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Evaluation of Cellular Wound Healing Using Flow Cytometry and Expanded Polytetrafluoroethylene Implants

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Abstract

In wound healing studies that investigate therapeutic interventions, it is important to characterize cellular responses. In a randomized trial enrolling patients at risk for surgical infection, one goal is to phenotype cells within a polytetrafluoroethylene implant using flow cytometry and immunohistochemistry, together with standard hematoxylin and eosin based histology. Subcutaneous implants are removed 8–9 days postoperatively. To obtain single cells associated with the mechanism of wound healing, we initially used a mouse skin digestion protocol. We optimized this to increase cell yield and isolate sufficient cells for flow cytometry. The modifications increased the total cells recovered per subject from an average of 5.3×10^4 to 41×10^4 with an average viability of 80%. The immunofluorescent staining assay was verified for our samples, which have smaller cell sample numbers than tissue biopsies. Thirty-two samples were stained. Cells from the polytetrafluoroethylene tubes were isolated and stained positively with fluorescent-labeled antibodies to CD3, CD20, CD31, CD34, CD68, CD133 and VEGFR2. Flow cytometry data correlated with IHC data especially with respect to CD68. This antigen was the most prevalent in both cell analysis methods. Our findings demonstrate flow cytometry can be used with polytetrafluoroethylene samples as an additional evaluation method to document and describe cellular wound healing responses.

Keywords

Flow cytometry; wound healing; polytetrafluoroethylene implants; method

INTRODUCTION

Relatively few models facilitate the study of healing in acute, surgically induced wounds in humans. Clinical evaluation, tissue biopsy, wound fluid collection or the use of implants placed and removed later for analysis are most commonly used in acute wound healing studies. For several years, visual wound inspection has been used to document clinical healing response or complications such as infection. Specific wound rating systems determine degree of wound disruption or infection.(1,2) In addition, characterization of cell and tissue responses enhances understanding of healing responses.(3) The latter is of particular interest when testing specific clinical interventions to understand how manipulation of the wound environment influences wound cell responses. Wound biopsy or the use of subcutaneous implants retrieved at specific times post injury permit study of the complex cellular interactions that in part define the healing response.

Insertion of a small tube of expanded polytetrafluoroethylene (ePTFE) is an established and accepted minimally invasive method for studying wound healing in humans.(4) Commonly used methods for ePTFE implant analysis include standard histological staining and immunohistochemistry (IHC), as well as enzyme linked immunoabsorbent and other assay methods to determine protein content. Cellular infiltration throughout the ePTFE tubing may be regionally heterogeneous.(5) Because morphology-based studies may rely on the examination of randomly selected cross sections of the ePTFE implant, the pattern and composition of cellular infiltrates throughout the implant may not, as a result, be well represented. Obtaining information on cells harvested from the entire implant may be more informative.

We are conducting a randomized clinical trial testing an intervention that may reduce surgical site infection and improve healing outcome in patients at high risk for surgical wound complications. The intervention we are testing hypothetically increases surgical site perfusion, immune cell recruitment and angiogenesis. Wound samples are obtained using the implanted ePTFE model. We were interested in using flow cytometry because it offers the advantage of analyzing cell responses within a larger ePTFE portion or the entire implanted tubing sample. A goal of the flow cytometry studies is to phenotype the cellular response that occurs under conditions of the experimental therapy compared to standard postoperative care. In the context of this RCT we are focusing our studies on endothelial and immune cells, elements that are particularly relevant to re-establishing capillary networks and wound bacterial defense. Markers of progenitor cells (CD133), endothelial cells (CD31, CD34 and VEGFR-2), macrophages (CD68), T cells (CD3) and B cells (CD20) were selected for the study of the respective angiogenic and immune responses as measured 9 days after surgery. This paper describes our method for the use of flow cytometry, including modifications specific to the ePTFE material and the nature of wound samples obtained when using this technique.

