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Quantitative Histopathology for Evaluation of In Vivo Biocompatibility Associated with Biomedical Implants

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Abstract

In the current chapter, digital morphometric analysis (DMA) was used to quantify two markers of biocompatibility around commonly used biomaterials. In the field of biomaterial evaluation for biocompatibility, more sophisticated methods are now being used to precisely characterize the elicited response from the surrounding tissue towards the implanted material. One reason for this is due to the fact that many newer biomaterial innovations are incorporating pharmaceutical agents (e.g., drug eluting stents and drug eluting balloons). Therefore, as described in many of the other chapters in this book, components of toxicology and pharmacology are being evaluated along with biocompatibility.

In this chapter, expanded polytetrafluoroethylene (ePTFE) was compared to polypropylene (PP) for inflammatory and foreign body response. Each material was implanted into dorsal subcutaneous spaces and evaluated after 2, 4, and 12 weeks. Each sample was reacted with an antibody to cluster of differentiation-68 (CD-68). The resulting slides were scanned and evaluated using DMA in order to obtain accurate, reproducible, and consistent results. Expanded PTFE demonstrated a lower overall weighted inflammatory score when compared to PP across all timepoints. This chapter describes the use of DMA as a novel approach to measure the inflammatory score that is associated with a specific biomaterial. Current and future medical devices will need to use various analytical tools to comprehensively assess device, biomaterial, or a combination therapy's biocompatibility. The next chapter further describes how quantitative data from histology and immunohistochemistry assessments can be coupled with quantitative polymerase chain reactions (PCR) as assessment tools for product development.

Key words Quantitative histopathology, Digital morphometric analysis (DMA), Biocompatibility, Biomedical devices, Medical devices, Biomedical implants, Medical implants, Expanded polytetrafluoroethylene (ePTFE), Polypropylene (PP), Inflammation, Inflammatory score, Foreign body response

1 Introduction

All materials elicit a tissue response when implanted into the body; therefore, when designing and evaluating new medical devices, the materials must undergo extensive biocompatibility testing. Biocompatibility is defined as the "ability of a material to perform with an appropriate response in a specific application" (1). The host tissue receiving an implant experiences a wound healing process that includes inflammation, foreign body reactions, and fibrous encapsulation (2). When normal tissue is disrupted, a healthy organism must be able to repair itself through the process of

wound healing. The normal wound healing model is characterized by four phases; *hemostasis*, *inflammation*, *proliferation,* and *remodeling*. These phases are not mutually exclusive, overlapping to various extents.

When biomaterials, either synthetic or biological, are implanted into the body, there is an altered response to wound healing (3, 4). A polymer-induced healing response initiates inflammation and a modified wound healing process through the initial implantation surgical procedure. It is understood that different polymers evoke varied wound healing responses that depend on the biocompatibility of each of these materials. However varied these responses are, there are some similarities in their healing characteristics and their deviations from normal wound healing.

Differences between various polymers and the elicited healing response first occur in the inflammatory phase of wound healing. The primary goal of inflammation is to neutralize or destroy an injurious or foreign agent as well as provide a fluid medium for the migration of repair cells (leukocytes and fibroblasts) to the area. Acute inflammation is relatively short lived, lasting minutes to days, and is characterized by polymorphonuclear leukocytes (PMN) and accompanied edema (2). Chronic inflammation can last much longer and remains localized to the implant site. In chronic inflammation the macrophage may very well be the most important cell based on the number of biologically active products it produces (2). Typically macrophages will persist during the presence of a foreign object, whether it is bacteria or implanted materials (3, 5).

