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Demasking of epithelial cell adhesion molecule (EpCAM) on circulating epithelial tumor cells by Tween®20 treatment in breast cancer patients

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Abstract

Background: The epithelial cell adhesion molecule (EpCAM) embedded in the plasma membrane of circulating epithelial tumor cells (CETC) is used for detection and enrichment of circulating tumor cells in peripheral blood and as a target for anti-epithelial antibodies elicited during immune response in anti-tumor immunization. Although an efficient immune response against EpCAM can be generated, the clinical application of such approaches has not been successful so far and the detection of circulating epithelial cells is highly variable. One reason for these discrepancies may be that not all circulating tumor cells are equally accessible for the specific antibody. A possible reason might be masking of EpCAM by glycoproteins or membrane lipoproteins preventing antibody binding.
Methods: We have tested the application of detergents as demasking agents known to be successful in demasking red blood cell epitopes and determined how and in which way they affect integral membrane proteins and membrane lipids.
Results: The results showed that the polysorbate Tween®20, a non-ionic detergent like organic solvent is able to demask EpCAM on CETC and makes it better accessible to its specific antibody retaining at the same time full cell viability.
Conclusions: The data presented in this study suggest that EpCAM is present on part of circulating tumor cells in a masked form and that it is possible to demask EpCAM on CETC of breast cancer patients using Tween®20 treatment. But further studies are needed to elucidate the mechanism of demasking.

Keywords: circulating epithelial tumor cells; demasking by Tween®20 treatment; EpCAM; laser scanning cytometry®.

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Introduction

Solid malignant tumors are one of the most frequent causes of death in the developed world. It is, however, rarely the primary tumor which determines the fate of the patient but the development of distant metastases. Thus, the dissemination of tumor cells to secondary sites is the most critical step in cancer progression. This dissociation of tumor cells from the primary lesions is driven by different factors including accumulated multiple genetic and epigenetic changes. Additional genomic events increase invasiveness of the tumor cells which can subsequently progress to form to metastases (1). Different approaches have been developed to track such cells on their way to metastasis formation (2, 3) and, indeed, the presence of circulating tumor cells in the bloodstream of cancer patients has been recognized for over a century (4). Due to the epithelial nature of most solid tumors circulating tumor cells can be identified in peripheral blood by the expression of epithelial cell adhesion molecule (EpCAM), an intercellular adhesion molecule overexpressed by a variety of carcinomas (5). Because of their easy accessibility circulating tumor cells would be an ideal tool for disease surveillance (6–8). Furthermore, it has been shown that EpCAM plays an important role as prognostic and predictive factor (9, 10). EpCAM is considered to be one of the most frequently and most intensely expressed tumor-associated antigens known and one of the most immunogenic proteins to which antibodies were generated in mice (5). Although there are several antibodies available known to have a high affinity and specificity for human EpCAM the molecule on the membrane of circulating epithelial tumor cells (CETC) may not always be adequately accessible for the specific antibody (11). So far, most immunologic approaches for tumor therapy addressing minimal residual disease have not been successful and only recently it has become definitively clear that one of the most promising approaches using the antibody edrecolomab was not effective for the adjuvant treatment of stage III colon cancer (12). In contrast, an anti-EpCAM-CD3-bispecific antibody (13) and trifunctional antibodies (trAbs) have been engineered to recruit and activate different types of immune effector cells at the tumor site in ascites (14). A possible reason might be masking of EpCAM on peripherally circulating tumor cells by membrane proteins preventing antibody binding (11, 15). However, antibodies (16) and chemotherapeutic agents like Paclitaxel (Taxol®; Bristol Myers Squibb, Princeton, NJ, USA) have been shown to modulate EpCAM expression (17). Although the increase in detectability of EpCAM on the cell surface may, in part, be a consequence of an inhibition of normal cycles of antigen