MATERIALS and METHODS

Sample

The study was reviewed and approved by the University of Washington, Human Subjects Division, Seattle, Washington, and the Internal Review Board of Stevens Hospital, Edmonds, Washington. Patients 18 years or older who are undergoing elective, open colon or bariatric surgery procedures are eligible for enrollment. Patients are excluded if they are taking glucocorticoids exceeding 5mg prednisone per day or equivalent, have a serum albumin below 3.0, or renal failure with serum creatinine >2.5 mg/dL. All patients agreeing to participate gave informed, written consent for the study. Flow cytometry studies were performed on 32 ePTFE samples.

ePTFE Procedures

The ePTFE (International Polymer Engineering, Tempe, AZ) test wounds are created by inserting two 10 cm ePTFE tubes subcutaneously, adjacent to and within 1cm of the abdominal surgical incision at the end of the surgery. Each ePTFE implant is attached to an 8cm surgical Keith needle that is used to place the tube subcutaneously. Once inserted, one end of the tube is left exposed and sutured to the epidermis and used for subsequent retrieval. The secured tubes are left in place and covered with a sterile transparent dressing. The ePTFE implants are removed on postoperative day 8 or 9. Immediately following removal, the ePTFE is sectioned and prepared for transport to the lab. Samples are transported to the lab in 4 ml test tubes containing RPMI 1640 media plus gentamicin (both from Invitrogen). On arrival in the lab they are sectioned into 3cm pieces and transferred to a 50ml tube containing 7ml of enzyme solution (1mg/ml hyaluronidase (Sigma), 1mg/ml collagenase (Roche), DNase 150u/ml (Invitrogen) in RPMI 1640). They are stored overnight in a 4°C refrigerator. Cells isolated from all of one ePTFE tube and from a section of the second ePTFE tube are dedicated to the flow cytometry studies.

Cell isolation

Our method was based on the original published protocol by Wilson et al. (6) which successfully isolated viable mouse cells associated with wound healing from skin for flow cytometry. Initial processing of ePTFE samples resulted in lower cell numbers than anticipated. To optimize the recovery of cells, protocol modifications included adding an overnight enzyme solution incubation at 4°C, increasing the day one enzyme solution incubation period at 37°C from 45 minutes to 2.5 hours and implanting two tubes instead of one. Because the cell and tissue samples were within the ePTFE implant material it was necessary to scrape both the inner and outer surfaces of the ePTFE tube with a scalpel. Wash buffers were changed from PBS to RPMI 1640 + 5% FCS to give the cells more nutrients while being processed. The modifications resulted in an increase of total cells recovered per subject from an average of 5.3×10^4 to 41×10^4 . Additionally, isolated cells were frozen to allow for batching of subject samples and optimization of the technician time.

The following protocol was established after modification to increase cell yield. The ePTFE sample is incubated overnight at 4°C in 7ml enzyme solution. The next morning incubation continues for 2.5 hours in a water bath at a temperature of 37°C. The tubes and solution are then transferred to a round petri dish. The outside of each ePTFE section is scraped with a #10 scalpel and then sliced lengthwise; the interior cells are then removed by continued scraping as described above. The contents of the petri dish are transferred to a 50ml tube after passing the liquid through a 70-micron mesh cell strainer (Becton Dickinson). Cells are washed with RPMI 1640 + 5% FCS (Invitrogen) (R+5), and isolated by centrifugation at 250g. The isolated cells are resuspended in 1 ml R+5 and counted in a hemocytometer with 0.1% trypan blue dye in phosphate buffered saline (both from Invitrogen) to obtain cell viability. After final pelleting of cells in a microcentrifuge tube at 15000 RPM, the cell pellet is resuspended in 1ml freezing media (10% DMSO-FCS), and put into a cryovial (Nunc). The vial is then placed in a Nalgene freezing chamber, moved into a -70°C freezer for more than 4 hours and then stored in liquid nitrogen until stained.

Cell Staining

Initially, the immunofluorescent staining assay was verified by comparing actual and expected relative PBMC populations (expressed as a percentage of total PBMC) under small sample conditions. Specifically, PBMCs from a normal donor in numbers comparable to the amount of viable cells available from the ePTFE tubing were stained with our study panel of antibodies. Expected percentages of cells (measured as percent positive for lineage selective antigens) were obtained.

For study samples, isolated cells are thawed in a 37°C water bath, washed with R+5 and counted; $2-4 \times 10^4$ cells are stained per sample in microfuge or 4ml tubes. Samples are incubated with the fluorescently labeled antibodies FITC CD3, PerCP Cy5.5 CD20, PerCP Cy5.5 CD34, FITC CD31 (all from BD Biosciences), PE CD133 (Miltenyi), PE VEGFR2 (R&D systems) for three color staining, and their matching isotype controls on ice for 30 minutes, washed, treated with BD Cytofix or BD Cytofix/Cytoperm and then stained with intracellular PE CD68 (BD), where appropriate. Cells are then stored at 4°C until the flow cytometry analysis is performed. Flow cytometry is performed using the BD FACScan™ (Becton Dickinson, San Jose, CA) and analyzed with the WinMDI software program version 2.8.

