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13. ABSTRACT (Maximum 200 Words) Proteomic methods developed through the support of this grant have been used to describe a previously unknown relationship between the pulse width of infrared laser light (1540nm) and the degree of post-exposure corneal wound healing. Using standard histologic methods, in vitro exposure of corneal tissue to nanosecond pulse widths appeared less damaging when compared to millisecond pulse widths. However, using MMP-2 immunohistochemistry to detect subtle stromal remodeling, we discovered a markedly increased tissue response to nanosecond exposures when compared to millisecond exposures. This finding is important to the AFOSR mission because it demonstrates that significant tissue changes associated with wound healing can occur in the cornea following exposure to nanosecond pulse widths that are not detectable using standard histologic techniques. Tissue engineering methods developed through the support of this grant were used to produce organotypic corneal models that reduced the number of experimental animals necessary to conduct laser-tissue interaction studies. Through tissue engineering, our research group developed a validated experimental model to document the marked proliferative response of corneal tissue to low fluence 1540 nm laser radiation.					
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Introduction

Infrared lasers have widespread military applications (e.g., range finders, laser radar and telecommunications-fiber optics equipment). The infrared lasers operating at the 1540 nm wavelength (i.e., Erbium-glass lasers) are widely used because they are considered "eye safe." The designation "eye-safe" was established because all photon energy of the 1540 nm laser is considered absorbed in the anterior segment of the eye and does not pose a retinal hazard. However, the term "eye-safe" is not used in the current American National Standards Institute (ANSI) guide for the safe use of lasers (ANSI Z136.1-1993). A significant problem surrounding this consensus definition is that minimal permissible ocular exposure recommendations for all current infrared lasers operating in the wavelength range ≥ 1400 nm are based on experimental data obtained using 10.6 μm CO₂ lasers (ANSI Z136.1-1993). Evaluation of laser-ocular tissue interaction for infrared lasers below this wavelength (including the 1540 nm laser) must be done by extrapolation. With a lack of well-defined safety standards, minimal information on 1540 nm laser-ocular tissue interaction and few precedents to answer medical and/or legal questions with regard to potential personnel exposures, military and laboratory commanders may be constrained from using 1540 nm laser systems in mission-critical situations. An important aspect of this project was the production of *in vitro*, organotypic corneal models. Our research team has developed three dimensional, *in vitro* organotypic models for the cornea that we believe will provide the information necessary to address the problems of assay validation. The organotypic corneal models (corneal equivalents) consist of a collagen-based stromal matrix that contains corneal keratocytes and epithelial cells from the respective host species. The resulting three-dimensional models are histologically and biochemically analogous to their respective native tissues. This approach maximizes the potential for a good correlation between *in vitro* test results and the extrapolation of experimental data to human experience. Using methods outlined in this report, one rabbit cornea provided enough seed culture cells to produce 20-25 rabbit organotypic corneal equivalents. Human corneal tissue provided enough seed culture cells to produce 3-5 human organotypic corneal equivalents. No live animals were used in this research project as we obtained the rabbit eyes from a meat processing plant. Human corneal tissue was recovered from post-surgical specimens obtained from a local hospital.

The principal objectives of this study were:

- (1) Produce organotypic corneal equivalent tissue models.
- (2) Determine the biochemical and morphometric dose-responses of explant rabbit corneas following *in vitro* exposures to 1540 nm laser light.
- (3) Determine the biochemical and morphometric dose-responses of organotypic corneal models following *in vitro* exposure to 1540 nm laser light.

This final technical report represents our efforts from March 1, 2001- February 29, 2004.

USAF Relevancy

The USAF has a scientific mission and a congressional mandate to reduce the number of animals used in research and to protect USAF personnel from operational hazards. The use of *in vitro* methods to screen USAF compounds of interest and operational procedures (laser) is an important component of the AFOSR Predictive Toxicology Program. The transfer of organotypic model technology from our laboratory to the laboratories at Brooks AFB (Dr. Ben Rockwell) and the Uniformed Services University of the Health Sciences (Dr. Thomas Johnson) should provide USAF scientists with useful methods that are directly applicable to the AFOSR Predictive Toxicology Program.

