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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.

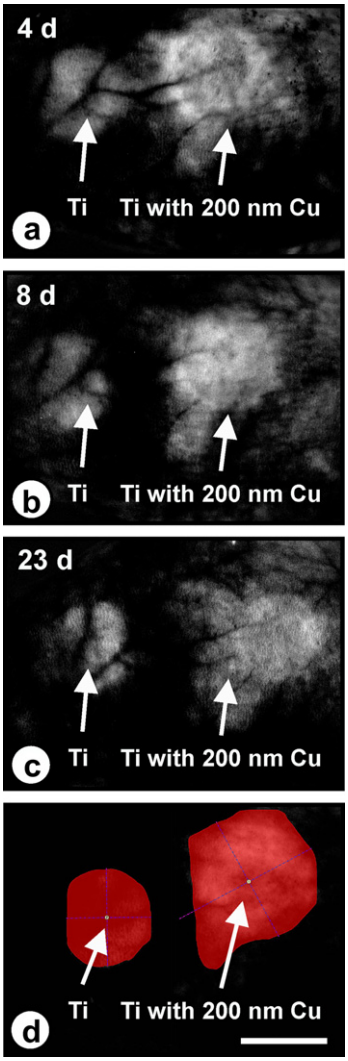

Fig. 1. Pure signal images of the apoptosis signal and the drawn regions of interest for quantifying the data. (a) Pure apoptosis signal 4 days after implantation. The titanium implant shows an apoptosis signal that is strictly limited to the area of the implant itself, whereas the apoptosis signal of 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.

2.9. F4/80 (macrophages) and NIMP-R14 (neutrophils) immunohistochemistry

The rehydrated sections were first mildly digested for antigen retrieval with 2% proteinase K (Dako) in tris buffered saline (TBS) for 20 min at 37 °C. After washing in TBS, sections were preincubated with a solution of 10% normal goat serum (Vector/Linaris, Wertheim-Bettingen, Germany) in TBS to block unspecific binding. The primary antibody was applied for 60 min at room temperature (rat-anti-F4/80 [clone CL:A3-1] or rat-anti-NIMP-R14, both from abcam, Cambridge, UK). The sections were washed three times in TBS (5 min each), followed by a 30 min incubation with a horseradish peroxidase labelled secondary antibody (goat-anti-rat, Dianova). Peroxidase activity was visualised using the liquid diaminobenzidine substrate chromogen system (Dako). In case of antibody-TUNEL double stainings, first the antibody staining and then the TUNEL staining was performed. The antibody detection was carried out then using a Cy3-labelled secondary antibody (goat-anti-rat, Dianova) which was applied for 30 min at room temperature. Sections were washed twice with TBS and distilled water and finally mounted with Aquatex (Merck).

2.10. Microscopy and photography

Photomicrographs were taken with a Zeiss Axioskop 40 microscope combined with a Zeiss AxioCam Mrc digital camera and Zeiss AxioVision software (all from Carl Zeiss AG, Oberkochen, Germany).

2.11. Cell counting and statistical analysis

Per every mouse and staining method one histologic slice was analysed. Positive cells were counted in a total area of 100,000 μm^2. As there were five animals per each implantation interval, the mean value and the standard deviation per each group was determined.

3. Results

3.1. Clinical observation of the implant related inflammatory process

At the day of implantation, the reddish copper layer of the coated titanium discs could be easily seen through the mouse skin. Swelling and a low-grade redness of the skin around the copper-coated implant could be observed from day 2 on and regressed after day 8. In contrast, the pure titanium implants did not evoke any signs of visible inflammation at any time. It could be observed easily through the skin throughout the complete study interval.

3.2. Detection of apoptotic cells with SR-FLIVO™

The SR-FLIVO™-signal could be detected directly above the pure titanium discs in all measurements (Fig. 1a–c). The initial signal intensity decreased from 455.71 scaled counts per second at day 4 to 194.03 scaled counts per second at day 8 after implantation. At day 23 after implantation, the signal intensity dropped to 122.86 scaled counts per second (Fig. 5a).