Immunohistochemistry

Implanted ePTFE samples are also evaluated using IHC. A portion of the second ePTFE tube was removed at the time of retrieval from the patient and was immediately fixed in 10% neutral buffered formalin. These samples are then processed and embedded in paraffin wax within 48 hours of retrieval in preparation for immunohistochemical analysis of macrophages, T-cells, and endothelial cells, using antibodies to **CD3** (clone PS1; diluted 1:100; Novocastra, Newcastle upon Tyne, UK), **CD20** (L26; 1:400; Dako, Carpinteria CA), **CD34** (QBEnd10; 1:100; Dako), and **CD68** (KP1; 1:16000; Cytomation).

Four micron sections are mounted on charged slides, dewaxed in xylenes and rehydrated to phosphate buffered saline (PBS; pH 7.4). Endogenous peroxidase activity is quenched by immersion of slides in 3% (v/v) hydrogen peroxide for 5 minutes and rinsed in PBS. Sections are then subjected to heat-induced epitope retrieval, using one of two techniques: **CD20, CD34, CD68** -- sections are immersed in 10mM citrate buffer pH 6.0 and heated in a commercial microwave for 15 minutes, allowed to cool to room temperature and rinsed in PBS; **CD3**-- sections are immersed in 1 mM EDTA pH 8.0 and heated in a microwave oven for 15 minutes, allowed to cool to room temperature and rinsed in PBS. Primary antibodies at the specified dilutions are applied to sections and incubated at room temperature for 2 hours in moisture chambers. After rinse in PBS, biotinyl horse anti-mouse antibody and avidin-biotin-peroxidase complex (Vector Elite ABC kits; Vector Laboratories, Burlingame CA) is applied in successive 30-minute room temperature incubations. After rinse in PBS, chromogenic development is performed by immersing sections in 3, 3'-diaminobenzidine tetrahydrochloride (0.84 mg/ml; Sigma Chemicals, St. Louis MO) as chromogen and hydrogen peroxide (0.02% v/v) as substrate. Slides are counterstained in Harris' hematoxylin. Appropriate positive (antibody) and negative (methodologic) controls are run for each stain batch; relevant internal controls for each target antigen are also evaluated.

For the evaluation of T- and B-lymphocyte populations present in the samples a stain is deemed positive if distinct membrane-based reactivity for the target antigen (CD3 and CD20, respectively) is identified on cells morphologically-consistent with lymphocytes and if no inappropriate staining is obtained in methodologic or internal controls. For macrophages, cytoplasmic reactivity for CD68 in a large cell population morphologically consistent with macrophage is deemed positive, with appropriate internal and methodologic controls. For endothelial cells, membrane-based reactivity for CD34 is deemed positive, with appropriate internal and methodologic controls. The latter applies to single cells in the infiltrate as well as the more obvious vasoformative elements, unless the single cell elements exhibited histologic attributes of hematology cells.

Individual cells are evaluated and categorized as positive using the criteria defined above. Each ePTFE tube cross section is evaluated in three domains; the outer half of the ePTFE implant (limited by the periphery of the tube and a parallel line at ½ the radius of the ePTFE material); the inner half of the implant (from the parallel line at ½ radius to the interior

central lumen edge of the ePTFE material); and the central lumen area. In most cases, the central lumen is devoid of cells; when present, cells are typically macrophages (CD68 positive), although T-lymphocytes (CD3 positive) are also occasionally present. Occasional spindled cells with histologic attributes of fibroblasts (and negative for all markers studied) appear to line the inner surface of the ePTFE tube. The number of cells staining positively for each marker was recorded for each domain, but was reported herein (see Table 2) as the sum of all 3 domains.

RESULTS

We obtained an average of 4.95×10^4 cells/cm ePTFE tubing. Of the cells recovered, a mean of 80% were viable at the time of freezing, as determined by trypan blue dye exclusion, which is a bit lower than some other fresh preps (e.g., peripheral blood mononuclear cells (PBMC) from blood) but is still acceptable. Across all samples, we document an average of 67% viability, after thawing from liquid nitrogen storage and before staining. Table 1 presents a summary of our staining results using flow cytometry, illustrating the mean percent of positively staining cells by cell type for the samples tested. Four examples of typical results from our ePTFE samples displayed on flow cytometry dot-plots and presented in Figure 1 demonstrate that we have been able to identify the cell types relevant to the aims of the present clinical study.

