopsy and perform experiments in an *ex vivo* setting. For high-throughput analysis, the obtained cells can be subdivided into numerous samples to test various concentrations (dose-gradient generator) and combinations of different chemotherapeutics. The objective of such a high-throughput analysis would be to rank different available chemotherapeutics based on their effectiveness for the specific tumor of a specific patient. In this way patient

survival can be increased and costs decreased. Specific for breast cancer patients, such an assay could be used for example to decide between a cyclophosphamide-methotrexate-5-fluorouracil (CMF) treatment or an anthracyclin containing agent. For this, a detailed validation of the chip in a clinical setting is greatly required.

Our future plans are first focused on optimizing the experimental set-up and be able to quantify apoptosis percentages, which is possible in conventional flow cytometry analyses (Wolbers et al. 2004a, b) and in multiparametric tests on chip (Gouaze et al. 2002; Mooney et al. 2002; Otto et al. 2003). Quantifying apoptosis makes it easier to correlate the obtained values with results published in literature, and undertake steps towards the clinic. This will increase the chemotherapeutic effectiveness and improve personal heath care. Although there is an increasing knowledge about the different chemotherapeutics and intracellular pathways, in our opinion one of the reasons for lack of chemotherapeutic effectiveness arise from the fact that there are hardly any predictive tests to match patients to chemotherapeutics.

4 Conclusion

In this paper the development towards a microfluidic chip which enables the cultivation of cells and the induction of apoptosis using a flow system is described. We found that MCF-7 cells could be cultivated statically for up to 7 days in a microfluidic PDMS-Pyrex chip. Upon induction of apoptosis with SSP, the characteristic aggregate-like phenotype disappeared, and MCF-7 cells were predominantly present in small PI positive clusters. Compared to untreated MCF-7 cells, SSP diminished the ratio of the fluorescence of the life-stain CAAM vs. the fluorescence of the death dye PI with almost a factor 2, indicating that apoptosis is induced. Next steps are focused on validating this device using clinically relevant hormone and chemotherapeutic agents in combination with patients' tumor cells, to prove the importance of such a device for the clinic.

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