The continuation of the chronic inflammatory response into a normal foreign body reaction is recognized by the chronic presence of foreign body giant cells (FBGC) with granulation tissue (2). With the chronic presence of these macrophages and foreign body giant cells, the late phase of inflammation may never resolve, causing the successive phases of normal wound healing to be hindered or never resolved. In the current study PP and ePTFE have been evaluated for the presence of macrophages and FBGCs. While it has been noted by Kellar et al. 2001 and Kidd et al. 2001, implantable materials need to be tested within the tissue the material is being designed for end use, the most common site for initial implantation during the development of a novel material is the subcutaneous space. Therefore evaluations of materials implanted in the subcutaneous locations were the focus of the current study. The subcutaneous space has been used extensively due to the relatively high-throughput, low-cost screening technique for the initial tissue response (6). This model also provides site-specific evaluation of the material to the biological interface that is often indicative of the healing that would be observed in other anatomical regions (7).

2 Materials and Methods

2.1 Slide Scanner

All glass slides were digitally scanned using the Aperio CS slide scanner with a 20× Olympus objective. At 20× magnification the Aperio scanner provides a digital image with a resolution of 0.5 μm/pixel (Aperio, Vista, CA).

2.2 Implants

The materials used were polypropylene mesh (Bard, Tempe, AZ) and thin-walled expanded polytetrafluoroethylene (Bard, Tempe, AZ). Four millimeter (4 mm) round punches were used for implantation into wild-type mouse models (129S1-Sv1mJ, Jackson Labs, Sacramento, CA). All animal studies were performed after approval of protocols by the Northern Arizona University Institutional Animal Care and Use Committee (IACUC). National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals were observed. Animals were housed in American Association for the Accreditation of Laboratory Animal Care approved facilities.

2.3 Histology and Immunohistochemistry

All explanted tissue samples were paraformaldehyde fixed, paraffin embedded, sectioned at 5 μm, and subsequently processed for immunohistochemistry. Sections were reacted with an antibody to cluster of differentiation-68 (CD-68) (Serotec, clone ED1, Raleigh, NC) used at a final dilution of 1:200. The primary antibody was visualized using a secondary antibody with a peroxidase reaction product recognition system (Universal mouse kit; Dako Inc., Carpinteria, CA). CD-68 is a protein that is expressed in the cytoplasm of activated macrophages and was selected for this analysis because of the high specificity in the current study because of the high specificity of the antibody resulting in a punctate cytoplasmic staining pattern (8, 9). This provides a distinct and clear positive signal for DMA.

2.4 Digital Algorithm

A commercially available algorithm was used to count the number of CD-68$^+$ cells (IHC Nuclear Image Analysis v9, Aperio, Vista, CA). The nuclear algorithm is a cellular counting algorithm which uses input factors based on cellular profiles. Cell parameters were defined by adjusting digital values including nuclear size, roundness, compactness, and elongation. These parameters are adjustable to assist the user with determining the appropriate amount of cellular segmentation. The user can adjust the color values based on the staining of interest. For example the user can use an "eye dropper" tool which chooses specific colors and gradients of color to use as the "positive stain" being measured as well as the background stain. The "eye dropper" tool then provides the user with a breakdown of the color into its red, blue, and green components. In the algorithm setup the user can also change the threshold

method in order to determine how the algorithm identifies the edges of the cell. This uses the colors that the user inputs and changes the way the algorithm segments or defines the cell. There is an "amplitude threshold" which adjusts according to the mean intensity of all the pixels and automatically thresholds to one sigma above the mean. The edge threshold method automatically adjusts the threshold according to the mean of edge pixels, using an edge finding method to identify the edge pixels and averages these values to determine the threshold. The manual threshold method uses an upper and lower limit set by the user to eliminate any unwanted background, but it will not automatically adjust to compensate for any lighter or darker staining between slides. The edge threshold method was used in the current study. The algorithm was adjusted using the parameters identified in the Aperio user's guide: (http://tmalab.jhmi.edu/aperiou/userguides/IHC_Nuclear.pdf).

3 Results

All values reported are averages ± standard error of the mean. All of the implants had been fully incorporated into the surrounding tissues at the time of explant.

3.1 Two Weeks

The ePTFE implants ($n = 5$) had an average CD-68 positive macrophage count of 442 ± 85.9; FBGC count of 21 ± 6.1. PP implants ($n = 4$) had an average CD-68 positive macrophage count of 2008.3 ± 213.8; FBGC count was 73.3 ± 10.7 (Fig. 1).