Technical Background

Objective 1- Produce organotypic corneal equivalent tissue models.

Cultures of corneal epithelial cells and keratocytes from native human and rabbit corneal specimens served as the seed cultures for the organotypic corneal equivalents. Corneal equivalents were produced in two steps. First, a liquid collagen/corneal keratocyte seed culture suspension was added to a Transwell (Costar) polycarbonate tissue culture insert contained within a 12 well tissue culture plate. The polycarbonate membrane of the insert served as a platform for the gelatinization of the stromal collagen and the growth of stromal keratocytes. The collagen/ keratocyte suspension formed a gel during incubation (37°C, 5% CO₂) and the keratocytes were grown in culture for 3-5 days. Second, a seed culture suspension of corneal epithelial cells was plated upon the collagen/ keratocyte gel and grown in culture for an additional 7-10 days. The tissue culture fluid level was slowly lowered over the incubation period until an epithelial cell-air interface was established.

Objectives 2 and 3- Conduct studies to determine the biochemical and morphometric dose-responses for laser-ocular tissue interaction.

Laser Exposures: All laser exposures were single pulse, 1540 nm laser light. Millisecond exposures were set at 0.8 millisecond pulse widths with a 0.6-micron diameter spot size and calculated energy densities from 25-100 J/cm² at 1/e². Nanosecond exposures were set at 500 nanosecond pulse widths with a 0.3-micron diameter spot size and calculated energy densities from 10-40 J/cm² at 1/e². The laser used in corneal exposures was manufactured by Laser Sight Technologies (Winter Park, FL). It had an Erbium:Glass rod and q-switched circuitry to produce pulse widths in the ms-ns range at a wavelength of 1540 nm. The pulse widths were measured using a Germanium detector (Thor Labs PDA 255) connected to a TDS 644B-digitizing oscilloscope (Tektronix). Energy measurements were made with a EPM 2000 detector (Molelectron) connected to a J-25 energy probe (Molelectron).

Histomorphometry: Digital images of the corneas were obtained (Kodak EDAS system), the tissues were frozen in OCT embedding medium (Tissue-Tek) and frozen sections were taken using a motorized cryomicrotome (Bright Instruments). Using the digital images of the post-exposure corneas projected onto a measuring grid, we could accurately locate the relative position of the circular laser lesion in the embedded tissue. Phase differential interference contrast microscopy (BK-2 microscope, Olympus) was used to evaluate unstained sections for relative position within the lesion, as well as, proper orientation and the occurrence of any sectioning artifacts. This allowed us to section through the lesion with micrometer precision and accurately resolves the inside edge, middle and outside edge of the laser lesion. All tissue sections for histomorphometric analysis of laser effects were taken through the middle of the lesion. Images of histologic sections were obtained using a Leitz Orthoplan microscope equipped with a SpotRT digital camera (Diagnostic Instruments). Alterations in the epithelial parameters (e.g., area of hyaline coagulative change vs. area of granular coagulative change) and stromal parameters (e.g., area of coagulative necrosis, number and distribution of keratocytes) were evaluated between millisecond and nanosecond treatment groups.

Immunohistochemical Analysis of Laser Exposed Tissue: Immunohistochemistry procedures were performed in CoverPlate Immunostaining Chambers (Shandon Scientific). Indirect immunostaining for matrix metalloproteinase 2 (MMP-2) was performed using mouse monoclonal antibodies (clone 42-5D11). Checkerboard titrations of primary mouse monoclonal antibodies and secondary biotinylated

goat anti-mouse IgG (Vector Labs) was used to establish appropriate dilutions of primary and secondary reagents. The Vectastain Elite ABC kit (Vector Labs) was used for immunohistochemical analysis, which was conducted following manufacturer's recommended procedures. Quantitative evaluations of the MMP-2 diaminobenzidine (DAB) immunohistochemical reaction products in the corneal epithelial and stromal layers were performed using Image Pro Plus analysis software (Media Cybernetics).