endocytosis and expression on the cell surface, it appears that the significant increases in surface antigen density detected cannot be accounted for solely by blocking of receptor internalization but might also be due to masking as mentioned above. Masking of surface antigens is a known phenomenon either by steric hindrance or from non-covalent associations with other proteins, both of which would be disrupted in vivo by proteases released into the blood by granulocytes, e.g., during the inflammatory phase of wound healing (18) or in vitro by detergent lysis (19). Detergents are widely used as agents for the detection, isolation, characterization, and purification of membrane proteins (20, 21). We, therefore, studied the effect of different concentrations and incubation times of the non-ionic detergent Tween®20 on EpCAM demasking. Tween®20 is a polyoxyethylene derivative of sorbitan monolaurate, and is distinguished from the other members in the Tween® range by the length of the polyoxyethylene chain and the fatty acid ester moiety (22). Polyoxyethylene and other non-ionic detergents are generally 'mild', which means that they solubilize membrane proteins without affecting important structural features and, in low concentrations, can leave the cell membrane intact (20). We here show that the incubation of blood samples with Tween®20 had significant influence on the specific surface binding of the respective antibody to EpCAM.

Materials and methods

Patient material

Anti-coagulated peripheral blood samples were drawn from 54 breast cancer patients (45 patients with primary breast cancer, nine patients with metastatic breast cancer; all treated with systemic chemotherapy) with informed consent using the S-Monovette® blood collection system (EDTA tubes) by Sarstedt (Nuernbrecht, Germany). Subsequently, the blood samples were used for the experiments or stored at room temperature for up to 5 days.

Cell preparation

All experiments were performed at room temperature. Samples of 1 mL of anti-coagulated peripheral blood were lysed by using 10 mL of erythrocyte lysis solution (Qiagen, Hilden, Germany) for 10 min, followed by a single centrifugation step for 10 min at 700 *g*. The pellet, containing white blood cells together with the tumor cells, was then resuspended in 500 µL of PE-buffer (PBS-buffer with 2 mmol EDTA). 100 µL of this cell suspension were incubated with 2.5 µL undiluted anti-EpCAM-FITC (as supplied by the provider) and 1 µL undiluted anti-CD45-PE (as supplied by the provider) for 15 min in the dark.

Antibodies

Antibodies were obtained from Miltenyi (Bergisch Gladbach, Germany). Leukocytes and CETC in the white blood fraction from lysed peripheral blood were incubated with the human monoclonal EpCAM antibody conjugated to fluorescein-isothiocyanate (anti-EpCAM-FITC, Clone HEA-125, isotype: mouse IgG1) and the human monoclonal CD45 antibody conjugated to R-phycoerythrin (anti-CD45-PE, Clone 5B1, isotype: mouse IgG2a).

Quantification of tumor cells by laser scanning cytometry®

In order to measure the number of CETC the sample was readjusted to 1 mL with PE-buffer without any additional wash step. One hundred microliters of this cell suspension were applied to a defined area on a poly-L-lysine treated slide (Menzel Gläser, Braunschweig, Germany). The settled cells were measured using a laser scanning cytometer® (LSC®, Compucyte Corporation, Cambridge, MA, USA) and displayed in scattergrams and dot plots (Figure 1). The microscope scans a defined area on the slide to which a certain volume of cell suspension has been applied. This allows the calculation of the absolute number of epithelial cells per mL of blood. Viability of the cells detected by laser scanning cytometry® was visually verified by looking for a clearly visible nucleus in transmitted light, a solely surface staining (6, 7) and propidium iodide (PI)-negativity. A typical cell detected by its green fluorescing cap is shown in Figure 2A.