3.2 Four Weeks

Expanded PTFE implants ($n = 4$) had an average CD-68 positive macrophage count of 487.5 ± 107.9; FBGC count of 3 ± 1.5. PP

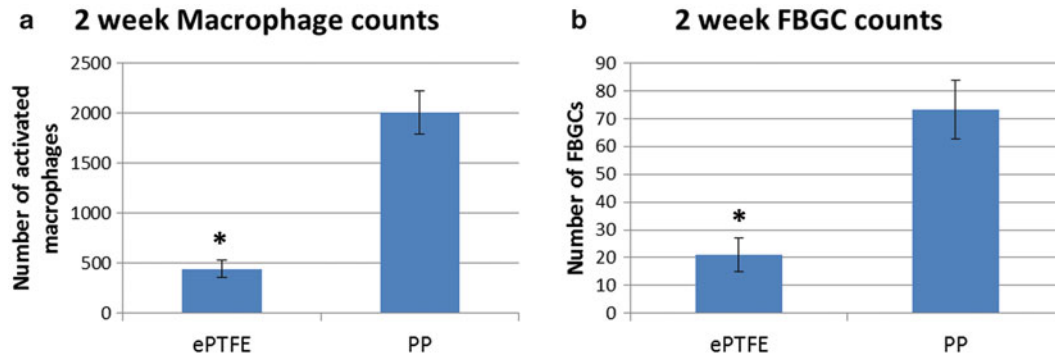

Fig. 1 Graphs depicting the number of cells counted around each material after being implanted for 2 weeks. (**a**) The average number of macrophages surrounding the ePTFE ($n = 5$) and PP ($n = 4$) implants *$p = 0.003$. (**b**) The number of FBGCs counted surrounding each implanted material *$p = 0.006$

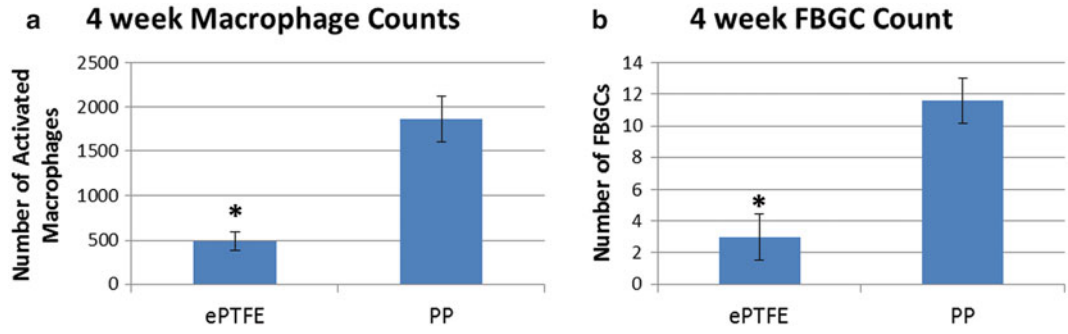

Fig. 2 Graphs depicting the number of cells counted around each material after being implanted for 4 weeks. (**a**) The average number of macrophages found surrounding the ePTFE ($n = 5$) and PP ($n = 4$) implants $^*p = 0.004$. (**b**) The number of FBGCs counted surrounding each implanted material $^*p = 0.004$