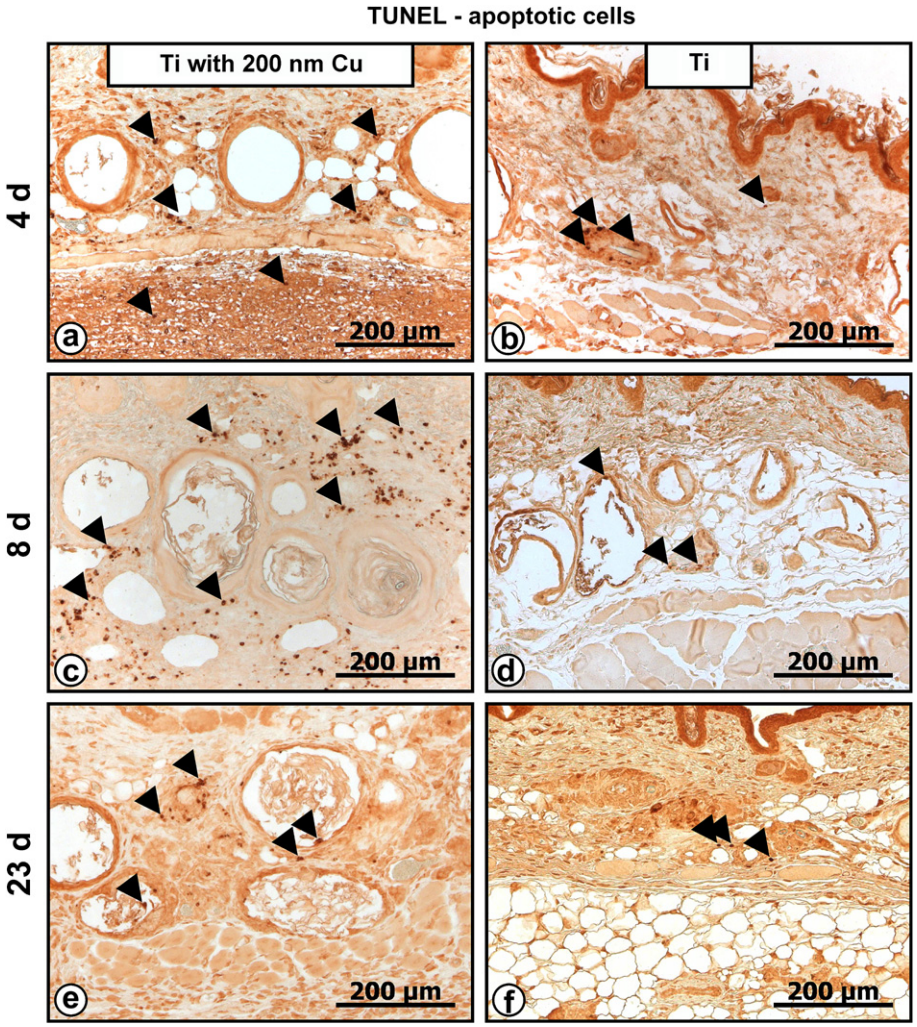

Fig. 2. Apoptotic cell-specific TUNEL staining of the implantation sites of the copper-coated discs (a, c, e) and the titanium discs (b, d, f) after 4 days, (a, b), 8 days (c, d) and 23 days (e, f) after implantation. There are much more apoptotic cells (some of them are marked by black arrowheads) in the tissues adjacent to the copper-coated discs compared to the titanium discs, especially after 4 and 8 days. Scale bars = 200 µm.