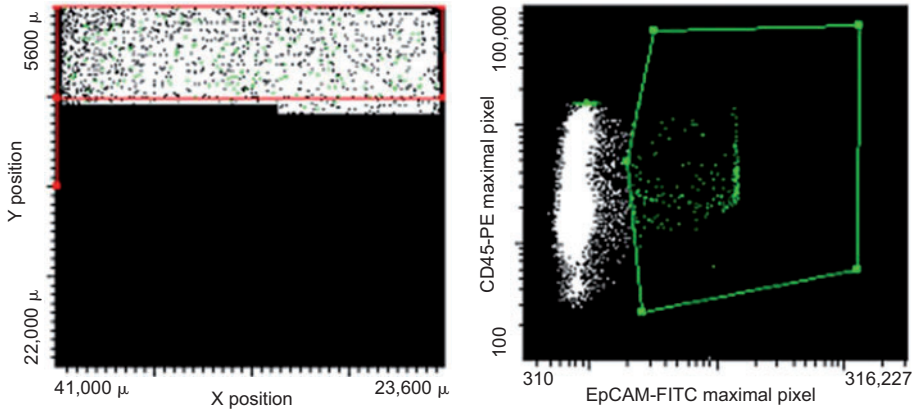

Figure 1 LSC® dot plot (left panel) and scattergram (right panel) of a blood sample.
The scanning stage on a defined area of a slide proceeds from one positive event to the next stopping at each position of a positive event and allowing the observer to control whether it represents a specific membrane stained cell as shown in Figure 2A,C. Each dot on the left side of the plot represents a cell for which the x and y coordinates are stored. The majority of cells are leukocytes (stained with anti-CD45-PE), showing orange fluorescence. Anti-EpCAM-FITC stained green fluorescing epithelial cells are gated in the green window (right panel). Cells in this window can be localized again, viewed, photographed and re-analyzed (6, 7).

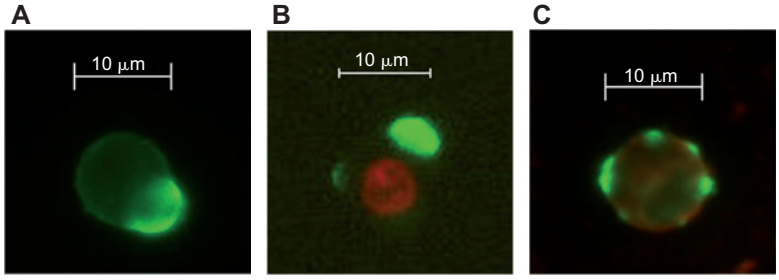

Figure 2 EpCAM positive circulating epithelial tumor cells.
(A) True EpCAM positive tumor cells can be identified by their typical cap-formed staining. (B) Dead CETC with intracellular red propidium iodide staining. (C) Multiple cap formation on CETC after Tween®20 treatment.

Demasking experiments

Demasking experiments were subdivided into four experimental series: A) Incubation experiments, B) Concentration gradient experiments, C) Demasking experiments for a period of three days and D) Inhibition of demasking by addition of protease inhibitors.

All demasking experiments were performed at room temperature immediately after drawing the blood sample and before red blood cell lysis. Sample volumes of 1 mL anti-coagulated peripheral blood were incubated with different concentrations of Tween®20 (Sigma Aldrich) for different experiments. Blood samples for experiment series A, B and C were stored at room temperature; blood samples for experiment series D were stored at 4°C.

For the first demasking experiments (pretests) eight blood samples of primary breast cancer patients and one blood sample of a metastatic breast cancer patient were used. Different Tween®20 concentrations and incubation times were tested (n=3 for 20 µL Tween®20, n=6 for 100 µL Tween®20). Simultaneously control samples of the same patients without Tween®20 treatment were carried out. Cell staining and quantification of the CETC numbers were subsequently accomplished as described above. Additionally, after scanning the sample the already evaluated cells were incubated on the slide with 10 µL PI as a marker of dead cells and re-evaluated again. Only cells with solely green fluorescent surface staining and PI-negativity were included in the analysis. Dead cells with intracellular red PI staining were excluded (Figure 2B). The difference in cell numbers before and after PI staining was recorded in percent.

Incubation experiments

For the detection of the optimal Tween®20 incubation duration sample volumes of 1 mL of anti-coagulated peripheral blood of a primary breast cancer patient were incubated with 20 µL Tween®20 for 2, 5 and 10 min at room temperature. The blood samples were subsequently lysed by adding 10 mL of erythrocyte lysis solution and centrifuged. Cell staining and quantification of the tumor cell numbers by LSC® were accomplished as aforementioned. Simultaneously a negative control sample of the same patient without Tween®20 treatment was carried out.

Concentration gradient experiments

For the concentration gradient experiments 21 blood samples of breast cancer patients were used (16 patients with primary breast cancer, five patients with metastatic breast cancer). Concentration gradient experiments were performed by adding 5, 10, 20, and 50 µL Tween®20 to 1 mL of anti-coagulated peripheral blood. These samples were incubated for 5 min. Cell preparation and quantification by LSC® were carried out as above mentioned. Simultaneously negative control samples of the same patients without Tween®20 treatment were carried out.

Demasking experiments for a period of 3 days

For the demasking experiments for a period of 3 days 14 patients with primary breast cancer patients were sampled in the first 3-day-experiment and three metastatic breast cancer patients were used in a second 3-day experiment.