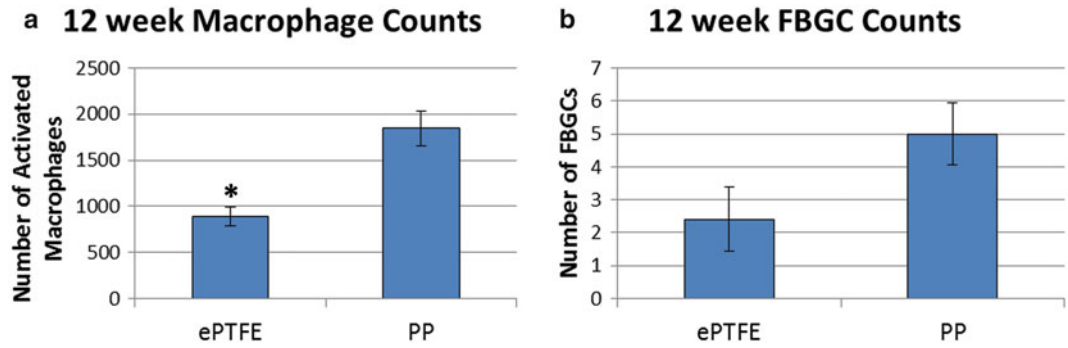

Fig. 3 Graphs depicting the number of cells counted around each material after being implanted for 12 weeks. (**a**) The average number of macrophages found surrounding the ePTFE ($n = 5$) and PP ($n = 5$) implants $^*p = 0.002$. (**b**) The number of FBGCs counted surrounding each implanted material. No significant difference

implants ($n = 5$) had an average CD-68 positive macrophage count of 1862.8 ± 259.5; FBGC count of 11.6 ± 1.4 (Fig. 2).

3.3 Twelve Weeks

Expanded PTFE implants ($n = 5$) had an average CD-68 positive macrophage count of 885 ± 102; FBGC count of 2.4 ± 1. PP implants ($n = 5$) had an average CD-68 positive macrophage count of 1844.2 ± 187.7; FBGC count of 5 ± 1 (Fig. 3).

In this study a very porous mesh material (PP) was being compared to a more solid material with less porosity (ePTFE). The mesh has a greater space between the woven material which could allow and possibly encourage macrophages to infiltrate and fill this space. To quantify the inflammatory/foreign body response, an equation was developed to provide weight to various staining intensities and provide a quantitative value to the macrophage and FBGC counts. This equation, the H-score, is

