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ORIGINAL PAPER

Biosensor incorporating cell barrier architectures on ion selective electrodes for early screening of cancer

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Abstract Angiogenesis occurs during the early phase of cancer. Recruitment of new blood vessels by existing cancer cells leads to the release of higher concentrations of cytokines as compared to cells in healthy individuals. Some of the common cytokines observed at higher concentrations, such as vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor and tumor necrosis factor-alpha, are also known to induce increased permeability across an endothelial cell monolayer. A whole-cell-based biosensor has been developed that can detect the presence of small quantities of the abovementioned cytokines individually and in different combinations. It was observed that the biosensor could differentiate between the cytokine concentrations observed in the sera of healthy individuals and cancer patients. The biosensor was also evaluated by exposing it to actual serum. These results demonstrated that the sensor can distinguish between healthy individuals and cancer patients and that the corresponding biosensor responses correlate with the stages of cancer.

Keywords Screening cancer · Angiogenesis · Cytokines · Cell-based biosensor · Ion-selective electrode

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Introduction

Millions of people die every year due to cancer all over the world, making it the second leading cause of death after heart disease. A major contributor to the large numbers of deaths is the absence of quick screening tools for cancer; in major cases, cancer is not diagnosed until the later stages of the disease, which reduces the chances of successful therapy. According to American Cancer Society, early screening of cancer can reduce cancer-related deaths by 50% both by diagnosing an individual at the early stages of cancer and by detecting individuals that show an inclination to develop cancer. As a matter of fact, mammography screening has contributed to a significant decrease in deaths of women suffering from breast cancer [1]. Moreover, recent years have witnessed profound decreases in mortality associated with uterine cancer because of the increased amount of screening using the Papanicolaou (Pap) test [2]. Besides specificity and sensitivity, another factor that plays a critical role when screening for cancer is the willingness of people to participate in a screening program. Increased participation is generally observed in tests which are easy to perform and safe as compared to complicated tests or tests which are uncomfortable and painful [3]. According to the American Cancer Society, while 79% of American women eighteen years of age or older undergo PAP tests and 70% of women older than forty years old undergo mammogram examination, only 40% of American women and men aged fifty years old or more take part in endoscopic screening, and only 20% participate in the fecal occult blood test (FOBT). Hence, recent research has been directed towards developing noninvasive techniques for the early detection of cancer.

The simplicity associated with the collection of blood samples has facilitated the development of diagnostic assays using serum or plasma biomarkers. One such method is the

detection of circulating tumor cells (CTC) in the peripheral blood of cancer patients. Studies have demonstrated that the detection of CTCs can be correlated with disease progression and therapeutic response [4–7]. One major problem associated with this method is the discrepancies associated with the detection rates, which in turn may be due to the differences in the methods used for enrichment and detection of CTCs. Another noninvasive method of cancer detection involves detection of DNA in blood samples [8, 9]. Studies have demonstrated the detection of genetic alterations such as mutations in the oncogenes and tumor suppressor genes, alterations in loss of heterozygosity (LOH), and the presence of microsatellite instability (MI) in tumors in the circulating DNA [10–13]. Another simpler approach involves the detection of the total DNA concentration in plasma. Studies have reported an increase in the total DNA concentration in plasma from cancer patients as compared to healthy individuals [14]. However, posttranslational modifications, which may be important in oncogenesis, cannot be detected by this method of cancer detection [15]. Since cancer is associated with altered expression of proteins and signaling pathways and eventually with altered levels of protein secretion in blood, detecting proteins in blood is a popular method of screening for cancer [15–19]. Some of the more common and successfully used biomarkers include prostate-specific antigen (PSA), cancer antigen 125 (CA-125) and carcinoembryonic antigen (CEA), mostly for prostate, breast and colorectal cancer. The detection and validation of these protein biomarkers depend mostly on traditional immunoassay methods such as ELISA or protein microarrays. These methods can be used to assay multiple proteins simultaneously and require very small sample volumes. However, protein arrays have several limitations, such as i) protein immobilization, ii) protein instability and denaturation, iii) the need for highly specific antibodies, and iv) problems associated with nonspecific binding [20].

One biological characteristic common to most cancers irrespective of origin is angiogenesis. To sustain a size beyond 2–3 mm and to progress from the benign to the malignant state, tumors must recruit blood vessels from existing vasculature [21, 22]. This in turn facilitates the metastasis or the spread of cancer [23]. Research has suggested that angiogenesis occurs during the premalignant phase of tumor growth, and inhibition leads to dormancy and ultimately death of cancer cells [24, 25]. Angiogenesis is regulated by the balance between pro- and antiangiogenic molecules. Tumor angiogenesis is believed to stem from increased secretion by tumor cells of different angiogenic molecules such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF) and tumor necrosis factor-alpha (TNF-α) [26, 27]. Increased secretion results in elevated levels of these molecules in the sera of cancer patients as compared to healthy individuals [28–39].

Because angiogenesis is not only common to most cancers but also occurs during the premalignant stage of cancer, we hypothesized that any physiological instrument with the ability to measure angiogenesis can act as a screening tool for cancer. A human umbilical vein endothelial cell (HUVEC)-based biosensor has been developed in our laboratory, which takes advantage of endothelial permeability dysfunction to detect small quantities of permeability-modifying agents [40–42]. The biosensor consists of a confluent monolayer of HUVECs seeded across the asymmetric cellulose triacetate (CTA) membrane of an ion-selective electrode (ISE). In the presence of the confluent monolayer, the passage of the marker ion, potassium, to the membrane surface is prevented, resulting in inhibited response from the sensor. When the permeability of the cell monolayer is altered by different permeability-modifying agents, potassium ion reaches the membrane surface, and hence the response of the sensor increases. The response thus obtained can be related to the concentration of these agents [40–42]. Earlier studies have demonstrated that the biosensor responds to the presence of the abovementioned angiogenic molecules, both individually and in combination, at concentrations observed in the sera of cancer patients [43].