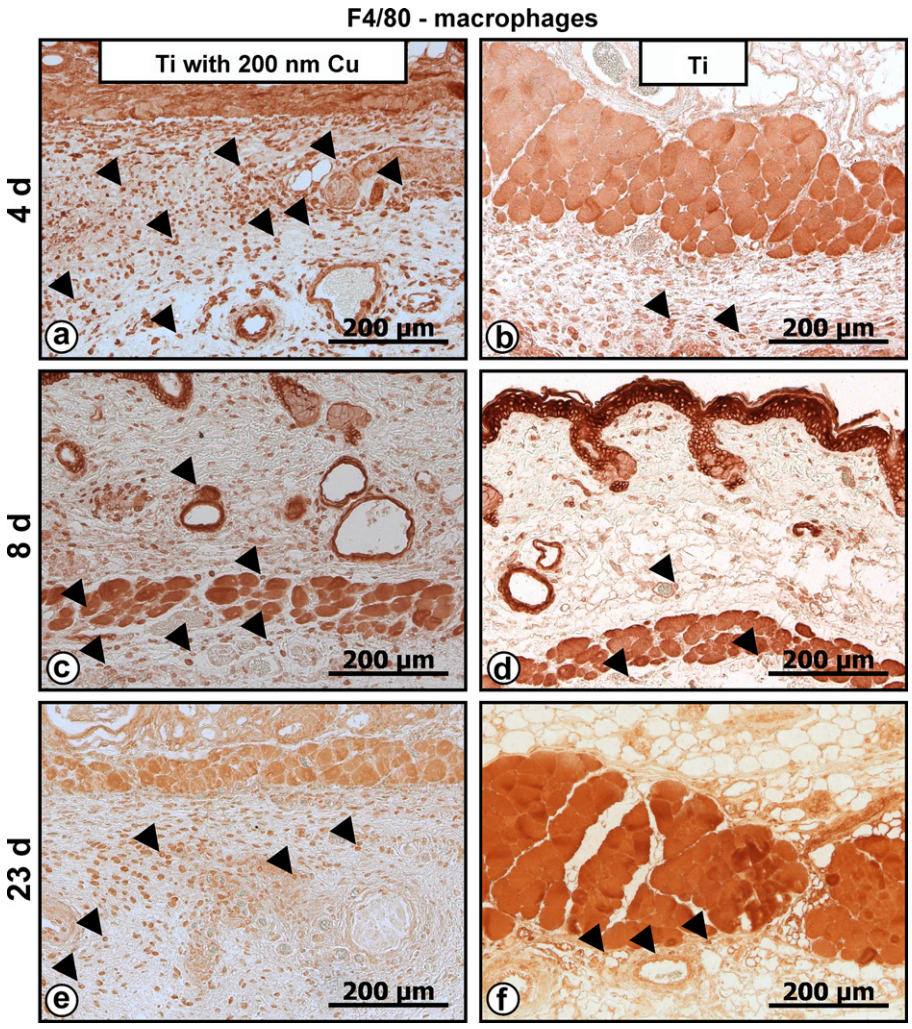

Fig. 3. Macrophage-specific F4/80-staining of the implantation sites of the copper-coated discs (a, c, e) and the titanium discs (b, d, f) after 4 days, (a, b), 8 days (c, d) and 23 days (e, f) after implantation. Especially after 4 days, there are much more macrophages (some of them are marked by black arrowheads) in the tissues around the copper-coated discs when compared to the titanium discs. Scale bars = 200 µm.

The SR-FLIVO™-signal could be detected above and adjacent to the copper-coated titanium implants (Fig. 1a–c) at all three time intervals. The initial signal intensity decreased from 693.45 scaled counts per second at day 4 to 659.05 scaled counts per second at day 8 after implantation. At day 23 after implantation, the signal intensity dropped to 331.82 scaled counts per second (Fig. 5a).

3.3. Coincidence of SR-FLIVO™-signal and presence of apoptotic cells

To investigate the cellular responses of the implant environments, we used apoptotic- neutrophil- and macrophage-specific markers for analysing cells of the FBR. As expected, the copper-coated titanium discs provoked a very intense FBR and the TUNEL staining shows very intense apoptotic processes (Fig. 2 and Fig. 5b). After 23 days, an approximately 300 µm thick foreign body capsule had been formed around these discs (data not shown). At day 4 after implantation, an average of 37.60 TUNEL-positive cells could be detected in the total analysed area of 100.000 μm^2. At day 8, the number of TUNEL-positive cells even slightly increased to 47.80 cells/area and decreased to 26.80 cells/area after 23 days. However, even at 23 days after implantation, there are much more apoptotic cells in the foreign body capsule around the copper-coated discs than in the capsule around the pure titanium discs.

The foreign body capsules around the titanium discs reached a thickness of approximately 150 µm (data not shown) and only very few inflammatory cells could be detected around the capsule. The number of apoptotic cells was very low throughout the whole observation period (day 4: 9,80 cells/area; day 8: 7.0 cells/area; day 23: 7.2 cells/area).

