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In Vivo Monitoring of Natural Killer Cell-dependent Clearance of Lung Metastasis Using Dynamic Positron Emission Tomography

CARSTEN KRUSCHINSKI[1,2*], YOSHIHISA HAMA[1*], THOMAS SKRIPULETZ[1,3], BERNHARD VASKE[4], WOLFRAM H. KNAPP[5], REINHOLD E. SCHMIDT[6], REINHARD PABST[1,7], STEPHAN VON HÖRSTEN[1,8] and MICHAEL HOFMANN[5]

[1]Institute of Functional and Applied Anatomy, [2]Institute of General Practice, [3]Department of Neurology, [4]Institute of Biometry, [5]Clinic of Nuclear Medicine, [6]Clinic of Clinical Immunology and Rheumatology and [7]Institute of Immunomorphology, Hannover Medical School, Hannover, Germany; [8]Experimental Therapy, Franz Penzoldt Center, Friedrich Alexander University Erlangen Nürnberg, Erlangen, Germany

Abstract. *Immunohistological methods indicated a rapid onset of cellular defence shortly after seeding of mammary adenocarcinoma cells into the lungs of F344 rats. The purpose of the present study was to monitor natural killer (NK) cell-mediated effects on tumour cell clearance in vivo, in this model of lung metastasis using dynamic positron-emission tomography (dPET). MADB106 breast cancer cells were labelled with 2'-[^{18}F]-2'-deoxy-D-glucose (FDG) then injected intravenously, after the F344 rats had been anaesthetized and placed in a PET scanner. NK cell-depleted and sham-treated control rats were investigated in parallel. The radioactivity per region of interest (ROI) over the lungs peaked at 60 s past injection and was followed by a slow decline over the observation time of 40 min in both groups. Statistical analysis using a linear mixed model revealed that release of radioactivity from tumour cells or tumour cell disintegration was significantly slower in animals after depletion of NK cells compared with controls. There was no significant tumour cell homing in organs other than the lungs. Early kinetics of tumour cells after injection were defined. PET with FDG was shown to be an adequate method to further investigate novel options for using cellular host defence mechanisms in cancer patients.*

The development of cancer metastases depends on multiple interactions within the microenvironment of the target tissue, including effects of cellular adhesion molecules, chemokines, and hydrodynamic conditions (1-5). In addition, the recruitment of leukocytes from the blood into the sites of tumour cell micrometastases represents a critical early step in the development of an effective local immune response (6). In recent publications, the role of cell types such as natural killer (NK) cells and monocytes has been demonstrated using immunohistological methods as being crucial in the context of metastatic seeding (7-9). Moreover, tumour cell seeding has also been studied using radioimaging techniques with ^{125}I-5-iodo-2'-deoxyuridine (10), in order to visualize the route of injected tumour cells and to demonstrate the role of NK cells in tumour cell clearance (11). However, these results were based on *ex vivo* measurements and an extrapolation of the radioactivity recovery over time.

We hypothesized that dynamic positron-emission tomography (dPET) of labelled tumour cells might provide a novel tool to monitor tumour cell clearance *in vivo* in a continuous time window, as has already been shown for stem cells (12). For the present study, 9',10'-dimethyl-1',2'-benzanthracene-induced MADB106 mammary adeno-carcinoma was selected as a variant of a cell line obtained from a pulmonary metastasis produced by the intravenous (*i.v.*) injection of MADB100 parental adenocarcinoma syngeneic with F344 rats (13). It represents a well-established model of lung metastasis (7, 8, 14). The purpose of the study was to visualize NK cell effects on tumour cell

*These Authors contributed equally to this study.

Correspondence to: Carsten Kruschinski, MD, MME, Institute of General Practice, OE5440, Hannover Medical School, Carl-Neuberg-Str.1, 30625 Hannover, Germany. Tel: +49 5115328159, Fax: +49 5115324176, e-mail: kruschinski.carsten@mh-hannover.de

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clearance and, thereby show the feasibility of PET for immune response studies in experimental oncology.

Materials and Methods

Animals. F344 rats were kept under barrier-reared conditions at the Central Animal Laboratory of the Hannover Medical School. They were kept in a specific pathogen-free facility at 25˚C under a 12 h light/12 h dark cycle (lights on at 07.00 am) with *ad libitum* access to food and water. Age-matched- 12 to 14-week-old female animals with a mean body weight of 180 g were used. The experimental design included one group of six NK-cell depleted animals and one group of six sham-treated controls. All research and animal care procedures were approved by the Review Board for the Care of Animal Subjects of the district government, Hannover, Germany, and performed according to international guidelines on the use of laboratory animals.

