Development of novel radiolabeled anti-CD20 antibody fragment for B-lymphocyte imaging in Non-Hodgkin's lymphoma patients

Summary of research proposal

B-lymphocytes play an important role in pathogenesis of Non-Hodgkin's lymphoma (NHL). NHL must be characterized with certainty before therapy is initiated. Approx. 90% of B-lymphocyte expressed CD20, therefore particularly suitable target for immunoimaging. Although an anti-CD20 probe is required for in-vivo imaging of CD20 positive B-lymphocyte infiltration. Radiolabeled monoclonal antibody (mAb) offers possibility to target tumour antigens for immunoimaging. However, intact antibodies are large proteins (~150 kDa) with long in-vivo half-lives and low penetration into tumours. Prolonged residence time in blood and subsequently extensive radioactive exposure to normal organs can be toxic and lead to organ failure. To overcome the limitations of intact antibodies, we planned to produce smaller antibody fragments (Fab;~50 kDa). Due to their small size, these fragments may exhibit improved tumour penetration and rapid clearance from the blood; and allows radiolabelling with positron emitter isotopes, which will allow a high resolution Positron Emission Tomography (PET) imaging. Moreover, the innovative PET imaging technique is quantitative, non-invasive, and can therefore be repeated over time, to follow-up dose-dependency and the time course of drug effects. Therefore, aim of this project is to develop and evaluate a new radiolabeled anti-CD20 Fab fragment for molecular imaging of tumour infiltrating CD20 positive B-lymphocytes.

KEYWORDS: B-lymphocyte infiltration, Anti-CD20 Fab fragments, PET imaging, Non-Hodgkin’s lymphoma, Therapy decision making.

Project description

- The subject and aim of the research

Response to therapy of NHL represents an important prognostic indicator and plays an important role in therapy planning, where an early detection of relapse of lymphoma may increase the chances of long term survival. The evaluation of possibly persistent minimal residual disease is also of great importance. Moreover, therapy planning and response to the therapy are highly dependent on accurate classification of the various types of lymphoma and staging. B-cells are involved in the production of antibodies, T-cell activation, pro-inflammatory cytokine production, and play an important role in the pathogenesis of NHL. These cells have been found in pathological infiltrates in affected tissues and, are implicated in disease progression. CD20 is expressed on approx. 90% of B-lymphocyte from blood and lymphoid organs, moreover, it homogeneously expressed on the cell surface with generally high antigen number (>100,000) per cell, therefore particularly suitable target for immunotherapy and immunoimaging. Anti-CD20 therapies for NHL patients include several FDA approved mAbs, e.g. Rituximab (Mabthera®), Tositumomab (Bexxar®), Ibritumomab Tiuxetan (Zevalin®) and Ofatumumab (Arzerra™).

These data highlighted the opportunity to use a radiolabeled anti-CD20 probe for in-vivo imaging of CD20 positive B-lymphocyte infiltration in NHL. Such a probe could allow non-invasive evaluation of severity and disease extension in NHL. This method could also be useful for patient selection and monitoring the efficacy of anti-CD20 mAb therapy. Therefore, the aim of this study is, to explore the possibility of radiolabelling of anti-CD20 Fab fragments for molecular imaging of NHL. This will help for the early detection, staging,
remission assessment, monitoring for metastatic spread and tumour recurrence, and assessment of CD20 expression prior to immunotherapy/ radio-immunotherapy.

- **Innovative aspect of research proposal**

Radiolabeled antibodies offer the possibility to target tumour surface antigens for immunoimaging (1-4). However, intact antibodies are large proteins (~150 kDa) with long serum half-lives and low penetration into tumours. The prolonged residence time in the blood and subsequently extensive radioactive exposure to normal organs can be toxic and lead to organ failure. To overcome the limitations of intact antibodies, we planned to use protein engineering methods to produce smaller antibody fragments (Fab fragments; ~50 kDa). Due to their smaller size, these fragments may exhibit improved tumour penetration and rapid clearance from the blood and non-target tissues through kidneys; and allows radiolabelling with positron emitter isotopes for PET imaging. Moreover, use of Fab fragments will reduce nonspecific binding that results from Fc portion interactions of the complete antibody, and therefore also elimination of Fc-associated effectors functions. Consequently, for *in vivo* imaging, Fab fragments produce lower immunogenicity in comparison with an intact antibody.

- **Research method(s) to be used**

*Phase I: Preparation of radiolabelled-anti-CD20 Fab*

**A. Production of Fab fragments from anti-CD20 monoclonal antibody**

We will use immobilized papain-agarose gel to produce Fab fragments of an anti-CD20 monoclonal antibody (Mabthera®, Roche). Papain is a nonspecific thiol-endopeptidase that enzymatically cleaves whole IgG just above the hinge region to create two separate Fab fragments and one Fc fragment per antibody molecule (5). Since, the papain protease will be used in immobilized form as beaded agarose resin, the digestion reaction will be easily stopped by removing the resin from the IgG solution; the final result will be digest enzyme-free Fab fragments.

**B. Quality control of anti-CD20 Fab fragments**

The purity and homogeneity of anti-CD20 Fab fragments will be determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. In addition, Fab fragments will be analysed by high performance liquid chromatography (HPLC).