Proteomic Analysis of Corneal Tissue: Frozen sections of corneal tissues between twenty-five and one hundred and fifty microns were obtained and placed on a pH 3-10 gradient polyacrylamide gel (CleanGel, Amersham Biosciences) containing appropriate ampholytes and 8M urea. The first dimension electrophoresis was performed at a constant temperature of 4°C using an isothermal controlled electrophoresis chamber (Model FB1001, Fisher) and a crossover regulated power supply (Model EC650, EC, Inc.). These first dimension gels were cut into strips and placed on top of 4-20% gradient SDS-PAGE mini-gels (8cm x 8cm) (Model 260, Hoefer Sci). The second dimension electrophoresis was conducted at a constant current of 40 milliamps per gel using a crossover regulated power supply (Model EC650, EC, Inc.). After the 2D-PAGE electrophoresis, gels were stained using a mass spectroscopy compatible silver stain kit (SilverQuest, Invitrogen). Digital images of silver-stained gels were captured using a digital gel documentation system (EDAS, Kodak) and stored for calibration against commercial isoelectric point and molecular weight standards (Invitrogen). Individual protein spots were excised from gels following the 2D-PAGE method listed above and processed by a sequential exchange of buffers followed by overnight incubation with sequencing grade trypsin (Sigma), extraction and desalting of the tryptic peptides (ZipTip, Millipore). Identification of individual protein "spots" in the two-dimensional gel was conducted using Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry.

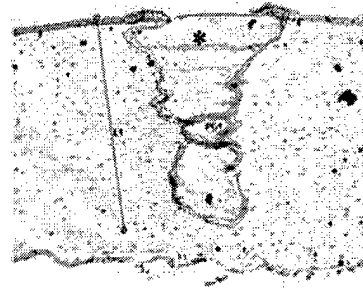
Summary of Accomplishments and Significance of the Research Project

Millisecond and Nanosecond Pulse Widths Induced Different Tissue Responses

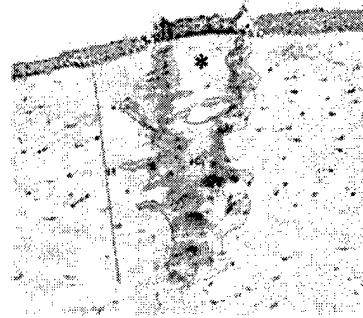
Studies supported by this project revealed marked pulse width dependence in the post-exposure corneal tissue response. The key-differentiating factors involved changes in the corneal epithelium, extracellular stromal matrix and stromal keratocytes. Exposure of rabbit corneal tissues to single pulse (0.8 millisecond pulse width) 1540 nm infrared laser light produced a marked coagulative necrosis of both the epithelium and the stroma. Because the laser was operating in the millisecond pulse range, we expected to see photothermal tissue decomposition and predicted that a coagulative necrosis would occur. We also noted histologic alterations in the stromal matrix within the beam path that we interpreted as matrix remodeling. To test this interpretation, we optimized an indirect immunohistochemical procedure to detect matrix metalloproteinase-2 (MMP-2) activity, which is an established biomarker of stromal remodeling. The result of the immunohistochemical analysis, in light of appropriate positive and negative controls, was that the MMP-2 reaction was mostly limited to the margins of the beam path in exposed tissues (Figure 1, Frame A). Exposure of rabbit corneal tissues to single pulse (500 nanosecond pulse width) 1540 nm infrared laser light produced a less severe coagulative necrosis of the tissues when compared to the millisecond exposures. We expected this result because nanosecond pulse widths should have less of a photothermal effect than millisecond pulse widths. However, we used MMP-2 immunohistochemistry to detect if subtle stromal remodeling had occurred. We were surprised to find a markedly increased MMP-2 immunohistochemical pattern (Figure 1, Frame B) than what we would have predicted from the H&E stains alone. Approximately half of the beam path was filled with MMP-2 reaction product. As far as we know, this is the first analysis comparing the stromal remodeling effects of an infrared laser operating in both the millisecond and nanosecond pulse widths.