Each 1 mL of anti-coagulated peripheral blood from the same patient from the first day of drawing the blood sample (day 0) until 2 days after drawing the blood sample (days 1 and 2) were incubated for 5 min with 20 µL Tween®20 followed by cell preparation, quantification and evaluation. Simultaneously negative control samples of the same patients without Tween®20 treatment were analyzed during the 3 days.

Inhibition of demasking by addition of protease inhibitors

For the experiments for the inhibition of demasking by addition of protease inhibitors blood samples of four primary breast cancer patients were used. For the inhibition experiments three arms of test series with anti-coagulated peripheral blood from the same patient were performed in parallel for a period of 5 days. In contrast to the previous experiments blood samples for protease inhibition were stored at 4°C to retain enzyme activity and were daily quantified by using LSC® as described above. The first test series consisted of untreated anti-coagulated peripheral blood (control samples). The second test series consisted of blood samples treated with 20 µL Tween®20 before CETC quantification. The third test series consisted of peripheral blood incubated with a protease inhibitor cocktail. The cocktail contained AEBSF [4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride, 50 mg/mL], Aprotinin (10 mg/mL in 0.01 M HEPES), Leupeptin (10 mM in H_2O), and Pepstatin A (1 mM in methanol) at a ratio of 10:1:1:1 and was added every 12 h to the stored blood sample to keep up enzyme activity during 5 days. The protease inhibitors were obtained from Roche (Mannheim, Germany).

Results

In previous experiments numbers of EpCAM positive cells have been shown to increase from day 0 to day 1 of storage of

the same blood sample at room temperature and then remain at constant numbers for up to 7 days (7). Therefore, we questioned the reason of this phenomenon and tested the application of Tween®20 as demasking agent.

In the first pretests 20 µL Tween®20 as lowest concentration and 100 µL Tween®20 as highest concentration were added to 1 mL anti-coagulated blood for 10 min immediately after drawing the blood sample. EpCAM positive cells could be identified immediately by their typical cap-formed staining (Figure 2A,C), whereas the simultaneous control samples without any Tween®20 treatment showed no EpCAM positive cells yet.

For the efficient use of Tween®20 and the determination of the optimal incubation time with minimal cell loss and the highest cell viability, blood samples were incubated with Tween®20 for 2, 5, and 10 min. Viable EpCAM positive cells were detected in each sample. Demasking of EpCAM occurred directly after addition of Tween®20 to the blood. Increasing numbers of EpCAM positive cells were observed with increasing incubation time (leveling off already at 10 min), however, the number of dead cells was unacceptably high at 10 min (data not shown). Since it is crucial to obtain high recovery rates of viable CETC and minimal cell loss (cell damage by Tween®20), 5 min were selected as optimal incubation time. Concentration gradient experiments were performed by adding 5, 10, 20, and 50 µL Tween®20 to 1 mL of anti-coagulated peripheral blood. Analysis of the samples showed that already minimal amounts of Tween®20 (5 µL) are able to demask EpCAM on CETC (data not shown), but the proportion of dead EpCAM positive cells was apparently lowest in the samples with 20 µL Tween®20 (Figure 3). Based on these concentration gradient results, the subsequent experiments were performed by incubating blood samples with 20 µL Tween®20 for 5 min.

To determine the sensitivity of CETC to Tween®20 and to monitor the increased accessibility of CETC during blood sample storage, experiments were performed with and without Tween®20 treatment from the same blood sample over 3 days. The averaged cell numbers were evaluated from in total 17 experiments (n=14 for primary breast cancer patients, n=3 for metastatic breast cancer patients). The results of the control samples without Tween®20 treatment showed increasing cell numbers from day 0 to day 1 and thereafter constant cell numbers (up to day 7; not shown). In contrast, samples treated with Tween®20 showed an immediate effect already on day 0, which was statistically significantly different from the control samples without Tween®20 ($p=0.002$ for day 0, $p=0.007$ for day 1). The results of the samples treated with Tween®20 showed stable cell numbers on days 0 and 1 and slightly decreasing cell numbers and a higher cell loss after day 2 (Figure 4A). It should be mentioned that this effect was restricted to breast cancer patients with primary cancer (n=14). In contrast, in patients with metastatic breast cancer (n=3) CETC were detectable immediately after drawing the blood sample (day 0). Therefore, the patient group with metastatic disease was evaluated separately. In patients with metastatic disease constant cell numbers were detectable up to day 2 without Tween®20 treatment whereas samples treated with Tween®20 showed high cell numbers also already on day 0 but decreasing CETC numbers on days 1 and 2 (Figure 4B). For this reason only non-metastatic primary breast cancer patients were included in all the following analyses on the effect of Tween®20 on CETC.