The present study is focused on measuring the responses of the sensor when exposed to the sera from healthy individuals and patients of different types and at different stages of cancer in order to explore the possibility that this biosensor could act as a quick screening tool for cancer.

Experimental section

Reagents Cellulose triacetate (CTA) pellets and all chloride salts were purchased from Aldrich (Milwaukee, WI, USA). Valinomycin was obtained from Calbiochem (San Diego, CA, USA). The plasticizer *o*-nitrophenyl octyl ether (NPOE) and the lipophilic salt potassium tetrakis(chlorophenyl) borate (KTClPB) were from Fluka (Ronkonkoma, NY, USA). Carbonyldiimidazole (CDI) and tris(hydroxymethyl) aminomethane (Tris) were from Sigma (St. Louis, MO, USA). Deionized water obtained with a Milli-Q Water Purification System from Millipore (Bedford, MA, USA) was used to prepare the aqueous solutions.

The synthetic RGD peptide sequence (Gly-Arg-Gly-Asp-Ser (GRGDS)) was obtained from Bachem (King of Prussia, PA, USA). Human umbilical vein endothelial cells (HUVECs) obtained from Cambrex Bioscience (East Rutherford, NJ, USA) were cultured in completely supplemented EGM-2 media system, also obtained from Cambrex Bioscience. Trypsin/EDTA, trypsin neutralizing solution (TNS) and

HEPES-buffered saline solution (HBSS) were also from Cambrex Bioscience.

Purified carrier-free $VEGF_{165}$, bFGF, HGF and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). The cytokines were dissolved in 0.1% w/v bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline (PBS) (GIBCO, Grand Island, NY, USA).

Serum samples from healthy individuals and stage III pancreatic cancer patients were obtained from Bioreclamation (Hicksville, NY, USA). All the remaining cancer serum samples, i.e., serum samples from stages 0, I, III and IV of breast cancer patients and stage I of ovarian and lung cancer patients, were obtained from Asterand (Detroit, MI, USA). Information on the ages of the subjects, the presence of steroid receptors (estrogen and progesterone), clinical diagnostics, etc., were obtained from the same sources.

Preparation of asymmetric cellulose triacetate (CTA) membranes Various studies have reported that functional membrane ion-selective electrodes (ISEs) can be prepared from hydrolyzed asymmetric CTA with valinomycin ionophore [44–47]. The hydroxyl groups present on the outer surface of the base layer facilitate further surface modification and biomolecule attachment [44, 46, 48]. The RGD-modified CTA membrane was prepared as described previously [40].

Seeding of cells onto the membranes Following RGD peptide attachment, the membranes were sterilized and were cut and mounted in Philips IS-561 electrode bodies. HUVECs at a cell density of 1×10^5 cells/mL were seeded onto the bottom surface of the membrane. The cells were then incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2 to ensure confluent monolayer formation on the membrane surface. HUVECs up to passage 5 were used for the experiments.

Evaluation of electrode response Following confluent cell monolayer formation, the control membranes were tested for inhibited ion response. The internal reference electrode was Ag/AgCl, and the internal filling solution consisted of 0.01 M KCl. The external reference electrode consisted of a double-junction Ag/AgCl electrode (model 90-02-00 from Orion Research, Inc., Cambridge, MA, USA) with an Orion (90-02-02) internal filling solution and 0.1 M Tris buffer (pH 7.5) in the outer compartment. Potentiometric responses were measured with a four-channel high-impedance amplifier interface (World Precision Instruments, Sarasota, FL, USA) connected to a Model 100 instruNet (Somerville, MA, USA) A/D converter. InstruNet software running on a Macintosh Power PC was used to analyze the data. To obtain the ISE response, a series of incrementally sized sterile KCl aliquots were added to the stirred sterile buffer solution. The measured potential (mV) was plotted against the logarithm of the concentration of the potassium ions in the bulk solution to generate the calibration plots. Overall responses obtained at 0.1 M KCl solution for the membrane with cells were compared with the responses obtained for the membrane alone; 0.1 M KCl was used because it is close to physiological ionic strength.

Earlier studies have demonstrated that the immobilization of RGD peptide on the ISE membrane surface does not give rise to a mass transfer resistance and affects the sensor response minimally [40]. It had also been shown that with the growth of cells, the overall ISE response decreases, eventually leading to almost completely inhibited response upon the formation of a complete confluent monolayer after 24 h [40]. After the confirmation of the inhibited ion response due to the formation of the confluent monolayer, the cell-seeded membranes were exposed to different cytokine solutions containing individual and combinations of cytokines (pH 7.2) in PBS for 1 and 3 h, and the response of the sensor was measured. The responses of the sensor to cytokines at concentrations found in sera of healthy individuals (i.e., 100 pg/mL VEGF, 500 pg/mL HGF, 5 pg/mL bFGF and 10 pg/mL TNF-α) were compared to the responses obtained when exposed to cytokines at concentrations found in the sera of cancer patients (i.e., 1000 pg/mL VEGF, 2000 pg/mL HGF, 40 pg/mL bFGF and 100 pg/mL TNF-α) [30, 33]. The cytokines were dissolved in 0.1% w/v BSA in PBS for these experiments. After confirming that the biosensor is able to differentiate between the different concentrations of cytokines, i.e., the concentrations of cytokines found in the sera of healthy individuals and in cancer patients, the responses were measured by exposing the cell-based biosensor to actual serum samples from healthy individuals and cancer patients at different stages of cancer.