3.4. Predominant cells during different phases of inflammation

Both the F4/80- (Fig. 3 and Fig. 5c) and the chloroacetate esterase- (Fig. 4 and Fig. 5c) stainings map the acute and the chronic phase of the foreign body reaction. Chloroacetate esterase-positive granulocytes are present mainly during the acute phase, especially around the copper-coated titanium discs. There, the numbers decreased from 30.80 cells/area at day 4 to 19.50 cells/area at day 8 and 19.4 cells/area at day 23. Around the titanium discs, 21 cells/area (day 4), 15.2 cells/area (day 8) and 16.2 cells (day 23) were found (Fig. 4 and Fig. 5d). A comparable pattern showed the F4/80-positive cells. The highest values were detected around the copper-coated titanium discs. After 4 days, 74.06 cells/area could be detected. Then the numbers decreased to 34.03 cells/area (day 8), and 34.64 cells/area (day 23). Almost no F4/80-positive cells could be found around the titanium discs. After 4 days, only 9.50 cells/area could be detected, 15.80 cells/area after 8 days and 12.76 cells/

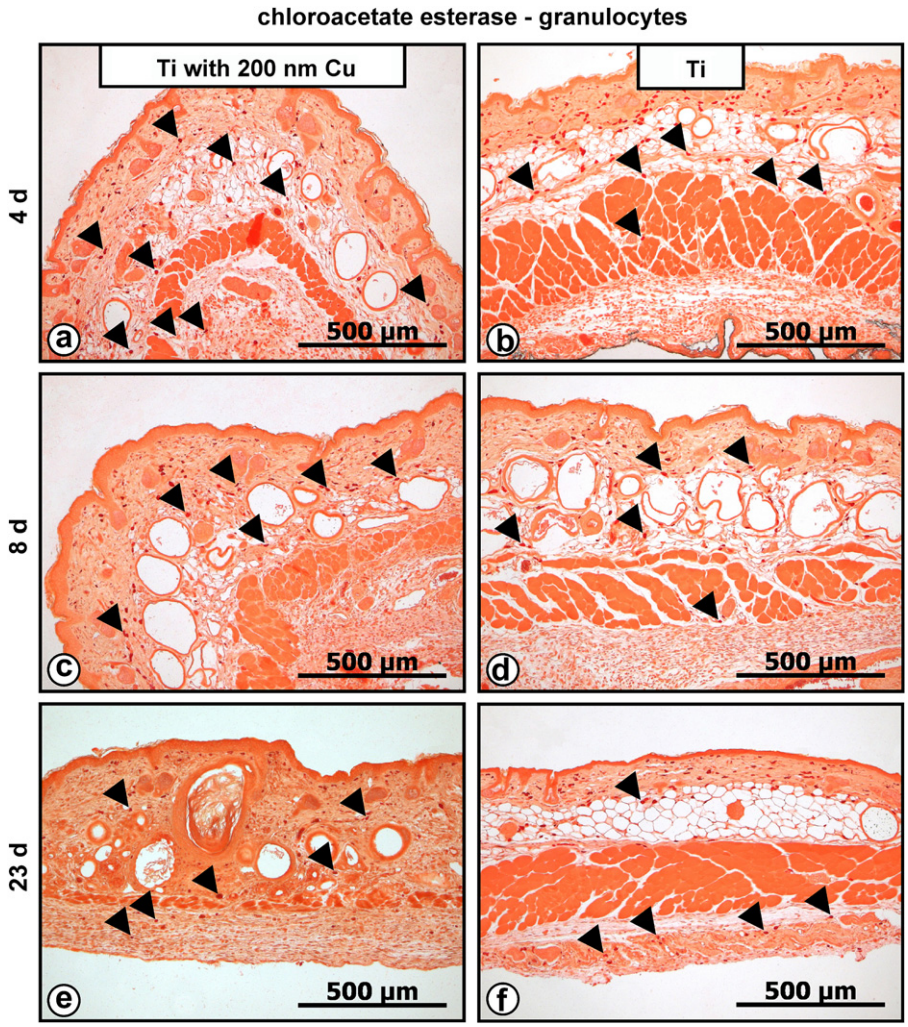

Fig. 4. Polynuclear granulocyte-specific chloroacetate esterase-staining of the implantation sites of the copper-coated discs (a, c, e) and the titanium discs (b, d, f) after 4 days, (a, b), 8 days (c, d) and 23 days (e, f) after implantation. There are more neutrophil cells (some of them are marked by black arrowheads) in the tissues adjacent to the copper-coated discs compared to the titanium discs. However, significant numbers of neutrophils can be also seen around the titanium discs. Scale bars = 500 µm.

area after 23 days (Fig. 3 and Fig. 5c). Double stainings of TUNEL with neutrophil-specific antibody NIMP-R14 and macrophage-specific antibody F4/80, respectively, revealed that during the acute phase of inflammation mainly neutrophils are apoptotic (Fig. 6a), whereas during later stages, mainly macrophages are apoptotic (Fig. 6b).

