Correlation of Circulating Tumor Cells with Tumor Size and Metastatic Load in a Spontaneous Lung Metastasis Model

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Abstract. Background: The prognostic value of circulating tumor cells remains unclear since, in principle, most tumor cells are unable to survive in the bloodstream. The aim of the study was to establish a system that can be used to investigate the metastatic process in more detail, with emphasis on circulating tumor cells. Materials and Methods: Human colon carcinoma cells (HT29) were transplanted into severe-combined-immunodeficient (scid) mice. The metastatic load in the blood was investigated using the human-specific carcinoembryonic antigen (CEA) as target for quantitative polymerase-chain-reaction (PCR). Results: A close correlation between the weight of the primary tumor and the number of circulating tumor cells was detected \(r=0.7240; p<0.0001\). Moreover, the number of circulating tumor cells and the actual number of spontaneous lung metastases was related \(r=0.8283; p<0.0001\). Conclusion: A tumor xenotransplantation model is presented that allows for a detailed investigation of the metastatic process in three different compartments: the primary tumor bed, the bloodstream and the target organ of metastatic residency.

Once in the bloodstream, candidate metastatic cells have to extravasate (i.e. to pass arterial and capillary systems) to reach various target organs. Only cells entirely successful in all these processes will develop into clinically detectable metastases. The metastatic efficiency of circulating (i.e. already positively selected) tumor cells is poor and they remain vulnerable or prone to apoptosis (1). Consequently, the frequency of clinically overt metastatic disease suggests two possibilities. Either a fairly large number of malignant cells must circulate at a given time or, alternatively, if leaving the tumor bed is the rate-limiting step for metastasis, the number of circulating tumor cells required for advanced disease could be fairly small. For a number of reasons, this important question is difficult to answer in humans. Since tumor-specific gene expression does not exist, a sufficiently sensitive detection of single cancer cells by molecular methods requires reverse transcriptase PCR (rtPCR) to amplify "tissue-associated" gene expression. However, low-level background transcription of these genes exists in normal cells, which interferes with the diagnostic specificity (2,3). To elucidate the key processes of metastasis, a model system is required to observe the cascade as a whole. The severe-combined-immunodeficient (scid) mouse xenograft model has been shown to be suitable and to provide clinically relevant information (4-6). In this system, human cancer cells are injected subcutaneously into scid mice. Thereafter, a solid tumor at the implantation site as well as metastatic deposits in the lungs are formed. This model has already revealed a significant correlation between the weight of the primary tumor and the number of spontaneous lung metastases (7). Quantitative data regarding the metastatic spread of tumor cells into the peripheral blood, however, are lacking to date. In scid mice, human-specific gene targets (e.g. carcinoembryonic antigen (CEA)) should allow for the selective detection of small numbers of circulating cancer cells with no interference from murine sequences. Also, gene copy numbers can be measured and translated into cell numbers, which is not possible with tests measuring gene expression. This system should allow the investigation of tumor behavior in three
different compartments: the primary tumor bed, the bloodstream and the target organ of metastatic residency. This information is of particular interest, both with respect to molecular diagnostics and to potential therapeutic strategies that aim at single tumor cells. Here, a system that can be used to study the metastatic process in more detail will be described.

Materials and Methods

Cell lines. The human colon cancer cell line HT29 was purchased from the European Cell Culture Collection (Wiltshire, UK). The cells were cultivated in vitro under standard cell culture conditions in RPMI-1640 medium (Gibco/Life Technologies, Karlsruhe, FRG) supplemented with 10% heat-inactivated fetal calf serum, glutamin, penicillin and streptomycin at 37°C in a humidified incubator with 5% CO2 and were prepared for inoculation by trypsinization. Viable cells (5 x 10^7) were suspended in 1 ml cell culture medium. An aliquot of 200 ml of this suspension was injected subcutaneously between the scapulae of each scid mouse.

The human lymphoma cell line K562 (ATCC, Manassas, VA, USA) was used for the generation of a CEA standard curve, since the cells are non-adherent and can be counted reliably. Briefly, cells were cultured as described above, harvested using Accumax medium supplement (PAA Laboratories, Linz, Austria) and counted in a GEN-S hematology analyser (Beckman/Coulter, Krefeld, FRG). After adjusting for cell densities, the samples were stabilized in 5 M guanidinium-isothiocyanate (GITC) solution as described elsewhere (8).

Scid mice. Sixty specific pathogen-free male BALB/c scid/scid (scid) mice (in-house breeding), aged 9-14 weeks, were used. The animals were kept in filter top cages and were provided with sterile water and food ad libitum. All manipulations were performed under aseptic conditions inside a laminar flow hood. Twelve mice were sacrificed every ten days between day 15 and day 55 to obtain different tumor sizes. The experiments were approved by the local animal welfare committee.