In the context of demasking experiments for a period of 3 days there were patient samples which neither showed EpCAM-positive cells at day 0 nor at days 1 or 2. They also did not show EpCAM-positive cells after Tween®20 treatment and therefore were used as negative controls. These CETC negative patient samples clearly indicate that EpCAM positive cells can only be demasked if such cells are present in the sample and that Tween®20 treatment does not cause unspecific antibody binding, e.g., to leukocytes. We never

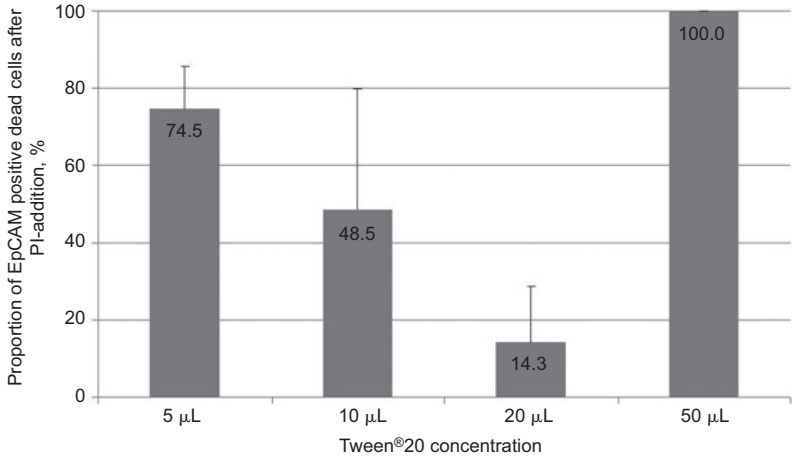

Figure 3 Diagram of cell loss of different Tween®20 concentrations (fraction of EpCAM positive dead cells after PI-addition in %). Concentration gradient experiments were performed by adding 5, 10, 20, and 50 µL Tween®20 to 1 mL of anti-coagulated peripheral blood. Analysis of the samples showed that already minimal amounts of Tween®20 (5 µL) are able to demask EpCAM on CETC, but cell loss was apparently lowest in the samples with 20 µL Tween®20. Error bars (SD) represent results from 21 experiments on blood from primary and metastatic breast cancer patients.