4. Discussion

The purpose of our present study was to introduce a non-invasive method using an *in vivo* fluorescence imaging system to follow apoptotic processes in the context of foreign body reactions. These processes are crucial responses of the host tissue to implants and determine the biocompatibility of biomaterials. Copper-coated titanium discs served as inflammation-inducing positive controls and pure titanium discs served as negative controls, since titanium based implants are widely accepted since they induce a minimal foreign body response after implantation. Visual examination as well as histological analysis revealed an inflammatory process connected with apoptosis above and around the copper-coated titanium discs. The tissue was intensively infiltrated with inflammatory cells, mainly neutrophils and macrophages. In these cases, the apoptosis signals were significantly higher around the copper-coated implants when compared to the pure titanium implants at any time after implantation. The data from the *in vivo* imaging system using SR-FLIVO™ and the histological data using the TUNEL staining showed coincident patterns. Due to the dissolved copper-ions from the disc surfaces, the apoptosis signal could be detected above and adjacent to the copper-coated titanium implant, whereas the signals around the non coated titanium discs were more localized and less intense. However, there are also some differences between the fluorescence signal and the histological results. The TUNEL staining showed an increase of apoptotic cells around the copper-coated titanium implant at day 8 after implantation when compared to day 4, whereas the SR-FLIVO™ data showed a slight, but continuous decrease. This variation could be due to the different targets of the detection methods. SR-FLIVO™ is caspase-3- and caspase-7-specific [12], both enzymes are involved in the initiation and execution of apoptosis. Moreover, activated caspases are found especially at the beginning of apoptosis, which is reflected by the higher SR-FLIVO™ signals both at day 4 and day 8 after implantation. Whereas the TUNEL staining detects fragmented DNA, which might also occur in late apoptosis stage as an effect of necrosis or as a preparation artefact [14,15]. Performing the *in vivo* measurements, the intensity of the apoptosis signal above and around the copper-coated implant as well as the apoptosis

