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In vivo fluorescence imaging of apoptosis during foreign body response

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ABSTRACT

Quantification of apoptotic tissues during inflammatory processes induced by biomaterials is challenging *in vivo*. Here we present a non-invasive method using a fluorescence imaging system which facilitates intermittent snap shots of the current state of local apoptotic tissue. For this purpose, apoptotic cells around two different subcutaneously implanted materials (titanium discs and copper-coated titanium discs) in hairless but immunocompetent mice were quantified after 4, 8 and 23 days of implantation. For validation, the results of fluorescence signals were compared to the histology of the inflammatory tissue using apoptotic-specific TUNEL-, macrophage-specific F4/80-, neutrophile-specific NIMP-R14- and chloroacetate esterase-staining. We could demonstrate that the fluorescence signals were well suited to quantify the extent of apoptosis *in vivo* and this is a good indication for the biocompatibility of biomaterials. This study shows that non-invasive monitoring of tissue processes following the implantation of biomaterials is possible *in vivo* and may help to reduce the number of animals in studies addressing biocompatibility.

1. Introduction

The foreign body reaction (FBR) is a mandatory, very complex and dynamic inflammatory process resulting from the immunological reactions originating both from injured tissues and the presence of a grafted biomaterial or an implant [1]. The purpose of the FBR is the effort of the organism to reduce the injurious stimuli and to initiate the healing process. In general, immigrating granulocytes and especially neutrophils dominate during the first days. This acute inflammation phase is proceeded by the chronic inflammation or healing phase which is characterised by the infiltration of macrophages, the presence of polynuclear foreign body giant cells and the formation of a fibrotic encapsulation of the biomaterial [2,3]. All these events are accompanied by apoptotic processes which are necessary to clean the injured and inflamed tissue and to support the healing and regeneration mechanisms. However, the FBR and apoptosis are is very difficult to follow *in vivo*, because of insufficient techniques and often hardly accessible implantation sites. Moreover, the intensity and phase lengths of the FBR are changing according to the biocompatibility of the implant and the reactions of the host tissues. Traditionally, tissue reactions are assessed via histological techniques at various time intervals requiring multiple animals for the whole study period. Therefore, an *in vivo* imaging system would be very helpful to assess tissue reactions and to follow the FBR in the living animal continuously.

Here we present a non-invasive method using a fluorescence imaging system that quantifies the extent of apoptosis *in vivo*. For establishing this method we used copper-coated titanium discs as FBR inducing controls. Copper is a cytotoxic element that induces apoptosis [4,5]. We compared the induced FBR with the tissue response induced by discs of pure titanium. By comparing this method with data from classical histological stainings, we can show that the non-invasive fluorescence imaging system can be a valuable tool in biocompatibility studies.

2. Materials and methods

2.1. Animals and study design

The animal experiment was conducted under an ethic committee approved protocol in accordance with German federal animal welfare legislation (Ref.-No. 33.9-42502-04-08/1499) and in accordance with the National Institute of Health guidelines for the use of laboratory animals.

Experiments were performed in female hairless but immunocompetent Crl:SKH1-*hr* mice aged 17–18 weeks with an averaged body weight of 29.03 ± 2.3 g (Charles River Laboratories, Sulzfeld, Germany). Each mouse was housed individually, received a standard diet of Altromin 1324 (Altromin Spezialfutter,

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Lage, Germany) *ad libitum* and had free access to drinking water. The animal husbandry had a temperature of 22 ± 2 °C, a relative humidity of 60 ± 5%, and was illuminated by artificial light 12 h a day starting at 7 am.

Fifteen mice were assigned randomly into three experimental groups ($n = 5$, each). The first group was imaged *in vivo* and then sacrificed for histology at day 4 after implantation. The second group was imaged *in vivo* after 4 and 8 days and then sacrificed, the third group was imaged *in vivo* after 4, 8 and 23 days and then sacrificed. During the whole study period, all mice were monitored clinically every day by experienced veterinarians according to van Griensven [6].

2.2. Implant materials

The complete production and processing of the implants was done at the North Carolina A&T State University. The copper-coating of the electropolished titanium discs was done by unbalanced DC sputtering method using an ATC Model 1800 F magnetron sputtering system. 99.995% purity copper target was used for depositions. Samples were cleaned thoroughly using acetone, 10N HNO_3, deionised water and methanol. In-situ RF plasma cleaning was applied prior to deposition for 30 min at the power of 15 W and air pressure of 20 mTorr. Deposition was performed on one side of the titanium discs at room temperature, at DC power of 200 W, an air working pressure of 2 mTorr and a deposition rate of 0.41 nm/s resulting in a 200 nm thick copper layer. The titanium implants (controls) were 8 mm in diameter and 1000 nm thick, the copper-coated implants (implants for intensive FBR) were 8 mm in diameter and 1200 nm thick [7–9]. All implants were gamma-sterilised prior to implantation (BBF Sterilisations service, Kernen-Rommelshausen, Germany).

2.3. Implantation procedure

Animals were placed in an abdominal position on a heating mat and anesthetised by intraperitoneal injection of xylazine 2% (10 mg/kg body weight; Rompun®, Bayer Health Care, Leverkusen, Germany) and ketamine 10% (100 mg/kg body weight; KetaminGräub®, Albrecht, Aulendorf, Germany) and the dorsal skin was cleaned according to chirurgical guidelines. Two longitudinal incisions of 1 cm each were made in the median line through the full thickness of the skin. Subcutaneous pockets between the facies of the dorsal muscles and the subcutaneous tissue were created and the implants were placed in these pockets. The skin was closed with resorbable surgical suture material (Vicryl, Ethicon, Johnson & Johnson, Neuss, Germany).