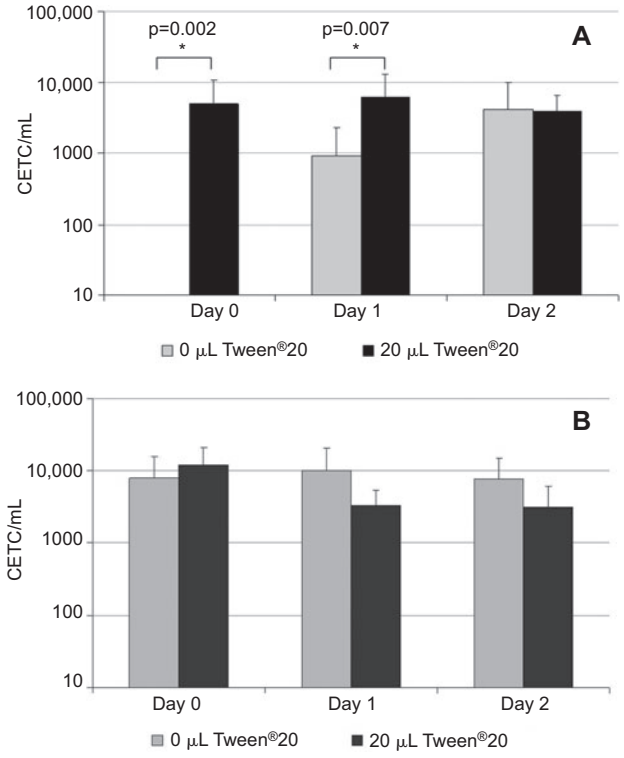

Figure 4 Course of cell numbers of blood samples of patients with breast cancer with and without Tween®20 treatment from day 0 to day 2.
(A) Patients with primary breast cancer. The results of the control samples without Tween®20 treatment showed increasing cell numbers from day 0 to day 1 and thereafter constant cell numbers. In contrast, samples treated with Tween®20 showed an immediate effect on the first day with stable cell numbers on days 0 and 1 and slightly decreasing cell numbers and a higher cell loss after day 2. Error bars (SD) represent results from 14 experiments on blood from primary breast cancer patients. A two-sided t-test analysis was performed to evaluate the p-values and to accentuate the significant difference between control samples without Tween®20 treatment and blood samples treated with Tween®20. (B) Patients with metastatic breast cancer. In patients with metastatic breast cancer CETC were detectable immediately after drawing the blood sample (day 0) without any Tween®20 treatment and constant cell numbers were detectable up to day 2. Samples treated with Tween®20 showed high cell numbers also already on day 0 but decreasing CETC numbers on days 1 and 2. Error bars (SD) represent results from three experiments on blood from metastatic breast cancer patients.

observed additional EpCAM staining on CD45 positive leukocytes after Tween®20 treatment.

In all experiments we observed a preferential elimination of a major part of leukocytes after Tween®20 treatment of the blood samples. This leads to a relative enrichment of EpCAM positive cells in the sample and an increased agglutination of cells. The microscope, still, correctly identifies every positive event even if it is cell clumps. All EpCAM positive cells showed clearly defined green fluorescent caps. Compared to the untreated control samples multiple cap formation on CETC was more often visible in Tween®20 treated samples but without loss of EpCAM intensity (Figure 2C).

It is known that certain surface epitopes are sometimes not immediately accessible to their corresponding antibodies due to masking effects. We hypothesized that masking is due to proteins and demasking might be a result of the activity of proteases in blood. Therefore we tried to prevent demasking by addition of protease inhibitors. In the three arms of the test series protease inhibition and Tween®20 demasking were performed in parallel in anti-coagulated peripheral blood from the same patient at 4°C. The untreated control samples showed increasing cell numbers over the first days (days 0–2) which was delayed as expected due to incubation of the samples at 4°C and reached a constant cell number for the last 2 days (days 3 and 4). Samples of the second test series treated with Tween®20 showed the well-known initial high numbers of viable CETC (which were set 100%) and decreasing cell numbers over the 5 days. In contrast, CETC were not detectable in the blood samples incubated with the protease inhibitor cocktail (Figure 5). Leukocyte staining with CD45-PE was always detectable even under these conditions.

Discussion

EpCAM (CD326) is a transmembrane adhesion protein that is frequently expressed at the basolateral membrane of the majority of epithelial tissues and is found to be overexpressed by a great variety of squamous cell carcinomas (5, 23, 24), including prostate, breast, colon, gastric, ovarian, pancreatic and lung cancer (10, 23). The overexpression of EpCAM in primary breast cancers appears to be associated with an increased malignant potential (9, 24) demonstrated by the fact that silencing the EpCAM gene expression decreases the proliferation, migration, and invasion potential of breast cancer cell lines in vitro (24). EpCAM has also been shown to be expressed on CETC and can be recognized by anti-EpCAM-FITC on the basolateral surface of the CETC, but compared to primary and metastatic tissues the EpCAM expression has been reported to be approximately 10-fold lower on the cells shed into the circulation, suggesting that loss of cell-cell adhesion is a prerequisite for tumor cell dissemination (25, 26). We have shown that it is possible to monitor the number of CETC during therapy and to use them to control the therapeutic efficacy of systemic therapy (6–8). In breast cancer patients we have shown that the CETC belong to the tumor and respond to therapy in an identical fashion as the tumor (7) and resistance to therapy predicts the development of metastatic relapse (8).

EpCAM positive cells have been regarded as a meaningful target for immune therapies (27) but, although an efficient immune response against tumor-associated antigens like EpCAM could be generated (24, 28), antibody-mediated therapies and the clinical application of such approaches has not been successful so far (12). Further, tests using the EpCAM epitope for magnetic bead enrichment showed that obviously not all circulating tumor cells are equally accessible for the specific antibody in breast cancer patients (7). Publications on the detectability of circulating epithelial cells after magnetic bead enrichment report highly variable numbers (7, 29–32).