Figure 1. Histomorphometry of MMP-2 Antibody Reaction following Millisecond and Nanosecond Laser Exposure.

Frame A-0.8 Millisecond Pulse Width Exposure. Note Normal appearing distribution of keratocytes within the Upper beam path (*). L1=depth at center of lesion (line offset for better visualization)=378 microns; PG1=area of DAB reaction product=30,399 microns².



Frame B-500 nanosecond Pulse Width Exposure. Note a Marked absence of keratocytes within the upper beam path (*) when compared to Frame A. L1=depth at center of lesion=202 microns; PG1=area of DAB reaction product=48,200 microns².



The number of keratocytes within the beam path is another remarkable feature that appears to differentiate millisecond from nanosecond pulse widths. Stromal keratocytes appear normally distributed in the superficial layer of the stromal matrix within the beam path of the millisecond laser (Figure1, Frame A). However, the majority of stromal keratocytes in an analogous position within the nanosecond beam path appear to be missing (Figure1, Frame B). Although we currently do not have an explanation for this change, the lack of a necrotic cell field suggests that the keratocytes may have been removed by apoptosis. A similar phenomenon of keratocyte apoptosis has been described in the superficial stroma following excimer laser surgery. This work was presented at the Photonics West Meeting (SPIE) in January 2002.

Proteomic Methods Were Developed to Describe the Laser-Tissue Interaction

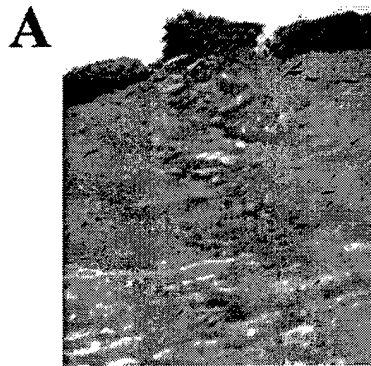
We have developed a novel proteomic method using histologic sections as the tissue source in conjunction with tissue isoelectric focusing (TIF). TIF is a direct recovery procedure and does not require homogenization or extraction of the specimen. The major advantage of this approach is that protein profile evaluation can be made with reference to the corresponding structural detail in a tissue section. TIF methods are particularly useful in laser-tissue interaction studies because the laser causes a small, discrete lesion that is surrounded by histologically normal tissue. We believe that the homogenization and extraction steps in standard proteomic methods would have difficulty separating the protein "signal" from the background "noise." With the TIF procedure, we can section through the lesion with micron precision and explore the tissue response in far-lesion, near-lesion and central-lesion tissue zones. We initiated proteomic studies of the laser- corneal tissue interaction in collaboration with Dr. Frank Witzman, a colleague at Indiana University and an AFOSR investigator. The total protein concentration present in the tissue sections used for TIF analysis was much less than optimum for the standard proteomic methods used in Dr. Witzman's laboratory. In addition, standard proteomic methods

involve tissue homogenization, thus, they provide limited information on protein distribution in the different cellular compartments of a heterogeneous tissue such as the cornea. In order to address these issues, we developed a mini-gel electrophoresis system for two-dimensional SDS-PAGE/ MS analysis of tissue isoelectric focusing TIF specimens. Identification of individual protein "spots" in the two-dimensional gel is performed using Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry and conducted in the Protein Sciences unit of the University of Illinois Biotechnology Center. Following a review of the literature, we believe that our method is the first application of TIF and two-dimensional SDS-PAGE / MS for proteomic analysis. This represents an important advancement from the perspective of toxicology and pathology because we can detect protein changes associated with focal tissue damage contained within histologic sections. Standard proteomic methods depend upon homogenization of the tissue prior to analysis and protein changes associated with focal lesions (such as those caused by a laser) are difficult to detect.