At a final concentration of 0.1 M KCl, the electrode response was measured for the following conditions: (a) the membrane without cells and without cytokines/serum, to obtain the baseline response of the sensor; (b) the membrane with cells and without cytokines/serum, to confirm the inhibited response of the sensor due to the confluent monolayer of cells; (c) the membrane with cells and with cytokines; (d) the membrane without cells and with serum, to test the effect of serum on the membrane of the sensor, and; (e) the membrane with cells and with serum samples.

To account for slight variations between fabricated membranes, the final data are reported as the ratio of the response obtained at 0.1 M KCl for the ISEs with HUVECs and cytokines/serum to the response obtained for the same membrane without HUVECs and cytokines/serum.

Statistical analysis For all experiments, data are reported as mean ± SEM. Statistical analyses were carried out using one-way ANOVA and the Student–Newman–Keuls test for

post hoc comparisons of the means with $P<0.05$. SigmaStat v 3.1 software was used for these analyses.

Results

Biosensor response to the different cytokines in PBS

Earlier studies demonstrated that exposing the cell-based sensor to individual and combined cytokines at concentrations observed in the sera of cancer patients for 1 and 3 h resulted in a significantly increased response from the sensor [43]. In the present study, the biosensor was exposed initially to the cytokines individually and in combination at concentrations observed in the sera of healthy individuals for 1 and 3 h, and the response of the biosensor was compared to the responses obtained when exposing the sensor to cytokines at concentrations found in cancer patients. As shown in Fig. 1a, the biosensor responded to the presence of the individual cytokines at concentrations found in healthy individuals. A response ratio of 0.04 was obtained for the control (cells only). Even though theoretically, under ideal conditions, no response would be expected from the biosensor in the absence of cytokines, the very low response ratio can be attributed to the presence of a few gaps between the cells, even in a confluent cell monolayer. The response of the sensor to the individual cytokines after 1 h exposure time was not significantly different from the value obtained from the control, except in the case of VEGF. This is because, at these low concentrations, the ability of these cytokines to induce gaps in-between the cells is very low. Upon exposing the biosensor to VEGF, HGF and TNF-α individually for 3 h, a response ratio of around 0.1 was obtained, which was significantly different from that obtained for cells only. Upon exposing the cell-based biosensor to the cytokine combinations of VEGF and TNF-α (V+T), VEGF, TNF-α and HGF (V+T+H) and VEGF, and TNF-α and bFGF (V+T+b) for 1 and 3 h, even though an additive response was not obtained, the responses obtained in the presence of combined cytokines were higher than those obtained by exposing the sensor to individual cytokines. Exposing the cell-based sensor to a combination of all four cytokines (V+T+b+H) for 1 and 3 h results in profound increases in the sensor response to 0.12 and 0.17, respectively. The responses thus obtained were significantly different ($p<0.001$) from the responses obtained from cells only.

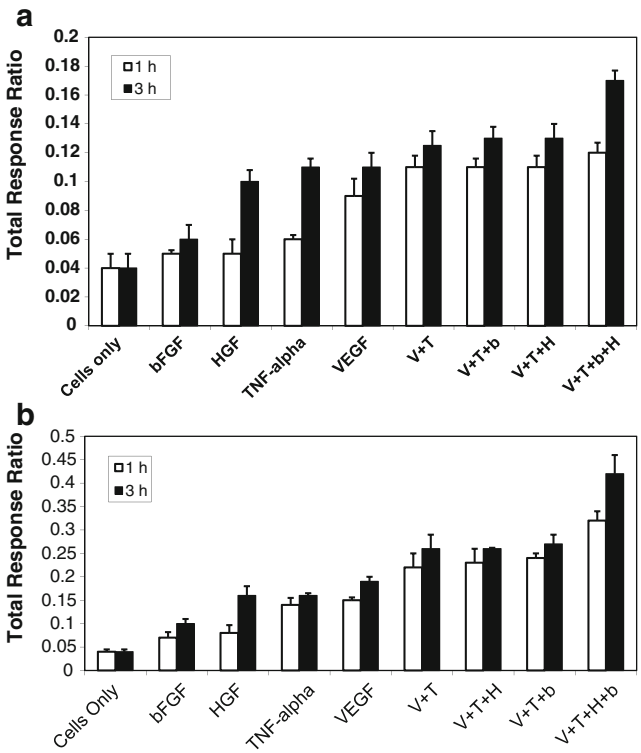

Fig. 1 Comparison of the ISE sensor responses obtained following the exposure of the sensor to individual and combinations of cytokines at concentrations observed in the sera of healthy individuals (**a**) and cancer patients (**b**) for 1 and 3 h. The *total response ratio* is defined as the ratio of the responses of the cell-based sensor with or without cytokines to the ISE response obtained at 0.1 M KCl for the membrane without cells and without cytokines. (Error bars represent SE $N=3$)

Figure 1b shows the responses obtained by exposing the sensor to these cytokines individually and in combination for 1 and 3 h at the concentrations observed in the sera of cancer patients. The responses of the sensor to the individual cytokines were higher than the responses obtained at concentrations corresponding to healthy individuals. For example, exposure of the cell-based biosensor to 1000 pg/mL VEGF for 3 h resulted in a response ratio of 0.19. When the sensor was exposed to 100 pg/mL VEGF for 3 h, a response ratio of 0.11 was obtained. Exposing the sensor to a combination of the cytokines resulted in a profound increase in sensor response compared to the responses obtained from individual cytokines. Furthermore, the responses obtained upon exposing the sensor to a combination of all four cytokines for 1 and 3 h was significantly higher than the responses obtained for the cells only.