NK cell depletion. Two days prior to tumour cell injection, animals of the NK cell-depletion group received 2.0 mg/kg of the anti-natural killer (NK) cell monoclonal antibody NKR-P1 (CD161) (14) *i.v.* and intraperitoneally (*i.p.*) as previously described (7). Control rats were injected *i.v.* and *i.p.* with the same volume of 1:20 diluted mouse serum. Complete depletion was confirmed by subsequent immunocytology of blood samples 48 h after antibody treatment. Controls exhibited normal cell numbers, as described in a previous study (7).

Preparation of MADB106 tumour cells and labelling protocol. Cell culture and injection of tumour cells were conducted as described before (7, 8, 14). Cultured MADB106 tumour cells were labelled by 30 min incubation at 27˚C in glucose-free phosphate buffered saline (PBS; Sigma-Aldrich, Steinheim, Germany), pH 7.4, with 2'-[^{18}F]-2'-deoxy-D-glucose (FDG; DHZ Bad Oeynhausen, Germany) in the presence of insulin (1 U/10 ml, Novo Nordisc, Germany) and purified from non-incorporated activity by sequential washing and centrifugation. The supernatant of the purified cells contained less than 1% of free radioactivity. Quantification of vital cells was performed by manual counting using a Neubauer chamber and trypan blue. *In vitro* incubation of the purified cells for 1 h at 37˚C in glucose-free buffer led to a release of less than 3% of radioactivity in the supernatant. Vitality rate prior to labelling was 97.5±1.5% and at post labelling it was 96±2.3%. Separate incubation of samples of labelled and non-labelled cells for 9 h at 27˚C did not lead to differences in vitality (labelled: 88±3.2%, non-labelled: 90±2.5%). The radiation dose to the tumour cells during loading with FDG and purification was estimated by use of the nodule module of MIRDOSE 3.1 software (Stabin MG, USA) for a spherical volume of 10 ml and a homogeneously distributed activity of 100 MBq ^{18}F.

In vivo monitoring of the tumour cell biokinetics by dPET. Altogether, six NK cell-depleted rats and six controls were investigated. Animals were anaesthetized using isofluorane inhalation and an *i.v.* line was introduced into the lateral tail vein. They were positioned in a holding device allowing optimum spatial resolution (4.5 mm intrinsic axial resolution as measured by phantom studies) in a calibrated clinical PET scanner (HR+, Siemens/CPS, Knoxville, USA), 3D mode, matrix 256, filter 2.0, iterative reconstruction by ordered subset expectation maximisation (OSEM; Siemens, Knoxville, USA). The maximal distance of animals from the centre of the field of view was 10 cm. Each animal was injected with 0.5 (±0.2) MBq radioactivity in 1.0×10^6 of MADB106 tumour cells. Sequential scans were taken from 0 to 40 min post injection (*p.i.*) (frame duration: 10×30 s, 10×60 s, 5×300 s, 2×600 s). After reconstruction of the whole-body images, time-activity curves of the lungs were generated by means of the region of interest (ROI) technique. ROIs were positioned over the lungs, the brain and the bladder. Mean uptake values were obtained.

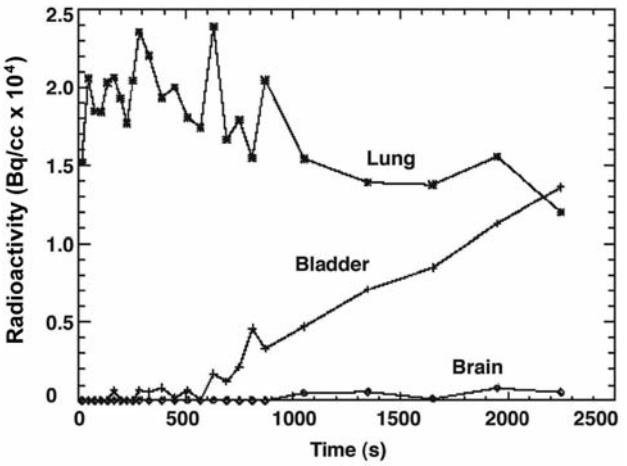

Figure 1. *Early biokinetics of 2'-[^{18}F]-2'-deoxy-D-glucose (FDG) loaded MADB106 tumour cells in different organs of natural killer (NK) cell-depleted rat after inoculation. Radioactivity over the lungs peaked quickly within first 60 s, followed by a slow decline. Onset of accumulation of radioactivity in the bladder was found about 10 min post inoculation No significant brain uptake occurred during the observation time.*

Statistical analysis. The analysis of NK cell lytic activity towards tumour cells was calculated at each time point as a percentage relative to the peak activity. The statistical methods applied were according to Brown and Prescott (15). All statistical calculations were carried out using the PROC MIXED program from SAS Software package Release 8.1 (SAS Institute Inc., Cary, NC, USA). Significance tests for fixed and random effects were also performed within PROC MIXED. Specifically, for the estimated fixed effects, three tests were conducted. Data represent the means±standard error of the mean (SEM).

