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STUDY OF THE HUMAN CHRONIC WOUND TISSUE: ADDRESSING LOGISTIC BARRIERS AND PRODUCTIVE USE OF LASER CAPTURE MICRODISSECTION

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Abstract

Direct procurement of tissue samples from clinically presented chronic human wounds is a powerful approach to understand mechanism at play in an actual problem wound. While such approach suffers from limitations related to lack of reproducible conditions across wounds, something that we are used to the laboratory while studying wounds on experimental animals, the direct study of human wound tissue helps recognize the right questions to ask in the laboratory. Going back and forth between human wound and experimental animal studies helps steer studies on experimental wounds in a clinically relevant direction. In this article, we describe critical factors that need to be considered prior to planning a study involving human wound samples. In addition, we describe an approach to capture wound hyperproliferative epithelium (HE) from chronic human wound biopsies using laser capture microdissection (LCM). LCM is a new technology applicable to a broad range of clinical research and represents a catalyst of sophisticated translational research.

1. Introduction

Wound healing studies designed to address mechanisms or causality, specifically at a molecular level, lag far behind other medical conditions because even the most sophisticated animal wound model fails to capture the complexities associated with a clinically presented chronic wound. It is indeed difficult to control for the multitude of conditions that impact clinical wound healing outcomes. On the other hand, studies on experimental wounds are empowered to dissect underlying mechanisms in a well-controlled manner. The risk here is that one may deconvolute a complex mechanism only to discover that the mechanism is of little clinical relevance. Thus, it is imperative that the direction of experimental studies be supervised by findings of studies examining the clinically presented chronic human wound. This strategy should help unveil mechanisms underlying wound chronicity and those findings would be clinically relevant therefore laying the foundation for clinically valuable solutions. In this article, we share the essence of what we have learnt as experimental wound biologists as we sought to undertake studies of the chronic human tissue. We hope that you

will find this article useful to recognize and address some of the logistic as well as scientific barriers that are integral to the system.

2. Infrastructure

Availability of appropriate infrastructure is critical to the success of wound research studies involving chronic wound patients.

2.1 Wound center: a catalyst

Continuous access to chronic wound patients (as they visit over time) and their comprehensive medical records (etiology, comorbidity, medication, outcome etc) represents a critical barrier to performing patient-based wound healing research. At most health care facilities, wound care is not recognized as a specialized clinical discipline. In such setting, wound patients are treated across a wide range of clinics making tracking of such patients very difficult. Few medical centers, including ours, are fortunate to have a Comprehensive Wound Center that covers both outpatient as well as inpatient services. This not only helps us access chronic wound patients but also enables us to track them as they are discharged from inpatient facilities.

2.2 Multi-center studies

Multicenter collaborations are useful in boosting the volume of patient enrollment. Lack of standardized wound care protocols among participating centers may lead to variation in data and sample collection, thus confounding results. Adoption of standardized protocols for routine wound care in participating centers is another key catalyst that drives patient-based wound research. At Ohio State, we are fortunate to have wound centers at five different locations in Columbus under the banner of our Comprehensive Wound Center. These clinics care for over 1500 patient visits a month. Wound care practices across these locations are guided by standardized algorithms.

2.3 Personnel training

As in most clinics, abrupt introduction of research into clinical operations is disruptive and alienates the clinical staff. The first steps in successful introduction of the research culture to a high volume clinic is to make sure that the entire team of clinical staff understands that research and education are integral components of clinical care. This process requires extra effort of already overburdened clinicians and thus must be appropriately incentivized. Next, personnel directed at clinical operations should not be additionally burdened with research tasks. Separate full-time effort must be budgeted and the clinics must be staffed with clinicians dedicated to the research mission. Such research staff must be trained to observe regulatory and compliance requirements. Topics to be addressed include the informed consent process, confidentiality, identifying and managing risks, protocol development, protocol adherence and ethical conduct of research as approved by the IRB. Standardized DVD-based training is desirable. Appropriate oversight of such staff by designated principal investigator(s) is required. Furthermore, within the perimeters of the clinic, dedicated research space must be identified and made available for patient handling and sample collection. To keep the clinical team motivated, it is useful to add a research agenda item on

each and every clinical operations meeting. This is a good venue to update the group on research progress and also to recognize the most productive clinicians who have helped recruit most subjects. This is also a good opportunity to find out which clinicians may want to voluntarily elevate their contribution to research to a point where they want co-authorship of publications. A few such cases soon get viral and become a big motivation factor for other clinicians – many of whom soon join in. Such transformation of culture in the clinics is a critical driver of effective research. Above all, it needs to be recognized that such change in work culture cannot be drilled bottom-up by passionate researchers who know a clinician or two at the wound clinic. Such transformation must be initiated with the complete buy in of top hospital leadership who need to be convinced to integrate clinical and research productivity as parts of one score card.