Since all of the cytokines are elevated during cancer, it was important to compare the responses of the sensor to the presence of all four cytokines at concentrations corresponding to both healthy individuals and cancer patients. This comparison is illustrated in Fig. 2. As can be seen, the sensor was able to differentiate between the two concentrations at both 1 h and 3 h exposure times ($p<0.05$). Because 1 h of exposure time was sufficient to generate significant differences between the responses at the two different concentrations, it was decided that an exposure time of 1 h would be used for the subsequent serum studies.

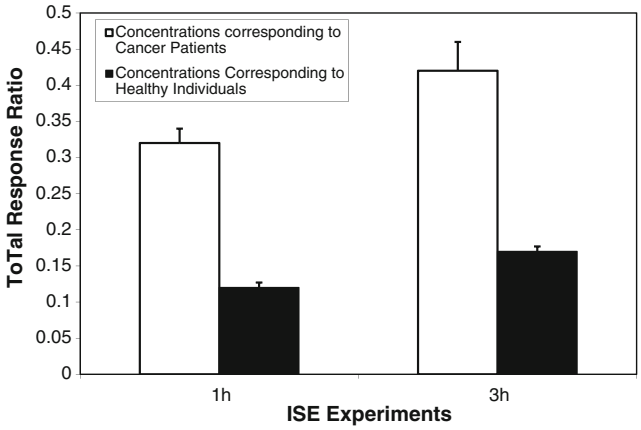

Fig. 2 Comparison of the ISE sensor responses obtained following exposure to the combination of all four cytokines at concentrations observed in the sera of healthy individuals and cancer patients for 1 and 3 hr. *Total response ratio* is defined as the ratio of the response of the cell-based sensor with cytokines to the ISE response obtained at 0.1 M KCl for the membrane without cells and without cytokines. (Error bars represent SE $N=3$)

Biosensor response to the sera of healthy individuals

Prior to exposing the cell-based biosensor to serum samples, it was important to investigate the effect of serum on the membrane of the biosensor. To do this, the sensor without any cells was exposed to the sera of healthy individuals for 1 h. Figure 3 compares the response profiles of the sensor without cells and with and without serum. As seen, the response profiles with and without serum were very similar. When the overall response obtained at 0.1 M KCl for the membrane exposed to the serum for 1 h was compared with that obtained for the control, it was observed that more than 99% of the sensor response was recovered, indicating that the serum does not effect the membrane surface.

Since the average response ratio of 0.12 was obtained by exposing the biosensor to the combination of the maximum concentrations of the four cytokines observed in the sera of healthy individuals, we used this ratio as the maximum cutoff limit for healthy individuals. Next, the cell-based biosensor was exposed to 15 serum samples from healthy individuals for 1 h. Figure 4 demonstrates the responses obtained from the biosensor. A significant difference was observed between the responses to the serum samples from healthy individuals and that of the maximum cutoff limit of 0.12 ($p<0.05$). As shown, the responses obtained for all of the samples were less than 0.12. The responses ranged from 0.06 to 0.11, with a mean of 0.09. No significant differences were observed when comparing gender and age ($p>0.05$).

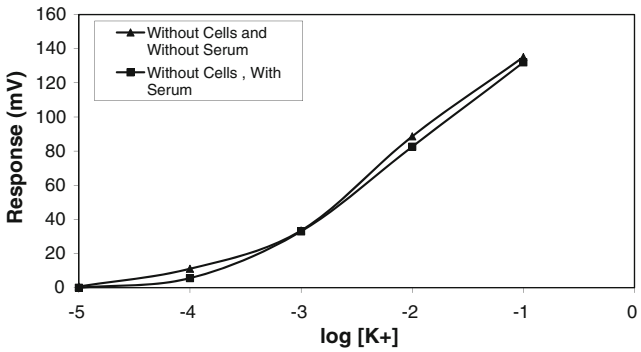

Fig. 3 Response vs. log K^+ for the ISE membranes without cells and without serum and without cells and exposed to serum from healthy individuals for 1 hr. This plot represents one experiment. The experiment was repeated twice more, giving similar results

Biosensor response to the sera of cancer patients

The biosensor was exposed to serum samples from pancreatic, breast, ovarian and liver cancer patients. Figure 5 shows the responses of the sensor to different cancer samples. As observed, in all cases except one, the responses were above the maximum cutoff limit for healthy individuals. The sample for which a lower response was obtained corresponds to a stage 0 breast cancer patient.

Figure 6a illustrates the responses obtained from the biosensor when exposed to the serum samples from stage III breast and pancreatic cancer patients and healthy individuals. When comparing these results, it was observed that the responses for cancer patients were significantly higher than those obtained for healthy individuals as well as from the maximum cutoff limit for healthy individuals ($p<0.05$). Furthermore, no significant differences were obtained between the responses to the sera of stage III cancer patients and the responses obtained by exposing the sensor to the combination of all four cytokines at concentrations observed in the sera of cancer patients ($p>0.05$). Also, no