Results

Early biokinetics of tumour cells. Tumour cells accumulated instantaneously in the lungs and did not spread to other organs. After tumour cell inoculation, the radioactivity per ROI peaked over the lungs within 60 s *p.i.* then declined slowly (Figure 1). Significant accumulation of radioactivity in the bladder began after 10 min. Uptake of radioactivity by the brain was negligible throughout the course of the experiment, indicating minor release of free FDG into the blood. Typical PET images are shown in Figure 2.

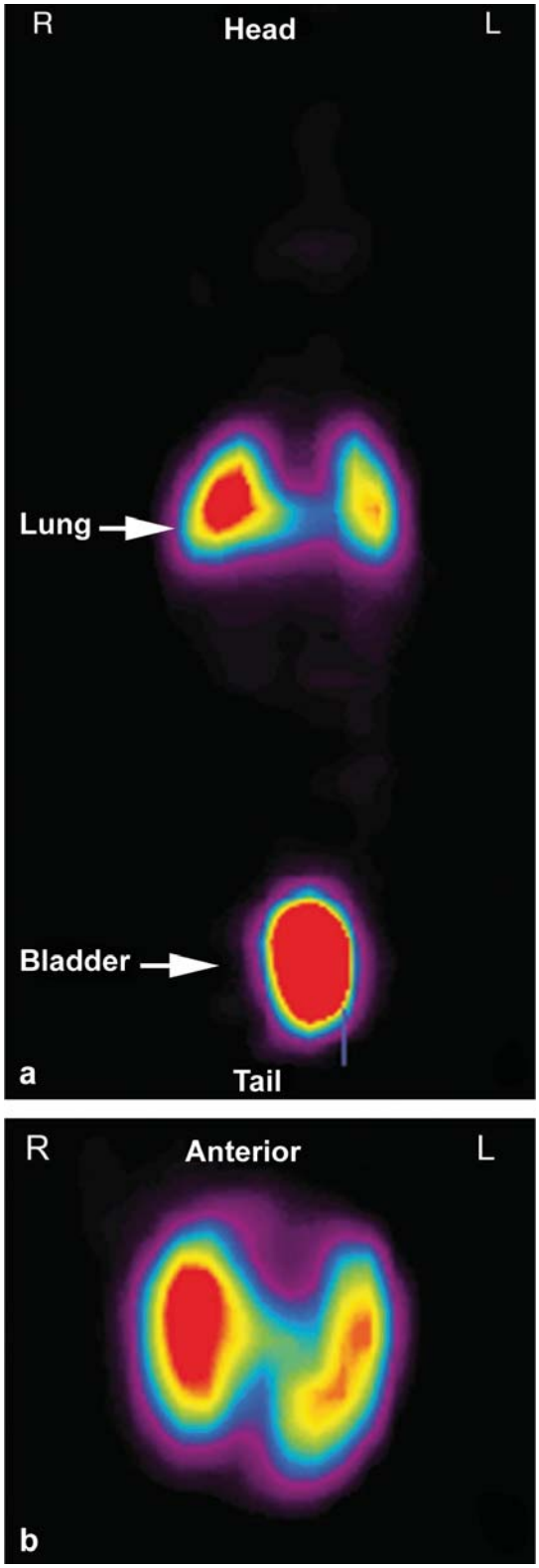

Figure 2. *Coronal (a) and transaxial (b) positron emission tomography (PET) slices showing the biodistribution of 2'-[^{18}F]-2'-deoxy-D-glucose (FDG)-labelled MADB106 tumour cells 40 min post inoculation. Only lung and urinary bladder showed accumulation of activity.*