Immunohistochemistry was performed on 30 ePTFE samples from the same study patients. Cells positive for CD3 and CD68 were present in all samples, CD20 positive cells were identified in 33% of the samples and CD34 positive cells or small vascular structures in 20%. Table 2 presents the mean positive cell counts by cell type identified in immunohistochemical evaluation.

DISCUSSION

A number of events in the wound healing process can be demonstrated by human wound healing models that evaluate chemotaxis, inflammation, fibroplasias, angiogenesis and matrix synthesis. The ePTFE model has the advantage of being minimally invasive and generally acceptable to patients undergoing surgery. The ePTFE technique provides the opportunity to create a test wound and study cellular responses including measures of newly forming wound matrix. The ePTFE implant has a 90–120 μ m pore size that permits cell entry, connective tissue deposition and subsequent angiogenesis. Tissue within the ePTFE tubing is readily available for analysis, allowing for examination of early or later biologic processes in the healing sequence, depending on the timing of removal. Typically, implants are left in place for up to 7–10 days.

Cell infiltration of the ePTFE reflects the temporal progression of healing showing an early predominance of leukocytes followed by the influx of macrophages and fibroblasts.(3,5) Frequently reported measures of responses corresponding to tissue repair and evaluated using ePTFE implants include hydroxyproline content, granulation tissue architecture and cellular infiltration (7,8,9,10,11). Various cells consistent with wound healing responses have been identified within ePTFE implants using IHC and other staining methods. In studies where ePTFE tubes were removed 7 to 14 days after insertion, neutrophils, macrophages, lymphocytes and fibroblasts have been present as well as collagen and new vessels. (3,7,8,12) In addition to healing responses, the implanted ePTFE tubes also act to some extent as a foreign body, as demonstrated by the presence of multinucleated giant cells within the histiocytic infiltrate in standard histologic sections (seen in our samples).

Using the flow cytometry protocol described we have been able to successfully identify the cell types we anticipated would be present in the ePTFE samples. The percent of cells identified by flow cytometry is highest for macrophages. Since the macrophage is a predominant cell in healing, this finding in our samples, removed 9 days after surgery, is consistent with the healing response. (13) In flow cytometry, CD31 and VEGFR2 positive cells were identified. These are both expressed to varying degrees by histiocytes and macrophages. Based on IHC CD31 analysis of a selection of samples (data not shown), macrophages were the most common CD31 positive cell. This may explain the higher mean percent of cells staining positively for CD31 compared to the CD34 results in the flow cytometry analysis. It is thus likely that the CD34 results seen with flow cytometry are a better indication of endothelial infiltration of the ePTFE.

Flow cytometric analysis is a commonly reported method in recent wound healing studies using animal models.(14,15,16) To our knowledge, performance of flow cytometric analyses optimized for analysis of ePTFE implant wound model samples has not been previously reported. A flow cytometry protocol applied to excised, full thickness mouse skin wounds at varying post wound time points was developed by Wilson et al. (6) and is the model on which our studies were based. In their studies, flow cytometry was a reliable cell quantification method identifying suprabasal and basal keratinocytes, bone marrow derived leukocytes, and granulocytes. In our current clinical trial, hematoxylin and eosin histology and immunohistochemistry provide confirmation of cell types and important data on both cell morphology and distribution of cells in the ePTFE matrix. The cell types identified with IHC are consistent with our flow cytometry results, though they reflect a smaller sample of the ePTFE.

Obtaining sufficient tissue or cells for wound healing analysis using human samples is a realistic concern and must be considered with the use of any human wound healing model. We adapted the current protocol from the mouse skin biopsy model of Wilson et al.(6) with particular emphasis on increasing the cell yield from the ePTFE implants as described in methods. Modifications to their protocol were needed because of differences between intact skin biopsy samples and ePTFE tissue samples. To optimize our protocol, we initially focused on two areas: obtaining a single cell suspension that preserved sufficient number of viable cells, and validating the immunofluorescent cell staining method for small cell number samples.

Modifications included increasing enzyme incubation periods, procedures for mechanical cell recovery and changing wash buffers. These resulted in an average 8-fold increase in mean total recovery. Even so, cells were still lost during the recovery process. Because cells were not stained prior to freezing, we are unable say if cell loss due to a freeze-thaw cycle was proportional or differentially greater in one specific cell type. In future studies, this shortcoming could be addressed by evaluating cell populations prior to freezing in representative samples. Alternatively, flow cytometry studies could be performed on fresh ePTFE samples, should the limitations of personnel resources that require delayed evaluation of samples (and thus, the need for freezing samples for their preservation) be mitigated. In our previous experience of flow cytometry studies using (PBMC) samples, freezing has not affected cell types differentially. Thus, we assumed that this would be similar for cells obtained from ePTFE implants, but this assumption should be verified.