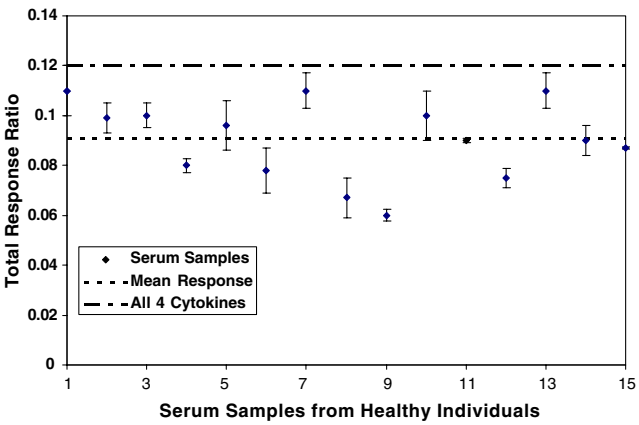

Fig. 4 Response ratios obtained when exposing the sensor to the sera from healthy individuals for 1 h. Serum samples from 15 healthy individuals were used in these experiments and each point represents one individual. Each serum sample was measured three times. *Total response ratio* is defined as the ratio of the response of the cell-based sensor with serum to the ISE response obtained at 0.1 M KCl for the membrane without cells and without serum. (Error bars represent SE)

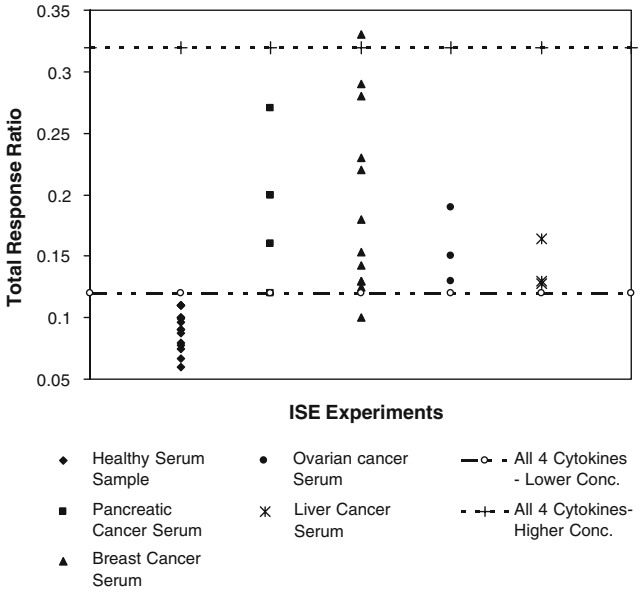

Fig. 5 Comparison between the response ratios obtained when exposing the sensor to the sera of cancer patients for 1 h and the responses obtained from healthy individuals. *Total response ratio* is defined as the ratio of the response of the cell-based sensor with serum to the ISE response obtained at 0.1 M KCl for the membrane without cells and without serum. A total of 23 cancer patients were used in this study, and each point represents one patient. Each serum sample from a cancer patient was measured once

significant difference was obtained between the responses to the sera from patients with different types of cancers. This indicates that the biosensor is able to differentiate between healthy individuals and cancer patients at stage III, but that it does not distinguish between the two cancer types.

To explore the ability of the biosensor to act as an early screening tool for cancer, it was then exposed to the serum samples from stage 0 and I breast cancer patients and the results were compared with those obtained for sera associated with the later stages of breast cancer. As shown in Fig. 6b, the responses obtained from the biosensor were significantly higher when exposed to sera from stage I breast cancer patients than sera from from healthy individuals. When the biosensor was exposed to sera of stage 0 breast cancer patients, the average response obtained was very close to the maximum cutoff limit for healthy individuals (0.13 vs. 0.12) ($p>0.05$). Such an observation can be justified by the fact that while stage 0 cancer—the precancerous state or in situ stage—corresponds to the stage of the tumor when the concentrations of the cytokines in the sera of these patients are not significantly elevated, the maximum cutoff limit of 0.12 corresponds to the responses obtained by exposing the biosensor to the combination of the maximum concentrations of the four cytokines observed in the sera of healthy individuals. However, when comparing these responses to the average responses from the healthy individuals, a significant difference between the two was obtained. The responses obtained at stage 0 and stage I were not significantly different from each other. To further confirm the ability of the biosensor to act as an early screening tool for cancer, the biosensor was exposed to stage I serum samples from ovarian and liver cancer patients and the results were compared to those obtained from sera from stage I breast cancer patients and healthy individuals. As shown in Fig. 6c, significant differences were observed between the responses to sera from cancer patients (irrespective of type of cancer) and sera from healthy individuals, indicating the potential of the biosensor to act as early screening tool for cancer.