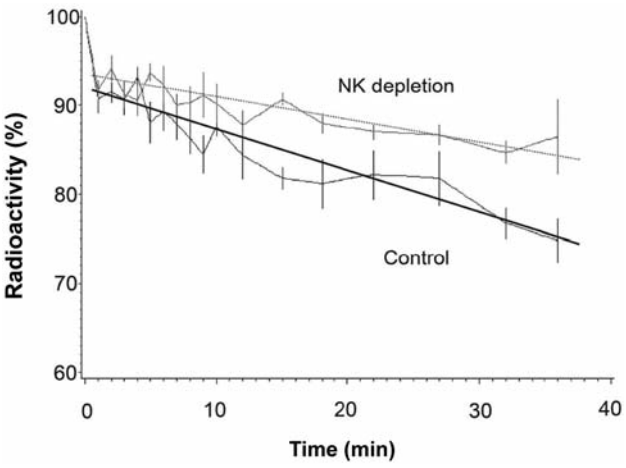

Figure 3. *The graph shows the kinetic changes of the mean values (±SEM) and the linear fit of the radioactivity as percentage of the initial activity in the lungs of each group.*

Tumour cell retention in the lungs. More than 95 % of the injected radioactivity was found in the lungs of all animals within 3 min *p.i*. The retention rates of radioactivity, however, differed in both groups of animals. The release of radioactivity from the tumour cells within the observation period of about 40 min in the NK-depleted group was lower than that in the controls (by 56%; $p<0.0001$) as shown in Figure 3 (about 15% *vs*. 24% of initial activity). Regression curves were calculated (mixed linear model): y(radioactivity)=92.7-0.49×t (time) [Controls] and y=93.5-0.27×t [NK-depleted animals]. The difference in intercepts of the curves at t=0 was not significant (p=0.4).

Discussion

By using dPET and the MADB106 model of lung metastases we showed that tumour cells accumulate in the lungs within seconds and do not seed to other organs. Tumour cell release from the lungs was found to start immediately after FDG-labelled tumour cell inoculation and was significantly slower in the NK cell-depleted group than in the control group.

The crucial point for translating these observations to cell kinetics relevant for immunology is whether intracorporal activity distribution represents distribution of tumour cells. Another question is whether the observed release of radioactivity from the lungs represents the net release of cells or whether a significant influx (or reflux) term has to be considered.

One reason for decoupling of activity from cells could be radiolysis of tumour cells. Therefore, we estimated the absolute dose to cells. Using 100 MBq FDG in a spherical volume of 10 ml, the radiation dose from deceleration of positrons and from annihilation of 511 keV photons was less

than 100 mGy. In addition, vitality testing prior to and post labelling did not show any degradation of cells. Furthermore, metabolic trapping of FDG is not reversible in the tumour cells used (16). Therefore, the low concentration of free radioactivity as released *in vitro* confirms the quality and stability of cell preparation.

Another reason for decoupling of activity from cells could be lysis of cells after incorporation, *e.g.* by splenic macrophages or NK cells. This lysis would result in release of FDG-6'-phosphate available for the kidneys, which could explain the appearance of activity in the urinary bladder. Theoretically, FDG-6'-phosphate could be reconverted to FDG by glucose-6'-phosphatase present in kidneys or liver. The presence of free FDG in serum would result in brain uptake of activity. Within the observation period of our experiments, however, there was no such significant uptake.

Apart from urinary bladder activity there was no activity accumulation in other organs. Therefore, and since there is no evidence for the appearance of free FDG in serum, one can assume that there was no prolonged afflux of activity to the lungs. Therefore, the rate of activity release from the lungs can be supposed to represent disintegration or release of tumour cells.

Thus, activity kinetics as studied with dPET provide information complementary to that derived from histological methods as they represent continuous observation and measurement within the entire organism.

From an immunological point of view, the findings of the present study are in line with previous results showing the preventative role of NK cells in metastatic seeding. NK cells have been shown to control the development of various experimental tumours (1, 13). In addition, defence mechanisms against MADB106 adenocarcinoma cells critically depend on NK tumouricidal activity (7, 17). Early studies by van den Brink demonstrated specific infiltration of NK cells in developing metastatic lesions 10-14 days after tumour inoculation (9). Our recent studies based on immunohistological quantification of vital dye-labelled tumour cells and co-localized NK cells in the lung vascular bed (7) suggest that NK cells trigger tumour target-specific responses as soon as within the first hour after tumour inoculation *in vivo*. As shown by depletion experiments, a lack of NK cells drastically increased the number of tumour cells retained in the lungs within the first hour (7). These initial NK cell-mediated processes appear to be critical for clinical outcome since the formation of cancer metastases dramatically increased under NK-depleted conditions.

In conclusion, early kinetics of tumour cells after injection were defined. There was no significant tumour cell-homing to organs other than the lungs, and tumour cell release or disintegration was significantly slower in animals with depletion of NK cells compared to controls. PET with FDG revealed to be an adequate method to further investigate novel options for exploiting the cellular host defence mechanisms in cancer patients.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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