Gene amplification. For the target marker the human CEA gene was used, since no homologue to this gene has been identified in mice to date. In order to obtain DNA, representative blood samples were withdrawn from the mice by puncturing the venous plexus of the orbita after general anesthesia with ether. By this method, nearly 1 ml of blood could be collected from a 25-g mouse with a whole blood volume of approximately 2 ml. One hundred ìl of each sample was prepared for DNA extraction using the GFX DNA purification kit (Pharmacia Biotechnologies, Peapak, NJ, USA) according to the manufacturer’s instructions.

The primers and hybridization probes specific for human CEA, known to be expressed in the human colon cancer cell line HT29, were obtained from TIB Molbiol (Berlin, Germany): CEA63a: 5'-ACGGACGATTCAGCATCTGG-3'; CEA63b: 5'-CAGGAAA CAGAAACAGAGGAAAAG-3'; 1128CEA-WT-Fl: 5'-CAAAACA ACTGTCAGTCTTCCTGAAATGAAGAAACTACACC-Fluorescein; 1097CEA-WT-red: 5'-LC640-GGGCTGCTATATCAGA AACTCCACCACCCCAACCAG-Phosphate.

PCR reactions were performed in 20 ìl volume capillary containing 10 ìl of DNA, 0.1 ìl Taq DNA polymerase in the reaction buffer (x10 conc.), 0.3 ìM primers, 4.0 mM MgCl2, 0.5 mM BSA, 0.2 mM dNTPs and 20 ìM hybridization probes. Following initial denaturation for 30 sec at 95°C, PCR amplification was carried out at 67°C (annealing temperature) for

Figure 1. Hematoxylin/Eosin-stained metastasis (arrows) in the lung of a scid mouse (x100 magnification).

Figure 2. Typical PCR reaction curves after using human K562 cells diluted into murine DNA and human specific hybridization probes for CEA (2A). Calculated standard solution curve used for interpolating the signal strength of the blood samples in order to determine the number of circulating human tumor cells (2B).
Table I. Results of ten reactions using limiting dilutions of K562 cells demonstrating the influence of stochastic effects on tumor cell detection at the sensitivity limit. Serial dilutions below 40 K562 human cells in murine DNA per reaction led to false-negative results. Samples negative by single testing were measured three times to confirm that they were tumor-free. Also note the higher standard deviation of the cycle numbers at higher dilutions.

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Mean: 23.99  27.03  30.28  33.66  37.31
Stand.-dev.: 0.33  0.14  0.38  0.40  0.91

8 sec, 72°C (extension temperature) for 6 sec and 94°C (denaturation) for 5 sec for 50 cycles by real-time PCR on a LightCycler (Roche, Molecular Systems, Mannheim, Germany). To assess the analytical sensitivity to detect single human cells among a background of murine leucocytes, systematic studies were performed using aliquots of the internal standard. This standard solution was reconstituted using human K562 cells diluted into murine DNA prepared previously as a bulk preparation. The background mouse DNA concentration was adjusted to 15 ng DNA/µl buffer as determined by measuring the optical density of DNA solutions obtained from healthy mice.

Finally, the number of circulating human tumor cells was determined by interpolating the signal strength of the blood samples of the mice to an equivalent signal of the serially diluted K562 cells using LightCycler software 3.0.

Histology. The right lungs of sacrificed animals were removed, fixed in 4% neutral pH-buffered formalin and processed for wax histology (Figure 1). Every tenth section of the tissue blocks was stained with Hematoxylin/Eosin and 10 stained sections from the centre of each block were examined at a x100 magnification. Quantitative assessment of lung metastases was performed as described previously (7).

Statistical analysis. The results were expressed as means and the relationships between the different compartments were analyzed for significance by Pearson’s product moment correlation using Prism (GraphPad Software, San Diego, CA, USA). Furthermore, a t-test was used to determine whether (r) is significantly different from zero. Differences with at least p < 0.05 were considered to be significant.

Results

Tumor weight. In order to obtain different sizes of primary tumors, mice were sacrificed at various time points. At the end of the experiment the mean weight of the dissected tumors at the implantation site was 0.81 g with a range from 0.02 to 5.96 g.

Circulating HT29 cells. The probability of positive test results was proven by single tests using limiting dilutions of K562 cells (Figure 2A and B). The results of ten reactions are summarized in Table I. Positive reactions were always achievable when more than 4 x 10^1 cells/10 µl were investigated. At higher dilutions the probability to obtain a positive result decreased owing to stochastic processes governed by the Poisson distribution. Additionally, the variation of the crossing points increased in standard deviation. However, to avoid false-negative results, negative samples were analyzed three times. Altogether 43 out of 60 samples demonstrated a positive amplification of the CEA sequence. On average, 358 human cancer cells/2 ml blood were found, with a range from 1 to 3588 human cancer cells/2 ml blood. When the association between the weight of the primary tumor and the number of circulating HT29 cells was analyzed, a significant correlation became evident (r=0.7468; p<0.0001).