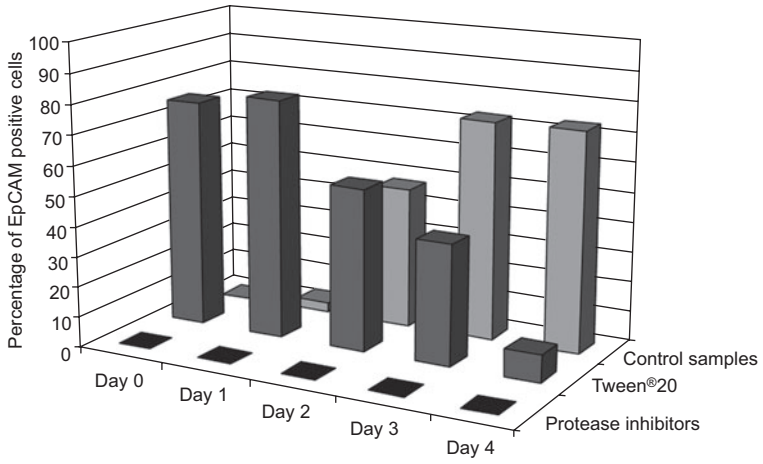

Figure 5 Course of averaged percentage of EpCAM positive cells of the different blood samples from day 0 to day 4.
In the three arms of the test series protease inhibition and Tween®20 demasking was performed in parallel in anti-coagulated peripheral blood from the same patient in the cold. The untreated control samples showed increasing cell numbers over the first days (days 0–2) and reached a constant cell number for the last 2 days (days 3 and 4). Samples of the second test series treated with Tween®20 showed the well known initial high numbers of viable CETC and decreasing cell numbers over the 5 days. In contrast, CETC were not detectable in the blood samples incubated with the protease inhibitor cocktail. The plot represents results from four experiments on blood from primary breast cancer patients.

These discrepancies may, in part, depend on methodological modifications (e.g., time and temperature of storing the blood samples, immunomagnetic cell enrichment, Ficoll separation). Further, fixatives and preservatives used in other cell preparation systems may also contribute to reduced detection of EpCAM on the cell surface (32). This led us to hypothesize that EpCAM on the surface of tumor cells in blood might be in part masked preventing antibody binding (33, 34). In contrast to conditions immediately after drawing a blood sample where only few EpCAM positive cells are detectable, this number increases considerably if the blood sample is left at room temperature until the next day with the cells still 95% viable (7). We have, therefore, tested the application of Tween®20 as demasking agent. Since it is necessary to retain the active conformation of the proteins (20) to obtain high recovery rates of CETC and minimal tumor cell damage we studied the effect of different concentrations and incubation times of the non-ionic detergent Tween®20 on EpCAM.

In the first pretests with Tween®20 added to anti-coagulated blood EpCAM positive cells could be identified immediately after drawing the blood sample. Whereas in the simultaneous control samples without any Tween®20 treatment our experience has been that no or low numbers of EpCAM positive cells are detectable immediately after blood drawing. Tween®20 demasked EpCAM positive CETC showed clearly defined green fluorescent caps on the cell surface. This demonstrates that Tween®20 is able to demask EpCAM and makes it accessible to its specific antibody without impairing cell viability. Optimal conditions for minimal CETC loss retaining at the same time full cell viability which is a prerequisite for specific binding of the antibody to the cells surface were subsequently established. We observed at the same time an accelerated tendency of the cells to agglutinate and this could be explained by the fact that EpCAM as an adhesion molecule results in increased cell-cell-adhesion considered a typical feature of EpCAM expression. To determine the sensitivity of CETC to Tween®20, experiments were performed without Tween®20 treatment showing increasing cell numbers of the blood sample from day 0 to day 1 and subsequently constant cell numbers for the next days. This confirms previous results by Pachmann et al. (7). In contrast, samples treated with Tween®20 showed constant cell numbers already from the beginning and later a slight decrease accompanied by an incremental loss of cell viability during the later time points. Therefore it can be assumed that Tween®20 has a demasking effect on fresh CETC but a damaging/toxic effect on aging CETC. Thus, Tween®20 which is definitively a suitable detergent to demask EpCAM on CETC during the first days after drawing the blood sample with minimal cell loss, but it is not the agent of choice for aged blood samples. In patients with metastatic breast cancer we observed CETC immediately after drawing the blood sample (day 0) probably due to the secondary tumor sites that might lead to a high dissemination of CETC into the peripheral blood. This may be the reason why less sensitive methods like those using magnetic bead enrichment can detect circulating tumor cells only in metastatic breast cancer patients (7). Based on the assumption that some surface epitopes are not immediately accessible to their corresponding antibodies due to masking effects by, e.g., serum proteins, we asked what contributes to demasking of surface epitopes in stored samples. It is well-known that granulocytes rapidly die in stored samples undergoing apoptosis and disintegration, releasing their granular contents like proteases into the external medium (35). Previous tests had shown that CETC survive longer than normal blood cells in a stored blood sample. After an initial increase during the first 1–2 days the number of epithelial tumor cells in the blood sample remains constant, while the number of