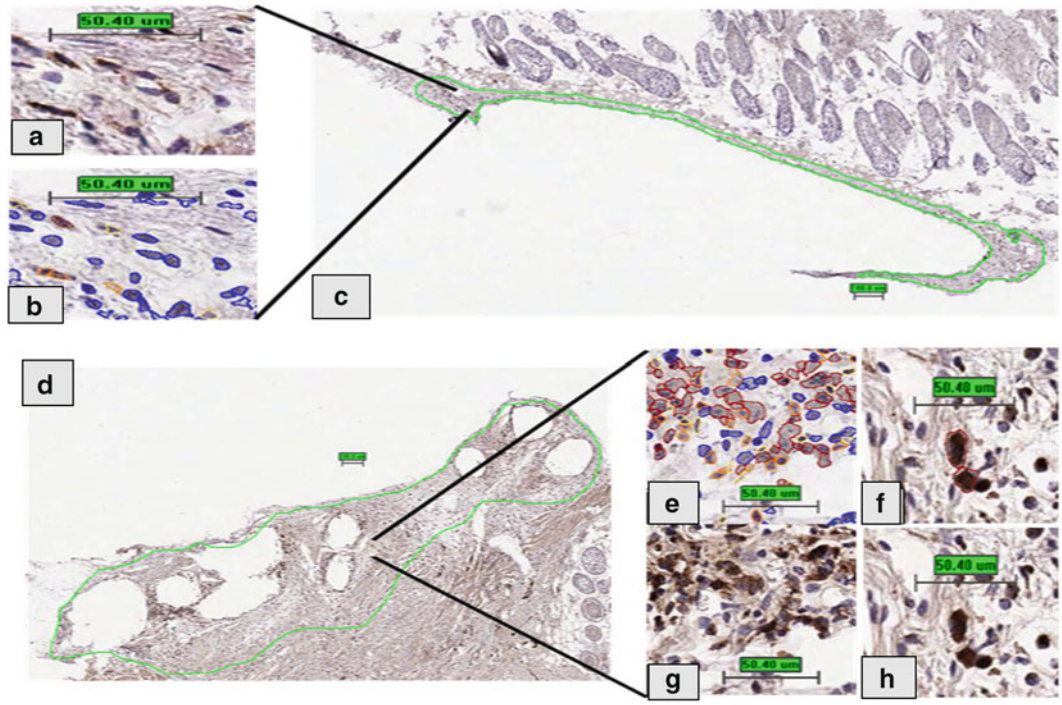

Fig. 4 Representative images of ePTFE and PP reacted with CD-68^{+} cells, showing the DMA false color markup. (**a**) ePTFE representation of the samples reacted with CD-68^{+} macrophages (scale bar = 50.40 μm). (**b**) False color markup of the nuclear counting algorithm, *red* = strong positive, *orange* = moderately positive, *yellow* = weak positive, and *blue* = negative (scale bar = 50.40 μm). (**c**) Macro-image of the region of interest around an implant of ePTFE. The material is not present and the majority of the measurement was performed on the superficial surface of the implant (scale bar = 100.8 μm). (**d**) Macro-image of the polypropylene implanted material (scale bar = 100.8 μm). (**e**) False color markup using nuclear counting algorithm to determine inflammatory response. *Red* = strong positive, *orange* = moderately positive, *yellow* = weak positive, and *blue* = negative (scale bar = 50.40 μm). (**f**) Micro-image of CD-68 reacted, activated macrophages (scale bar = 50.40 μm). (**g**) False color markup of FBGC in *red* (scale bar = 50.40 μm). (**h**) Micro-image of FBGC reacted with CD-68 (scale bar = 50.40 μm)

currently used by pathologists (10). The H-score is obtained by the formula:

$$(3 \times \text{percentage of strongly staining nuclei}) + (2 \times \text{percentage of moderately staining nuclei}) + (\text{percentage of weakly staining nuclei}) = \text{a range of } 0 \text{ to } 300$$

Strongly staining nuclei were represented by red in the false color markup in the digital algorithm; moderately stained nuclei were represented by orange in the false color markup; and weakly stained nuclei were represented by yellow. Combining the H-score calculations of the counted macrophages and FBGC and dividing by two yields a weighted inflammatory score (Fig. 4).

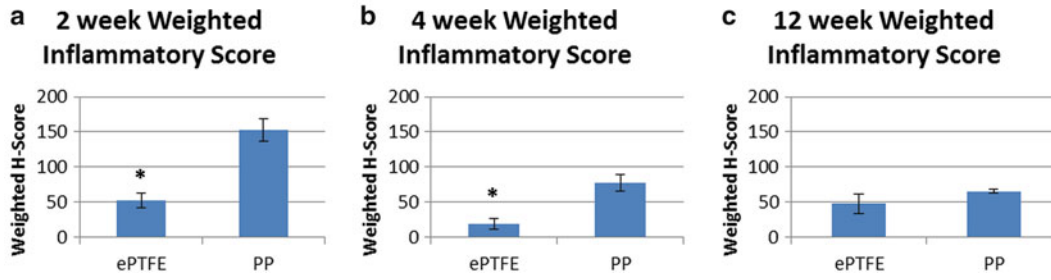

Fig. 5 Graphical representation of the weighted inflammatory score across all three timepoints. As FBGCs diminish over time, the PP weighted inflammatory score also decreases over time. (**a**) Two week weighted inflammatory score $*p = 0.003$. (**b**) Four week weighted inflammatory score $*p = 0.001$. (**c**) Twelve week weighted inflammatory score, no significant difference found

Table 1
Inflammatory index based on the weighted inflammatory score using the weighted H-score

Material	2 Weeks	4 Weeks	12 Weeks
ePTFE	Mildly reactive	Minimally reactive	Mildly reactive
PP	Moderately reactive	Mildly reactive	Mildly reactive

Expanded PTFE is mildly reactive at 2 and 12 weeks and negatively reactive at 4 weeks. PP is moderately reactive at 2 weeks and mildly reactive at 4 and 12 weeks

This will provide weighting to the presence of FBGs as well as a representative overview of the entire inflammatory and foreign body response in a single graphical representation (Fig. 5).