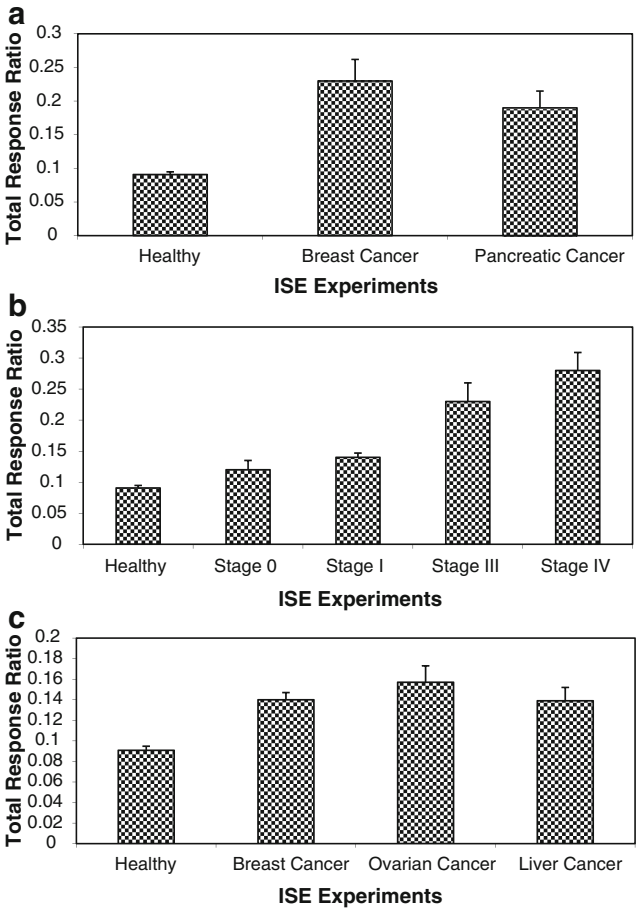

Fig. 6 **a** Comparison between the response ratios obtained when exposing the sensor to the sera of stage III pancreatic and breast cancer patients for 1 h and the responses obtained from the sera of healthy individuals. The values represent the mean of 15 healthy individuals and five pancreatic and three breast cancer samples. **b** Comparison between the response ratios obtained when exposing the sensor to the sera of breast cancer patients at different stages of cancer for 1 h and the responses obtained from sera of healthy individuals. The values represent the mean of 15 healthy individuals and three each of the samples from patients at different stages of cancer. **c** Comparison of the response ratios obtained when exposing the sensor to the sera of stage I breast, ovarian and liver cancer patients for 1 h and the responses obtained from sera of healthy individuals. The values represent the mean of 15 healthy individuals and three each of the different cancer types. *Total response ratio* is defined as the ratio of the response of the cell-based sensor with serum to the ISE response obtained at 0.1 M KCl for the membrane without cells and without serum. Error bars represent SE

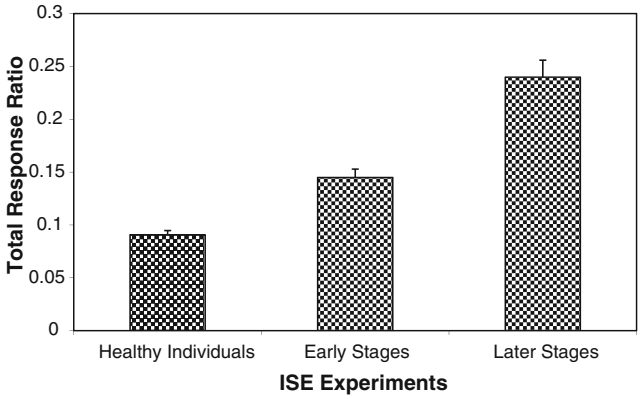

Fig. 7 Comparison of the response ratios obtained when exposing the sensor to the sera of early and later stages of cancer patients for 1 h and the responses obtained for sera from healthy individuals. *Total response ratio* is defined as the ratio of the response of the cell-based sensor with serum to the ISE response obtained at 0.1 M KCl for the membrane without cells and without serum. Stage 0 and I cancer patients were coupled together as "early stages" and stage III and IV as "later stages" of cancer. The values represent the mean of 15 healthy individuals, 12 individuals with early-stage and 11 with later-stage cancer. Error bars represent SE

However, no significant differences were observed ($p>0.05$) between the responses to the sera from patients with cancers of three different organs. Moreover, the responses thus obtained for stage I cancer patients were significantly lower than the responses obtained by exposing the sensor to the combination of all four cytokines at concentrations observed in the sera of cancer patients ($p<0.05$). This is due to the fact that the concentrations studied corresponded to the maximum concentrations of these cytokines observed in the sera of cancer patients. These concentrations also correspond to the later stages of the disease.

To further compare the sensor response to samples from healthy individuals to those obtained from cancer patients, stages 0 and I of different cancer types were coupled together as the "early stage" while stages III and IV were coupled together as "later stage" of cancer, and the responses to sera associated with the early and later stages of cancer were compared. These results are shown in Fig. 7, and they demonstrate that significant differences were obtained when comparing the responses to sera from healthy individuals to the responses to sera from patients at early and later stages of cancer. In addition, significant differences were obtained when comparing sera associated with early and later stages of cancer. This shows that the biosensor is not only able to act as an early screening tool for cancer, but that it can also differentiate between early and later stages of the disease. Furthermore, when these responses were compared with the responses obtained by exposing the sensor to all four cytokines at concentrations observed in the sera of cancer patients, unlike the sera for the early stages, no significant differences were observed for sera corresponding to later stages of the disease ($p>0.05$).

Discussion

It is well known that screening can significantly reduce cancer morbidity. Firstly, by diagnosing an individual at the early stages of cancer, screening increases the chances of successful therapy. Secondly, by diagnosing abnormal cells or lesions, screening can help to identify an individual that shows an inclination to develop cancer and can thus reduce the morbidity of cancer treatment. Research has shown that people are more willing to participate in easy-to-perform screening tests like blood tests as opposed to complicated or painful screening examinations like endoscopy or mammograms. Moreover, the fact that beyond 50 years of age an individual must undergo a number of tests to ascertain health status also reduces the individual's willingness to participate in screening programs. For example, a woman 50 years or more in age must undergo mammogram and endoscopic examinations as well as Pap tests to be certain that she is not suffering from breast, colon or uterine cancer. Similarly, a man 50 years or more in age should undergo endoscopic examination to screen for colon cancer and also digital rectal examination and PSA testing for prostate cancer. In addition, it is important for those patients that have been diagnosed with cancer and have received treatment to continue receiving these diagnostic exams in order to check for re-occurrence of the cancer. The cancer will often develop in a different part of the body other than the original site of growth. Hence, there is a need for not only a noninvasive diagnostic tool for cancer but a tool which can identify patients suffering from cancer, irrespective of origin. This has encouraged research into the detection and validation of biomarkers for cancer. Biomarkers are biological molecules which are present at different concentrations in healthy individuals and in cancer patients [49]. Some common biomarkers are circulating tumor cells (CTCs), circulating DNA and proteins. Differences in enrichment and detection methods of CTCs translate into major discrepancies in detection rates of CTCs, which in turn act as a major drawback of this cancer detection method. However, proteomics score over genomics for biomarker detection and cancer screening due to the ability to take into account posttranslational modifications, differential splicing of mRNAs, and functional regulation of gene expression [50]. Since there are hundreds of proteins with significantly different concentrations in healthy individuals and in cancer patients, a device will have a greater potential to act as a screening tool if it can detect a combination of proteins rather than an individual protein. However, the major challenge lies in selecting proteins, individually or in combinations, which will successfully act as biomarkers.