leukocytes decreases and other blood components like dying cells increase (Hekimian, unpublished). Thus, the concentration of released proteases increases in the blood sample during storage. Increased demasking of EpCAM on CETC might therefore be due to the activity of proteases in the blood. Consequently, the number of CETC recovered in the blood sample might rise proportionally to the concentration of proteases released into the blood by dying granulocytes until a constant concentration level is achieved. This effect could explain the primary surge of the CETC number during the first days after blood drawing and the constant cell number during the following days. In order to test this hypothesis, we tried to prevent demasking of EpCAM by addition of protease inhibitors. The results clearly demonstrate that the presence of proteases in blood samples has a strong influence on demasking of EpCAM on CETC and that this effect can be prevented by protease inhibitors. Our results also show that it is possible to initiate demasking of EpCAM by addition of Tween®20. In the protease inhibition experiments all samples were stored at 4°C to retain enzyme activity, leading to a decreased demasking in the control samples with a later increase in cell numbers on the first days after blood drawing. This effect could also be explained by the fact that granulocytes survive longer in a stored sample under cooling conditions leading to a slowed decay of granulocytes. The question arises whether masking of EpCAM on CETC might also play a role in vivo in resistance to antibody dependent anti-cancer therapy and whether proteases-induced demasking in, e.g., thromby contributes to metastasis formation. If the epitopes, to which the immune response has been elicited, are masked and not accessible in vivo, this may explain the inefficiency of most of the immune approaches so far. The concentration of proteases in the blood circulation is certainly much lower than in the closed system of a stored blood sample. This might be a reason why disseminated epithelial tumor cells are able to circulate unobstructed through the blood vessels and can remain in the circulation over long times. These cells may remain dormant, but might settle and grow into metastases, if they find appropriate conditions, even after years (36). It is well-known that size restriction in small capillaries may be one reason for accumulation of circulating tumor cells in organs like lungs, liver and bone marrow. Blood-flow patterns and other physical factors can influence the seeding of cancer cells to secondary sites and lead to arrest of CETC in the first capillary bed, whereas additional molecular interactions between the cancer cells and the surrounding environment influence the probability that the cells will grow there (2). Another possible reason for accumulation of tumor cells in distant organs could be a local increase of proteases demasking adhesion molecules on the surface of CETC. Leukocytes are carried by the blood flow through the capillaries and often arrest by adhesion to the walls of vessels with a larger diameter than the cells themselves (2) especially at sites of epidermal or epithelial injury, e.g., after surgery or tumor resection. During the inflammatory phase of wound healing, recruited leukocytes are the predominant cell type with granulocytes infiltrating and starting the debridement of devitalized tissue (18). To perform this task, granulocytes release a large variety of highly active antimicrobial substances, cationic peptides, and most of all proteases (18). This could contribute to uncovering of EpCAM on the surface of CETC, subsequent accumulation due to cell-cell-adhesion in the blood capillaries and invasion by-and-by into the surrounding tissue and thus initiation of the well-known local or scar relapses but also to distant metastases due to capillary defects.

In summary, the data presented in this study suggest that EpCAM is present on part of CETC in a masked form and that it is possible to demask EpCAM on CETC of breast cancer patients using Tween®20 treatment, even though further studies are needed to elucidate the mechanism of demasking. The consequences of masked EpCAM molecules and demasking at specific sites may have far-reaching implications on development of metastases. Thus, our investigations may contribute to the generation of hypotheses of metastasis formation by circulating tumor cells and, thereby, to the development of more effective antibody mediated therapies.

Conflict of interest statement

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