**C. Labelling of anti-CD20 Fab fragments with $^{18}$F for PET imaging**

Because of its smaller size, pharmacokinetics of the Fab fragments of mAb is usually sufficiently fast; therefore we will radiolabel it with positron emitter isotopes $^{18}$F (half-life 110 min).

Labelling of Fab fragments with $^{18}$F will be performed by conjugation of Fab fragments with N-succinimidyl 4-[$^{18}$F]fluorobenzoate ($[^{18}$F]SFB). $[^{18}$F]SFB is an activated ester of 4-[$^{18}$F]fluorobenzoic acid and is prepared via a 3-step process as previously described by Gu et al. (6). We will prepare $^{18}$F-Fab fragments by reacting $[^{18}$F]SFB with Fab fragments. Above method might be subjected to slight modification depending on results obtained.

*Phase II: Quality controls and in-vitro studies*

**D. Radiochemical purity, Structural integrity and Stability assay**

After labelling, HPLC will be performed to calculate the labelling efficiency. Possible modifications induced by the labelling process on Fab fragments will be tested by SDS-PAGE of the final product. The labelling stability will be assessed in plasma and saline at 37°C, by HPLC.

**E. in-vitro binding assay**

An *in-vitro* binding assay on Burkitt lymphoma cell lines will be performed in order to test
the binding affinity and calculate the $K_d$ of radiolabelled Fab fragments (7).

**F. Immune-reactivity fraction (IRF) assay**

An IRF assay will be performed for determining the fraction of immune-reactive Fab fragments after radiolabelling process, by linear extrapolation to conditions representing infinite antigen excess (7).

**Phase III: in-vivo studies**

**G. in-vivo biodistribution study**

Balb/c nude mice (n=12) will be injected in tail vein, with radiolabeled Fab fragments; $\mu$PET images will be acquired at different time points. Images will be analysed qualitatively and quantitatively. $\mu$PET images will be acquired under anaesthesia. After in-vivo imaging, mice will be sacrificed in groups (each group n=4) at different time-points and all major parts (heart, lungs, liver, spleen, kidney, stomach, small bowel, large bowel and blood) will be collected to count the associated radioactivity expressed as percentage of injected dose per gram of tissue (%ID/g).

**H. in-vivo specific binding of radiolabeled anti-CD20 Fab fragments**

We will perform an in-vivo blocking assay to assess to what extent the uptake and retention of radiolabeled anti-CD20 Fab fragments to CD20-positive cells could be displaced by an excess of unlabeled antibody. To this aim, nude BALB/c mice (n=8) will be subcutaneously xenografted with $20 \times 10^6$ CD20 positive Burritt lymphoma cells in Matrigel in the right thigh. As a control, mice will be implanted with the same volume of Matrigel, without cells in the left thigh. 4 mice will be injected i.v. with a mixture of a 100-fold excess of unlabeled anti-CD20 Fab fragments after that with a tracer dose of radiolabeled anti-CD20 Fab fragments; the remaining mice (n=4) will receive only radiolabeled Fab fragments. $\mu$PET images will be acquired. Afterwards mice will be sacrificed and organ radioactivity will be counted.

**I. in-vivo targeting experiment in Burritt lymphoma cells xenografted mice**

To evaluate whether the radiolabeled CD20 Fab fragments imaging signal correlates with the number of CD20 positive Burritt lymphoma cells in-vivo, we will perform a targeting experiment in Balb/c mice (n=12). Mice will be divided into 3 groups (n=4 each) and subcutaneously implanted with an increasing number of Burritt lymphoma cells in the left thigh ($4 \times 10^6$, $8 \times 10^6$, or $16 \times 10^6$cells, respectively). In the right thigh, mice will be implanted with Matrigel alone as control. Mice will be injected, in the tail vein, with radiolabeled CD20 Fab fragments, $\mu$PET images will be acquired after different time points. After the imaging experiment, an ex-vivo counting of the two thighs will also be performed.

**- Current state of knowledge in the field and how the planned research will advance this**

Both conventional morphological imaging modalities (computerized tomography, ultrasound and magnetic resonance imaging, and functional imaging: 67-Gallium scintigraphy, $^{18}$F-fluoro-deoxy-glucose positron emission tomography ($^{18}$F-FDG PET) and radioimmunoscintigraphy (RIS) play a role in establishing tumour stage, restaging residual masses and relapse, and therapy response monitoring. It is desirable to have an imaging tool available that could provide an index of the grade and proliferative activity of the tumour and also demonstrate early response to treatment even when the size of the tumour remains unchanged. Until recently the best available functional modality for the evaluation of treatment response and for the prediction of outcome in lymphoma patients was 67-gallium scintigraphy, however, this has now largely been replaced by $^{18}$F-FDG PET imaging, when available. $^{18}$F-FDG PET shows a higher sensitivity compared with RIS as the total tumour burden increases, but lacks specificity, which is the crucial advantage of RIS. In addition, RIS is of essential importance when treatment with radiolabeled monoclonal antibodies is considered.

In search for a more specific imaging approach, and possibly reduced radiation burden, we come out with an innovative project, which is proposed here.
References