2.4 Institutional Review Board (IRB)

The design, conduct and monitoring of a research activity involving human subjects is the responsibility of the Principal Investigator (PI) who provides a complete written description of the proposed research to an IRB and obtains appropriate approvals to conduct such research. No activity involving human subjects may begin until it has been reviewed and approved by an IRB. The primary responsibility of IRBs is to safeguard the rights and welfare of human research subjects. Therefore, a PI must provide enough information for the IRB to determine that human subjects will be adequately protected and that the research will be conducted in full compliance with federal regulations and policies. In order to approve a research activity involving human subjects. For specific details on IRB approval, please consult your own institution. Typically, the following sections need to be addressed:

1. Introduction
2. Objectives
3. Study Design and Methods
4. Inclusion and exclusion criteria
5. Monitoring Subjects and Criteria for Withdrawal of Subjects from the Study
6. Analysis of the Study
7. Human Subject Protections
8. Rationale for Subject Selection and procedures for recruitment (include a Targeted/Planned Enrollment Table)
9. Rationale for the involvement of special classes of subjects, if any, such as fetuses, pregnant women, children, cognitively impaired individuals, prisoners or other institutionalized individuals, or others who are likely to be vulnerable
10. Evaluation of Benefits and Risks/Discomforts
11. Adverse Event Reporting and Data Monitoring
12. Collection and Storage of Human Specimens or Data
13. Remuneration/Compensation

14. Consent and Assent Processes and Documents

15. References

Note: If the protocol involves subject enrollment at multiple sites, describe plans for ensuring appropriate IRB review and approval at each site.

2.5 Clinical-basic science researcher partnership

Obtaining tissue from human subjects with chronic wounds requires participation of not only physicians, but all clinical staff. It is therefore, important to create and maintain a culture at the wound clinics/centers that supports research by *a)* keeping an ongoing dialogue between research staff and all clinical staff on research related logistics as well as findings; *b)* minimizing disruption of clinical work flow because of research activity; *c)* incentivizing (e.g., academic credits or monetary reimbursement) physicians to participate in research; and *d)* developing a scorecard to monitor the impact of research on overall clinical revenues.

2.6 Wound data collection tools

The high degree of variability in clinical conditions among chronic wound patients means that a large number of subjects must be screened and stratified based on their medical history to identify comparable cohorts of patients suitable for productive data analyses. An integrated data management platform that can access and intelligently mine electronic medical records as well as research data records is of extraordinary value. It is recommended that local bioinformatics expertise be engaged to provide support in this regard.

3 Laser Capture Microdissection (LCM): spatially resolved molecular analyses of the wound tissue section

Biopsies collected from human cutaneous wounds presented in the clinic are highly heterogeneous in cellular composition. The small sample volume poses additional challenges in detailed molecular analyses. The composition of the tissue may vary from one collection to another complicating comparison of results derived from tissue homogenates. Thus, the utility of such tissue material is primarily limited to histological studies. Laser capture microdissection (LCM) is a powerful technique to collect specific single-cell or multi-cellular structures from within the tissue section¹. These tiny tissue samples lend themselves to subsequent molecular analyses¹. In 2007, we reported the first evidence demonstrating LCM-based microdissection of blood vessels from human wound tissue sections². The collected tissue elements were subjected to high-density microarray analysis and quantitative PCR-based validation of microarray data. Residual tissue in the single biopsy was used to perform immunohistochemistry aimed at validating microarray data. All of the above-said analyses were performed using no more than a single 3 mm punch biopsy from the chronic wound tissue. Comparison of results from blood vessels at the edge of chronic wound tissue with those of vessels from intact human skin demonstrated a striking contrast between the transcriptome of vessels collected from the two different locations². LCM is applicable to a broad range of clinical research and represents a catalyst of

sophisticated translational research. Here, we detail the approach to capture wound hyperproliferative epithelium (HE) from chronic human wound biopsies.