2.4. Multispectral acquisition and analysis system

The *in vivo* fluorescence imaging system *Maestro*™ from CRi (Minnesota, USA) is a special camera and software system that allows the imaging of whole small rodents. A liquid crystal tuneable filter prior to the CCD detector records the emission spectrum in 2 nm increments. The images taken at different wavelengths are assembled to a so-called "cube". The data within this cube can be used to define the individual spectra of both autofluorescence and specific fluorescence signals. The *Maestro*™ system is capable to magnify the area of interest, but it cannot resolve single cells. The penetration depth is generally increasing with higher excitation wavelengths and fluorophors with emission spectra in the near infrared region [10,11]. According to our observations, the fluorophor can be detected in the intestine which is several millimetres below the skin, thus subcutaneous observations are feasible with the *Maestro*™ system.

2.5. Tagging apoptotic cells in vivo by SR-FLIVO™

The dye SR-FLIVO™ (ImmunoChemistry Technologies, LLC, Minnesota, USA) is a cell-permeable, non-cytotoxic, red fluorescent and covalent-binding inhibitor of apoptosis-specific caspases [12,13]. The SR-FLIVO™ probe was prepared according to the user instruction and adjusted to the body weight of every single mouse. 100 µl SR-FLIVO™ was administered intravenously 60 min prior to imaging. Unbound dye is eliminated via the liver (about 1 h after administration). The apoptotic cells in the inflamed area exhibit a fluorescence, which can be easily monitored. Regions of interest (ROI) were created in order to quantify the fluorescence signal intensities of the corresponding implant (Fig. 1d). For *in vivo* imaging, the mice were anaesthetised as described above.

2.6. Histology

After 4, 8 and 23 days, respectively, the discs were removed and the surrounding tissues were explanted and fixed in 3.7% buffered commercial formalin (Otto Fischar, Saarbrücken, Germany) for 24 h. According to standard procedures, tissues were dehydrated and embedded in paraffin using an automated embedding system (Pathcentre Tissue Processor, Shandon, Dreieich, Germany) to assure optimal quality. Using a Leica RM 2155 microtome (Leica, Bensheim, Germany), 5 µm thin sections were cut, mounted on poly-L-lysine coated glass slides (Sigma, Taufkirchen, Germany) and dried for at least 24 h at 37 °C.

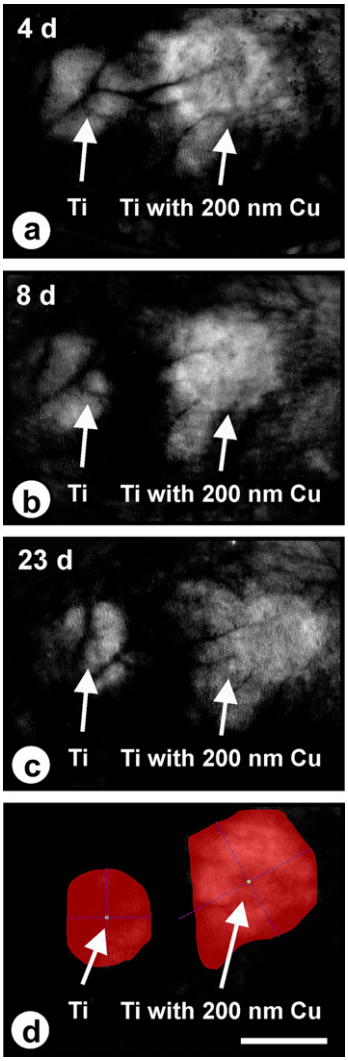

Fig. 1. Pure signal images of the apoptosis signal and the drawn regions of interest for quantifying the data. (a) Pure apoptosis signal 4 days after implantation. The titanium implant shows an apoptosis signal that is strictly limited to the area of the implant itself, whereas the apoptosis signal of the copper-coated implant is diffusely spread beyond the disc shape. (b) Pure apoptosis signal 8 days after implantation. The titanium signal as well as the copper-coated titanium signal declines and the shape of the copper-coated implant is more recognisable. (c) Pure apoptosis signal 23 days after implantation. The titanium signal as well as the copper-coated titanium signal further diminishes. (d) Regions of interest created around the two implants were used to quantify the apoptosis signal. Scale bar = 8 mm.

2.7. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL)

To identify apoptotic cells, the DeadEnd Colorimetric TUNEL System from Promega (Mannheim, Germany) was used. Shortly, biotinylated nucleotides were incorporated at the 3′-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase (TdT). Horseradish peroxidase labelled streptavidin was then bound to these biotinylated nucleotides. Peroxidase activity was visualised using the liquid DAB substrate chromogen system (Dako, Hamburg, Germany). In case of antibody-TUNEL double stainings, Cy2- labelled streptavidin (Dianova, Hamburg, Germany) was used.

2.8. Chloroacetate esterase stain (polynuclear granulocytes)

The rehydrated sections were first washed in distilled water and then incubated with naphthol AS-D chloroacetate (Sigma) in 4% pararosaniline (Chroma, Olching, Germany) and 4% sodium nitrate in 0.1 M acetate buffer for 120 min. Sections were washed in distilled water and mounted with Aquatex (Merck, Darmstadt, Germany). All cells containing red-brownish granules were regarded as positive. Control sections were incubated without the substrate. No staining developed in these controls.