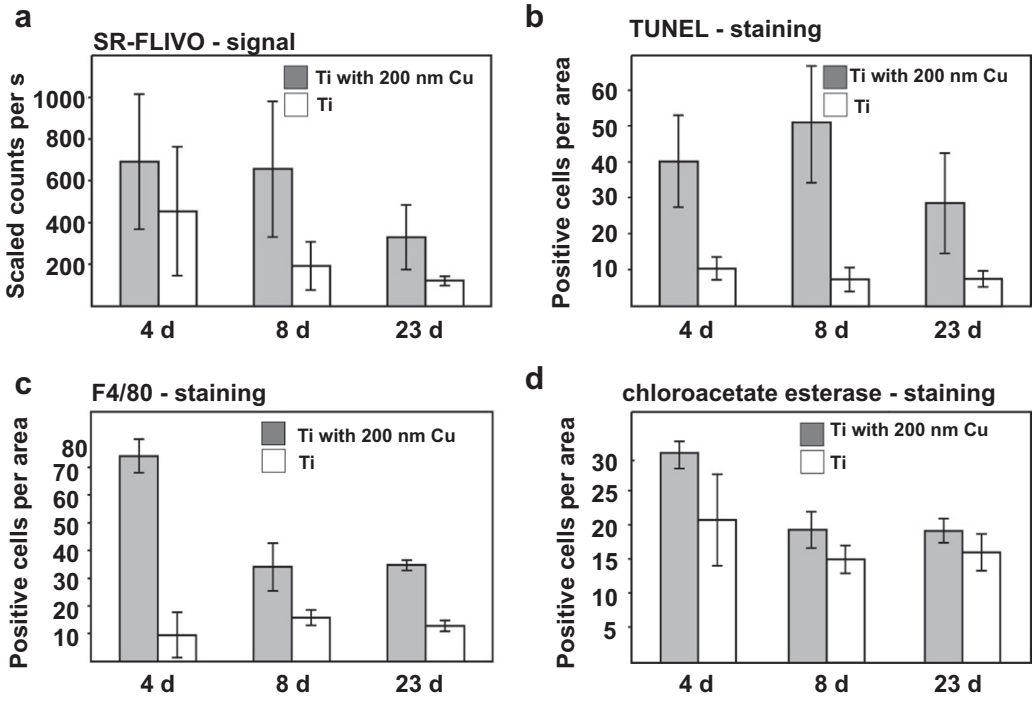

Fig. 5. Comparison of the SR-FLIVO™ signals (a), with the number of TUNEL- (b), F4/80- (c) and chloroacetate esterase- (d) Positive cells. The SR-FLIVO™ signal around the copper-coated discs seems to be enhanced with higher numbers of TUNEL-positive cells. The tissues around the titanium discs show much lower SR-FLIVO™ signals which is in line with the low number of apoptotic cells there. There are also much more F4/80-positive cells around the copper-coated discs, which decrease continuously with time. This time dependant pattern can be confirmed histologically by chloroacetate esterase-staining.

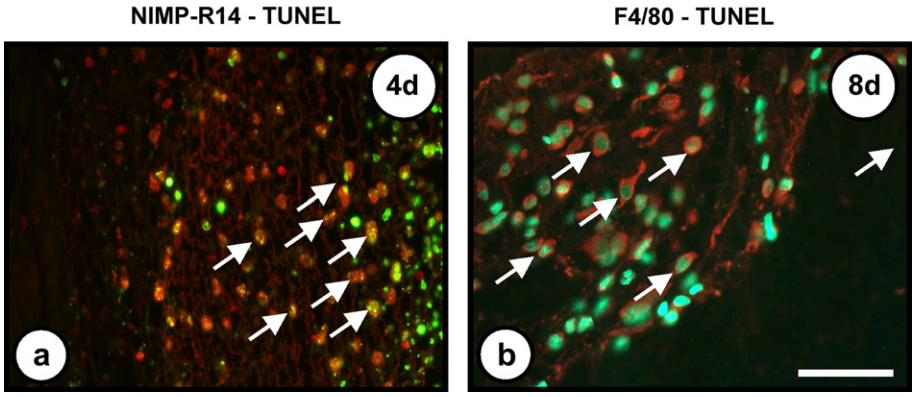

Fig. 6. Double stainings of neutrophil-specific antibody NIMP-R14 (a, after 4 days) and macrophage-specific antibody F4/80 (b, after 8 days; both detected with Cy3 in red) with TUNEL (detected with Cy2 in green) show that during the acute inflammation phase, mainly neutrophils are apoptotic, whereas during the chronic inflammation phase, mainly macrophages are apoptotic. Some of the double labelled cells are marked by white arrows. Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

signal above the pure titanium implant decreased from day 4 to day 23.

As a limitation of the fluorescence imaging method, an additional trauma is introduced by inserting the implants in a subcutaneous pocket by blunt dissection with scissors. Creating the implantation pocket for the discs, many cells were detached from their supportive matrix [3]. As reported by Anderson et al. [16,17], the distraction of an intact tissue evokes inflammation when placing a biomaterial in the *in vivo* environment and is followed by wound healing and a foreign body reaction as parts of the tissue response. However, if control implants (non coated discs) are compared to treatment groups (copper-coated implants) using the same surgical implantation method, a reliable comparison of the implant related apoptosis signal can be obtained.

This study demonstrates that the inflammatory response to implanted materials *in vivo* can be characterised non-destructively by using an *in vivo* fluorescence imaging system. This method may be very valuable to screen the foreign body reaction of novel biomaterials in which the time sequence of the foreign body reaction is unknown, such as with biodegradable metals [18].

5. Conclusions

The *in vivo* monitoring of apoptotic processes in the context of a foreign body reaction as a response to implanted biomaterials has been demonstrated and can be used to estimate the biocompatibility of a biomaterial. Furthermore, the fluorescent dye SR-FLIVO™ in combination with the Maestro™ System provides an excellent

opportunity to reduce the number of animals in biocompatibility studies.

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