We have determined that it is possible to isolate single cells from the implanted ePTFE tubing, removed eight to nine days after surgery, and identify them as components of the wound healing response by fluorescent label-based flow cytometry. We have consistently detected significant numbers of CD68 positive cells, which is to be expected with this experimental model and the timing of ePTFE retrieval on the 9th postoperative day. This is

consistent with other results reported for cells present at 7–9 days in acute, ePTFE wounds. (8,17,18) We have also been able to successfully label and detect additional cell types, including lymphocyte populations, endothelial and progenitor cells.

Implications for Future Studies

The combined use of flow cytometry and the ePTFE wound sample method produces a smaller than usual number of cells than is typically evaluated with flow cytometry. This needs to be taken into account in future studies. Flow cytometric analysis of ePTFE samples will yield more explicit information if a limited number of antigens most specific to the research aims are analyzed. If however, there are techniques in which the ePTFE placement and harvesting of intact cells could yield greater cell populations (between 10^6 to 5×10^6 cells), other uses of flow cytometry could be utilized in conjunction with the specific antigens listed above. The value of tissue morphology/immunohistochemistry in representative sections as an adjunct to flow cytometry is also confirmed by this study. Flow cytometric results mitigate the potential limitations of sampling in histologic studies, while the distribution of cell types within the graft determined by histology/IHC enhances the value of reliable cell counts. One area of particular importance is the wound healing process in which apoptosis is responsible for the removal of inflammatory cells and granulation tissue. In this case, one can use propidium iodide staining, coupled with flow cytometry analysis of double-stranded DNA in order to check for presence of sub-diploid DNA (A0) present in the cellular matrix.

Flow cytometry is a useful and productive means of identifying cell types involved in wound healing. Our results indicate that flow cytometry provides an additional method for assessing cellular responses to ePTFE. It offers the advantage of analyzing the healing responses within the entire ePTFE implant, and provides a tool capable of identifying intervention-associated or intervention-induced changes in cell or antigen-based phenotypes or even phenotypic differences between groups of patients with varying wound healing risk profiles. Treatment-related changes in phenotypes, in combination with morphologic and other assessments, could help identify conditions that favor a more vigorous immune response and reduced incidence of wound infection, leading to more robust adjuvants to wound healing and better clinical outcomes.

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Figure 1.

Representative flow cytometry dot plots from our samples illustrate positive results in detecting cell types that were expected to infiltrate the ePTFE implants, T cells, macrophages and endothelial cells. The four plots show cells labeled with antibodies to the following: (A) isotype control, (B) PE-CD68 (upper left quadrant), (C) FITC CD3 (lower right quadrant), and (D) FITC CD31 (lower right quadrant).

FL1-H, FL2-H and FL3-H indicate the log of the fluorescence intensity for detection channels of the flow cytometer.

Table 1

Flow cytometry results. Mean percent of cells staining positively for specific antibodies.

		Cell Type						
	Progenitor	B cell	T Cell	Endothelial	Endothelial	Endo/Macrophage	Macrophage	
Antigen	CD133	CD20	CD3	CD34	VEGFR2	CD31	CD68	
Mean	0.44	0.39	9.92	6.55	2.95	22.51	34.15	
SD	1.16	1.72	12.88	12.79	5.68	22.91	19.52	
N	7	20	21	16	7	15	32	

N-number of samples, SD-standard deviation. The number of samples for each antigen varies depending on the cell yield from each ePTE tube. In the majority of samples the number of cells did not permit staining with all antigens. Values represent the mean percent of cells positive for the antigen based on individual patient samples (designated by sample size). Analysis included samples with and without positive staining for the antigen.

Table 2

Immunohistochemistry results. Number of cells staining positively for specific antibodies (mean, SD, minimum and maximum number of cells identified).

	Cell Type			
	B Cell	T Cell	Endothelial	Macrophage
Antigen	CD20	CD3	CD34	CD68
Mean	.43	26	.76	430
SD	1.3	27	1.0	258
Minimum	0	1	0	54
Maximum	7	124	23	957
N	30	30	30	30