4 Materials & Methods

4.1 Chronic wound tissue biopsy collection

Materials

- 3mm biopsy tool
- (Optimum Cutting Temperature) O.C.T compound
- Aluminum foil
- Dry ice
- Cryo-molds
- Styrofoam box to store dry ice

Protocol

- **Preparing the wounds for sampling.** There is a lack of consensus on the correct way to prepare and sample a wound. We collect biopsies following routine marginal debridement of the wounds to avoid collection of dead necrotic tissue. Prior to biopsy collection, the wound bed is prepared with local antiseptics. Local anesthetics are administered according to patient needs. If the effects of specific anesthetics are being tested, it is necessary to avoid the use of local anesthetics.
- **Collection procedure.** Wound-edge tissue specimen is collected as 3 mm biopsy from the boundary (1 cm) between skin and open wound.
- **Sample storage and transport.** Samples are immediately rinsed with ice-cold saline, patted dry on Kimwipe® and then placed in collection vial containing appropriate collection medium (*e.g.*, OCT for cryosectioning) and snap frozen in liquid nitrogen as appropriate. The samples can be transported from clinic to laboratory in properly secured biohazard-safe containers under appropriate temperature conditions (*e.g.* in portable liquid nitrogen tanks). Shipments of any biological materials are regulated by the U. S. Department of Transportation (DOT) and the International Air Transport Association (IATA). Additional regulations may apply while shipping of wound samples in dry-ice.
- **Embedding of sample in OCT compound.** Place dry-ice in a thick-walled Styrofoam box. Place a cryo-mold on the dry-ice block. Add OCT compound to the cryo-mold. Place the biopsy in appropriate orientation to obtain cross-section of the wound tissue. Add more OCT to cover the tissue. Let the OCT solidify on dry-ice. Once solidified, the cryo-mold containing embedded tissue biopsies can be stored in liquid nitrogen vapor.

4.2 Cryosectioning

Materials

- PEN membrane Slides (Carl Zeiss Microimaging, Inc. USA)
- RNA Zap® (Applied Biosystems, Ambion, USA)
- Diethyl pyrocarbonate (DEPC) treated water (Sigma)

Protocol

- Mark side (non-glossy) of the slide that contains the membrane with pencil
- Place slide on clean paper towel
- Dip the membrane slide into RNA Zap® ten times
- Dip the membrane slide into DEPC water 10×
- Repeat with fresh DEPC water
- Flick the slide to remove excess water and let slide dry
- Take out the OCT embedded tissue block and mount on a cutting chuck. Perform sectioning using standard cryo-sectioning equipment. We routinely use 10 µm thick sections for LCM captures of hyper proliferative epithelium (HE) from wound tissues.

Notes: 1. It is essential to maintain the sample temperature below −20°C during the entire sectioning process.

2. before collection, the freshly cut section should be left for 5 min in cryosectioning equipment for appropriate cooling.

3. The slides containing tissue sections should be kept on dry ice immediately after they come out of cryo-sectioning equipment.

4. To get best results, cryosection no earlier than an hour before LCM

4.3 Rapid Staining – Visualize Tissue Architecture yet Preserve RNA Integrity

Truncated hematoxylin and eosin staining^{3–5}

Materials

- Hematoxylin QS (Vector Labs, USA)
- Eosin Y, 2% in 95% ethanol
- RNALater® (Ambion, USA)
- Nuclease-free water (Invitrogen, USA)
- Molecular biology-grade ethanol and Xylene (Sigma, USA)
- Mini-stainer jars (Ted Pella, USA)

Protocol

- Thaw and dry frozen slides by placing them in a box containing silica gel for 5 min. This should be performed in a biosafety cabinet to minimize contamination. Do not allow the silica gel to come into direct contact with the sections.
- Add RNALater® to cover the sections and incubate 4 mins.
- Add a drop of Hematoxylin QS on the section and incubate for 30 s.
- Wash the slide in DEPC water by gently dipping the slide up and down 5 times.
- Repeat wash with fresh DEPC water.
- (*Optional step*): Add a drop of 2% Eosin Y in 95% EtOH to the sections and incubate for 30 s.
- Wash in 95% EtOH by gently dipping the slide up and down; then let the slide sit in fresh 95% EtOH for a period of 1 min.
- Place the slide in 100% EtOH for 1 min.
- Place the slide in xylene for 1 min.
- Shake off xylene and let the slide dry in the hood by placing it in a box containing silica gel for 2 min.

Notes: 1. Use molecular-grade ethanol, RNase free solutions, DEPC treated water and fresh xylene during staining.

2. Reduce time of staining as much as possible to minimize RNA degradation.

3. Avoid storage of stained tissue. The staining should be performed immediately before LCM.

4.4 Laser capture microdissection (LCM) or laser microdissection and pressure catapulting (LMPC)

Materials

- PALM MicroBeam IV (Manufacturer: P.A.L.M. Microlaser Technologies GmbH, a company of the Carl Zeiss Group, Bernried, Germany)
- Stained tissue sections on a PEN membrane slide (see sections 4.2–4.3 for preparing stained tissues for LCM)

Protocol

- The PALM MicroBeam LMPC system consists of inverted microscope with a motorized stage and a ‘cold’ nitrogen UV laser that is not harsh on DNA/RNA or proteins. For cell or tissue element capture, the laser is focused on to the tissue section by adjustment of objectives. The laser traverses the glass slide from below and cuts the section from beneath (Figure 1).