Number of metastatic deposits in lung. The number of metastases was counted in H&E-stained sections according to Jojovic et al. (7). In the 60 animals an average of 258 metastatic deposits per right lung were found (range 0 to 7952). The number of metastases increased proportionally to the primary tumor weight (p<0.0001) with a correlation coefficient of r=0.7240. Furthermore, an association between the number of circulating HT29 cells in the peripheral blood determined by quantitative PCR and the number of metastatic deposits in the lung was seen. This correlation was also statistically significant (r=0.8283; p<0.0001).
Discussion

The aim of this study was to establish a sensitive and specific quantitative PCR allowing for the quantification of human colon cancer cells in the peripheral blood in a mouse model of spontaneous metastasis formation.

First a suitable marker to detect the presence of human cancer cells in a xenogenic background had to be determined. Human CEA was used as a marker marker, since no homologue to this gene has been identified in mice (9,10). In humans, CEA is mainly expressed in epithelial cells of the gastrointestinal tract. Moreover, its expression is up-regulated in a variety of carcinoma cell lines such as HT29. Hence, results in this xenograft model system cannot be influenced by low-level background gene amplification from non-malignant human cells after conventional PCR. This represents a clear advantage of this model system over the clinical situation where, in particular, the rtPCR-based approaches for the detection of minimal residual disease encounter relative lack of tissue specificity, illegitimate transcripts or non specific amplification due to processed pseudogenes (e.g. in case of cytokeratins) (11). As cytokines and growth factors have been shown to induce the mRNA expression of CEA and cytokeratins in human blood leukocytes (12), another factor for false-positive results has been eliminated using this xenograft model. Additionally, the use of an autochthonous target is preferable to using transfected marker genes, since incorporation of such genes into the nuclear DNA can alter the properties of a tumor cell line regarding its metastatic potential or aggressiveness (13). As the CEA gene is present in all human cells, this xenograft model is of use in future studies as it can be applied to any other human/scid mouse xenograft model system of spontaneous metastasis formation.

We found standardization and quality control to be of particular importance. For example, we used cells of the human lymphoma cell line K562 as standards, because they are non-adherent and can be counted reliably with routine flow cytometry methods. In addition, the limit of PCR sensitivity was assessed carefully. Specifically, forty K562 cells were reliably detected by single testing, while at higher dilutions an increasing rate of false-negative results was observed owing to stochastic processes governed by the Poisson distribution (Figure 3), as discussed previously (14). By testing negative samples three times, we increased the functional sensitivity to 4 cells/reaction in a sample volume corresponding to 0.5% of the circulating blood volume in the mouse. In contrast, stochastic effects during sampling are more pronounced in humans, since approximately 100 times less of the circulating blood volume is usually assayed in cancer patients.

The investigation carried out through a single blood collection at the experimental end point can be regarded as a snapshot, while the shedding of cancer cells that subsequently appear in the circulation may be an undulating process rather than a continuous one. Nevertheless, the results confirm that an association between the weight of the primary tumor and the number of metastatic cells within the peripheral blood exists ($r=0.7468; p<0.0001$). The larger the tumor, the more tumor cells are shed into the bloodstream. A further positive correlation also exists at the exit site of the tumor cells from the bloodstream since a significant association between the quantity of circulating tumor cells and the count of metastatic deposits in the lungs was evident ($r=0.8283; p<0.0001$). This contradicts the supposition that the circulating tumor cells found by PCR may not be metastatically competent, but merely indicate occult tumor burden. Further investigations are necessary to clarify whether the different steps of the metastatic cascade can take place independently, since one can imagine that tumors exist that shed high numbers of cells into circulation which are unable to extravasate into target organs. Therefore, the number of circulating tumor cells alone is not sufficient to specify the metastatic potential, whereas a ratio calculated by the count of circulating tumor cells and the number of metastatic deposits should be a useful tool to determine the aggressiveness and the organ affinity of various cell lines. It also remains to be determined whether candidate metastatic cells possess an a priori capability of adhesion and extravasation or, in contrast, whether these functions are developed under certain circumstances such as stress e.g. owing to mechanical shear forces. These findings demand that, with respect to cancer therapy, particular efforts should be made in inhibition of extravasation since physiological forces and a functional, active immune system seems to be sufficient to damage nearly all circulating tumor cells within seconds.

In summary, we have developed an in vivo system for the detection of metastatic processes in human tumors. As a first important result, we were able to demonstrate that cancer cells circulate in the blood and are capable of forming metastases. Due to the human-specific gene target and the quantitative design, this model system measures small numbers of tumor cells and should be transferable to any other human/scid mouse xenograft metastasis system. It is therefore ideally suited to investigate quantitative and qualitative changes of circulating tumor cells in metastatic transit and possibly specific changes in gene expression of these cells.

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References