The inflammatory score can then be indexed using the following criteria (10): See Table 1.

$$0 = \text{minimally reactive}\,[0\,\text{to}\,50],$$
$$1 = \text{mildly reactive}\,[51\,\text{to}\,100],$$
$$2 = \text{moderately reactive}\,[101\,\text{to}\,200],$$
$$3 = \text{strongly reactive}\,[201\,\text{to}\,300].$$

4 Discussion

The current uses of automated digital analysis have been focused on pharmacological and toxicological effects in histopathology; therefore, much of the literature surrounding digital pathology is driven by cancer and pharmacological research. In these fields automated microscopy and computerized processing have provided increased accuracy, quantification, and standardization (11).

Currently, biocompatibility assessments using histological techniques on explanted materials and associated surrounding tissue are determined utilizing manual methods, including using

photomicrographs of a selected number of high-powered fields of view and performing visual or digital measurements across these images (12). This allows bias to enter the analysis because the investigator can be drawn to areas that have a high concentration of staining while possibly ignoring areas with little or no stain. Therefore, the biocompatibility of the entire sample of material is not analyzed, and instead often only a narrow area is evaluated and reported on. Additionally, inter-investigator biasing can be an issue when more than one investigator performs measurements and sample counts. Depending on how these individuals were trained, they may interpret the histological features differently. Investigators may also perform manual evaluations over various periods of time. For example, manual evaluations for large studies may take a single or multiple investigators days or weeks to evaluate, increasing the likelihood of variations and biases that can change from day to day or week to week. Computational whole slide analysis removes these biases by performing measurements with the exact same inputs (and assumptions) across all samples being analyzed, consistently (13).

Digital analysis of histological samples represents a small, but important aspect of biocompatibility testing. By measuring the inflammatory and foreign body response of these devices, the material's biocompatibility can be evaluated. A significant advantage of performing digital analysis around biomaterials is that the investigator receives a more comprehensive overview of the entire material's biocompatibility response versus traditional manual methods that are currently used.

In the current study, two well-characterized and well-used materials in the biomedical industry were evaluated at three timepoints to assess the elicited inflammatory response, with each of these materials demonstrating varying tissue-biomaterial responses. Expanded PTFE was found to be mildly reactive at 2 and 12 weeks and minimally reactive at 4 weeks based on a weighted inflammatory response. PP was found to be moderately reactive at 2 weeks and mildly reactive at 4 and 12 weeks based on a weighted inflammatory response. Whole slide digital scans of IHC-reacted slides were created and digital morphometry was used to characterize the tissue-biomaterial interface with respect to inflammation. The results reported in this study are supported by previously published studies where ePTFE elicits a lower inflammatory response when compared to PP. Expanded PTFE has demonstrated a foreign body response present through 21 days (14). Other researchers have found no difference between the inflammatory response between PP and ePTFE in abdominal implants over 28 days (15). At 56 days it has been noted ePTFE has a greater healing response related to granulation tissue formation and the foreign body response (16). In other studies PP has not demonstrated a decrease in macrophage presence between 7 and 90 days (17). The Rosch study used high-

powered fields of 100 μm of the mesh; the current study uses DMA to present a more robust analysis of the tissue response surrounding the entire implant.

With an increasing number of new materials being created to support developments in science and medicine, whole slide digital scanning with algorithm-assisted morphometry could help increase the speed and accuracy of biocompatibility testing. Furthermore, these methods could help to reduce or eliminate inter-investigator biases while also providing a whole slide analysis versus limited fields of view analysis which would result in a more accurate assessment of biocompatibility. Finally, these techniques may help to improve the quality, accuracy, and reproducibility of biocompatible testing results, thus allowing a greater ability to directly compare results from different materials.

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