Angiogenesis is a biological characteristic that is common to most cancers, irrespective of their origin. For a cancer to progress from its benign state to a malignant state, recruitment of blood vessels from the existing vasculature

is required [21, 22]. This leads to increased secretion of cytokines, such as VEGF, HGF, TNF-α and bFGF, into the sera of cancer patients as compared to healthy individuals [28–39]. The concentrations of these cytokines can be measured by ELISA. Multiplexed technology enables one to measure several of these cytokines simultaneously but individually. However, the concentrations of these cytokines in the sera of cancer patients can vary not only on the basis of the origin and the stage of the cancer, but also from person to person, even when they suffer from cancer of the same organ and are at the same stage. Moreover, during the early stages of cancer, the concentrations of all of the cytokines are not elevated simultaneously. Hence, any physiological tool which can detect combinations of cytokines will be a better screening tool than a device which measures several of the individual cytokines simultaneously.

A whole-cell-based biosensor has been developed in our laboratory which takes the advantage of endothelial cell barrier dysfunction to detect small quantities of permeability-modifying agents [40–42]. Earlier studies with this biosensor showed that by measuring increases in cell permeability induced by cytokines such as VEGF, HGF, bFGF and TNF-α, it can act as an alternate assay for measuring angiogenesis. The concentrations of the cytokines used were those generally observed in the sera of cancer patients [30, 33]. In this study we showed that upon exposing the sensor to cytokines individually and in combinations but at concentrations observed in the sera of healthy individuals, lower responses were obtained from the sensor, indicating a dose-dependent response of the sensor to the presence of these cytokines. Upon comparing the biosensor responses to the combination of all four cytokines at concentrations observed in the sera of healthy individuals and cancer patients, it was observed that the biosensor was able to differentiate between the two concentrations. It was also shown that a response from the sensor can be obtained in just 1 h; in contrast, an ELISA requires 4–6 h.

When the biosensor was exposed to the serum samples from 15 different healthy individuals, the responses varied from 0.06 to 0.11 with a mean of about 0.09. The biosensor was then exposed to the serum samples from patients suffering from pancreatic, breast, ovarian and liver cancers. The results demonstrated that the responses of the biosensor to sera from patients with different cancers, irrespective of the stages, were significantly higher than the responses to sera from healthy individuals. Upon comparing the responses for sera from breast cancer patients at different stages of cancer to the responses to sera from healthy individuals, significant differences between the responses were observed. Moreover, when the responses of the sensor to sera from different stage I cancer patients were compared with those obtained for sera from healthy subjects, it was observed that irrespective of the origin, the responses of the sensor to cancer-associated sera were higher than those to sera from healthy individuals. Finally, stage 0 and I cancer were coupled together as "early stage" and stage III and IV as "later stage" cancer, and the responses of the sensor to sera from patients with early and later stages of cancer were compared with those obtained for sera from healthy individuals. The results indicate that the sensor was able to differentiate early stage cancer patients, irrespective of the origin of the cancer, from healthy individuals. The present study demonstrates that the biosensor can not only distinguish between healthy individuals and cancer patients, but that responses from the sensor correlates with the stage of the cancer.

In summary, these initial studies indicate that the HUVEC-based biosensor can differentiate healthy subjects from patients at both early and later stages of cancer within 1 h. These results bolster its potential to act as a screening tool for cancer. In addition, it is proposed that this biosensor could be used to screen for re-occurrence of cancer anywhere in the bodies of cancer patients that have already received treatment. While 1 h is a sufficient amount of time for the sensor to respond, the original studies using combinations of cytokines indicate that a 3 h exposure time gives a larger signal and hence may provide better differentiation between healthy individuals and cancer patients. Future studies will focus on this long exposure time to improve the difference in response.

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References

1. Tabar L, Yen M, Vitak B, Chen H, Smith R, Duffy W (2003) Lancet 361:1405–1410
2. Greenlee RT, Murray T, Bolden S, Wingo PA (2000) CA Cancer J Clin 50:7–33
3. Zitt M, Zitt M, Muller HM (2007) Disease Markers 23:51–71
4. Denis MG, Lipart C, LeBorgne J, LeHur P-A, Galmiche J-P, Denis M, Ruud E, Truchaud A, Lustenberger P (1997) Int J Cancer 74:540–544
5. Muller V, Stahmenn N, Reithdorf S, Rau T, Zabel T, Goetz A, Janicke F, Pantel K (2005) Clin Can Res 11:3678–3685
6. Sher Y-P, Shih J-Y, Yang P-C, Roffler SR, Chu Y-W, Wu C-W, Yu C-L, Peck K (2005) Clin Can Res 11:173–179
7. Nakagawa T, Martinez SR, Goto Y, Koyanagi K, Kitago M, Shingai T, Elashoff DA, Ye X, Singer FR, Giuliano AE, Hoon DSB (2007) Clin Can Res 13:4105–4110
8. Silva JM, Silva J, Sanchez A, Garcia JM, Dominguez G, Provencio M, Sanfrutos L, Jareno E, Colas A, Espana P, Bonilla F (2002) Clin Can Res 8:3761–3766
9. Herrera LJ, Raja S, Goodwing WE, El-Hefnawy T, Kelly L, Luketich JD, Godfrey TE (2005) Clin Chem 51:113–118
10. Mulcahy H, Lyautey J, Lederrey C, Chen X, Anker P, Alstead EM, Ballinger A, Farthing MJ, Stroun M (1998) Clin Can Res 4:271–275
11. Silva JM, Dominguez G, Garcia JM, Gonzalez R, Navarro F, Provencio M, Martin SS, Espana P, Bonilla F (1999) Can Res 59:3251–3256