- The microscope stage and UV laser are controlled by a PC (through RoboSoftware™), and a video camera allows the tissue sections to be displayed on the PC screen. Viewing of tissue is possible with all objectives.
- The captured tissue element is collected using Robomover Z, the robotic unit of PALM MicroBeam controlled by RoboSoftware™ with multipurpose functions (Figure 2). It is a collection device with the adaptors for the versatile capture vials such as single microfuge caps, multitude of microfuge tubes, multicap strips or plates. Different customized holders allow collection of samples from single or multiple object slides, LMPC–optimized slides or culture dishes. The Robomover Z can process up to three slides simultaneously.
- *Principles of LMPC.* In LMPC, the biological material is placed on a glass slide covered with a polyethylene naphthalate (PEN) membrane. The laser beam source is positioned and focused. A tightly focused laser beam is used to cut the PEN membrane and the tissue sample on it. A defocused laser beam is then used to “pressure catapult” the cut membrane with dissected cells of interest into a sample collector. Thus, cell retrieval is accomplished by LMPC approach against gravity. This is a non-contact method that prevents possible contamination by surrounding tissue as opposed to technologies that rely on gravity-driven “falling” of the dissected tissue element into the sample collector.
- Mount the stained tissue-containing slide on the slide holder on motorized stage.
- Load appropriate collection vial on to the Robomover. The collection vial should contain some collection solution. For gene expression studies, RNA extraction lysis buffer (see section 4.5 for details) is appropriate.
- Use appropriate objective to visualize the tissue and region of interest (ROI).
- Mark the ROI utilizing the pen tool feature of the Robosoftware™.
- Activate the laser to cut and catapult to the collection vial
- Collect images before and after the captures as well as of the collected tissue in vial (Figure 3).
- The laser focus, laser energy and speed of cutting needs to be optimized for specific type of tissues to obtain efficient tissue cutting and catapulting.

Notes. 1. It is recommended to optimize LCM conditions with a trial tissue to minimize loss of time.

2. For gene expression studies, the LCM process must not exceed 30 minute to secure high quality RNA integrity. We have reported a gradual loss in RNA stability in tissue sections as a function of time following staining⁴.

4.5 RNA Isolation^{24–6} (Figure 4)

Materials

- PicoPure RNA Isolation Kit (Arcturus)

- NanoDrop system (NanoDrop Technologies)
- Agilent bioanalyzer

Protocol

- Tissue elements are cut and captured in LCM collection vial containing RNA extraction buffer (PicoPure RNA Isolation Kit, Arcturus).
- Perform total RNA isolation from captured tissue elements using a small sample RNA extraction kit for example Picopure RNA isolation kit (Arcturus) following manufacturer's instructions.
- After isolation, the RNA quantity (Nanodrop) and quality (Bioanalyzer, Agilent) is measured to determine quantity as well as to ensure the integrity of the RNA. A RNA integrity number (RIN, 1 is worst quality vs 10 is the best) above 5 is considered as acceptable RIN to proceed with gene expression analysis studies.

Note: 1. The optimum tissue elements for gene expression studies vary with type of tissues. In general, 0.1–1 million sq micron tissue elements provide sufficient quantity of RNA to perform gene expression studies.

4.6 Reverse Transcription and Quantitative Real-time PCR^{2,4–6}

Materials

- Arcturus® RiboAmp® HS PLUS cDNA Kit (Invitrogen)
- TaqMan® Gene Expression Assays (Invitrogen)

Protocol

- The ARCTURUS® RiboAmp® HS PLUS cDNA Kit permits quantitative real-time PCR (qRT-PCR) based gene expression analysis from small (as little as 100pg) LCM captured of total RNA.
- Perform reactions using instructions included with the kit.
- The cDNA ready from reaction above can be used directly for qRT-PCR assay.
- TaqMan® Gene Expression Assays are specifically designed to determine gene expression via qRT-PCR. The kit comes with detailed instructions to perform the assays.

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Figure 1.
Major components of the PALM MicroBeam IV system.

Figure 2.

A representative screen shot of RoboSoftware™ Pro software to show tissue elements marked and ready for capture.

Figure 3.
LCM of human wound tissue hyperproliferative epithelium (HE). *Left.* Hematoxylin stained HE area marked for capture. *Right.* The same area shown after capture.

Figure 4.
Workflow of gene expression analysis from LCM captured human wound HE.