12. Shaw JA, Smith BM, Walsh T, Johnson S, Primrose L, Slade MJ, Walker RA, Coombes RC (2000) Clin Can Res 6:1119–1124
13. Anker P, Stroun M (2001) Leukemia 15:289–291
14. Leon SA, Shapiro B, Skiaroff DM, Yaros MJ (1977) Can Res 37:646–650
15. Kopf E, Zharhary D (2007) Int J Biochem Cell B 39:1305–1317
16. Srinivas PR, Verma M, Zhao Y, Srivastava S (2002) Clin Chem 48:1160–1169
17. Haab BB (2005) Mol Cell Proteomics 4:377–383
18. Nedelkov D, Kiernan UA, Neiderkofler EE, Tubbs KA, Nelson RW (2006) Mol Cell Proteomics 5:1811–1816
19. Zangar RC, Daly DS, White AM (2006) Expert Rev Proteomics 3:37–44
20. Sanchez-Carbayo M (2006) Clin Chem 52:1651–1659
21. Gimbrone MA Jr, Leapmean SB, Cotran RS, Folkman J (1972) J Exp Med 136:261–276
22. Folkman J (1995) Nat Med 1:27–31
23. Holmgren L, O, Reilly MS, Folkman J (1995) Nat Med 1:149–153
24. Folkman J (1985) Adv Cancer Res 43:175–203
25. Hanahan D, Folkman J (1996) Cell 86:353–364
26. Oppenheim J, Fujiwara H (1996) Cytokine Growth F R 7:279–288
27. Dunlop RJ, Campbell CW (2000) J Pain Symptom Manag 20:214–232
28. Sliutz G, Tempfer C, Obermair A, Reinthaller A, Gitsch G, Kainz C (1995) Cancer Lett 94:227–231
29. Cronauer MV, Hittmair A, Eder IE, Hobisch A, Culig Z, Ramoner R, Zhang J, Bartsch G, Reisigle A, Radmayr C, Thurnher M, Klocker H (1997) The Prostate 31:223–233
30. Dirix LY, Vermeulen P, Pawinski A, Prove A, Benoy I, Pooter CD, Martin M, Oosterom ATV (1997) Brit J Cancer 76:238–243
31. Sheen-Chen SM, Chen WJ, Eng HL, Chou FF (1997) Breast Cancer Res Tr 43:211–215
32. Heer K, Kumar H, Read JR, Fox JN, Monson JRT, Kerin MJ (2001) Clin Can Res 7:3491–3494
33. Sezer O, Jakob C, Eucker J, Niemoller K, Gatz F, Werneeke KD, Possinger K (2001) Eur J Haematol 66:83–88
34. Ueno K, Inoque Y, Kawaguchi T, Hosoe S, Kawahara M (2001) Lung Cancer 31:213–219
35. Yoshida N, Ikemoto S, Narita K, Sugimura K, Wada S, Yasumoto R, Kishimoto T, Nakatami T (2002) Brit J Cancer 86:1396–1400
36. Granato AM, Nanni O, Falcini F, Folli S, Mosconi G, De Paola F, Medri L, Amadori D, Volpi A (2004) Breast Cancer Res 6:R38–R45
37. Marci A, Versaci A, Loddo S, Scuderi G, Travagliante M, Trimarchi G, Treti D, Famulari C (2006) Biomarkers 11:184–193
38. Szlosarek P, Charles KA, Balkwill FR (2006) Eur J Can 42:745–750
39. Premkumar VG, Yuvaraj S, Vijayasarathi K, Govindaswamy S, Sachdanandam P (2007) BCPT 100:387–391
40. May KML, Wang Y, Bachas LG, Anderson KW (2004) Anal Chem 76:4156–4161
41. May KML, Vogt A, Bachas LG, Anderson KW (2005) Anal Bioanal Chem 382:1010–1016
42. Ghosh G, Bachas LG, Anderson KW (2007) Anal Bioanal Chem 387:567–574
43. Ghosh G, Mehta I, Cornette AL, Anderson KW (2008) Biosens Bioelectron 23:1109–1116
44. Cha GS, Meyerhoff ME (1989) Talanta 36:271–278
45. Cha MJ, Shin JH, Oh BK, Kim CY, Cha GS, Shin DS, Kim B (1995) Anal Chim Acta 315:311–319
46. Brooks KA, Allen JR, Feldhoff PW, Bachas LG (1996) Anal Chem 68:1439–1443
47. Sakong DS, Cha MJ, Shin JH, Cha GS, Ryu MS, Hower RW, Brown RB (1996) Sens Actuators B 32:161–166
48. Koncki R, Hulanicki A, Glab S (1997) Trends Anal Chem 16:528–536
49. Srinivas PR, Kramer BS, Srivastava S (2001) Lancet Oncol 2:698–704
50. Eyk JEV (2001) Curr Opin Mol Ther 3:546–553