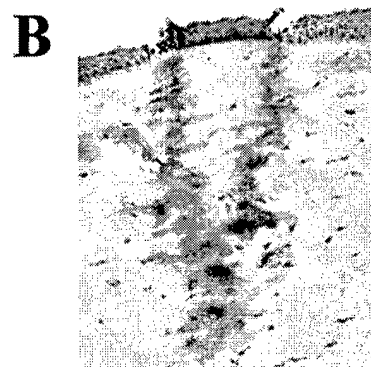
A previous report by our research group (SPIE Vol.: 4617; p26-29, 2002) used immunohistochemistry to determine that a component of laser-induced corneal wound healing involved an increase in MMP-2 tissue levels following laser exposure. Using the proteomic methods outlined above, we confirmed the immunohistochemical finding of increased tissues levels of MMP-2 and expanded the protein profile of the laser-corneal tissue interaction (Figure 2). Current studies are focused on using proteomic methods to identify additional corneal proteins in the molecular weight range between 20 and 40 kilo Daltons that are altered following laser exposure (Figure 3, Frame B).

Figure 2. Comparison of Histologic and Immunohistologic Sections with Tissue Proteomic Patterns

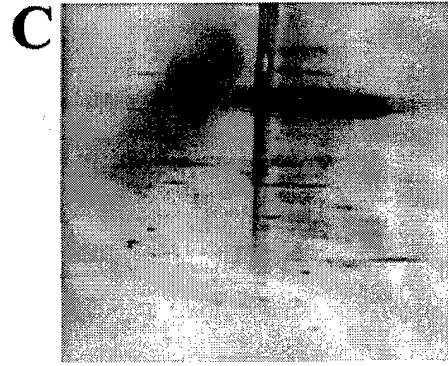
Frame A-Laser lesion stained using standard H&E stain.



Frame B-Serial section of laser lesion stained using antibody against MMP-2



Frame C-Two dimensional gel showing protein profile of control rabbit cornea



Frame D-Two dimensional gel showing protein profile of lesion area following laser exposure. Arrow indicates area of MMP-2 reactivity.

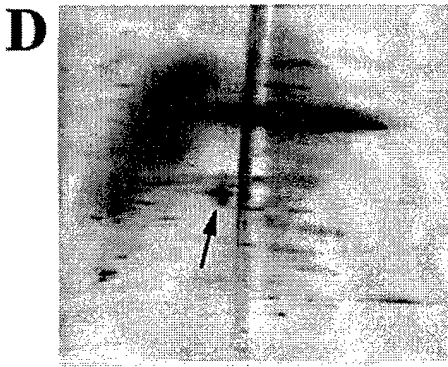
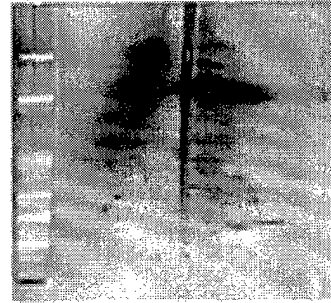
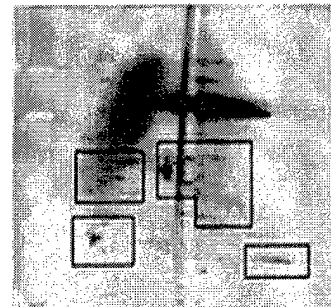


Figure 3. Comparison of Proteomic Profiles of Control and Laser-Induced Lesion Areas in an Experimental Rabbit Cornea.

Frame A. Representative Protein Profile from Control New Zealand White Rabbit Cornea.



Frame B. Representative Protein Profile Obtained from the Middle of a Lesion in a Laser Exposed Rabbit Cornea. Areas of interest indicated by overlying boxes contained proteins in the range of 10,000-50,000 daltons with isoelectric points in both the acidic and basic range.



These findings are important to the AFOSR mission because: (1) novel proteomic methods were developed that could compare tissue protein profiles with histopathology, (2) significant tissue changes associated with wound healing occurred in the cornea following exposure to nanosecond pulse widths that were not detectable using standard histologic techniques and (3) proteins of interest following laser-induced corneal damage occurred in the mid to low molecular weight range and were characterized by individual proteins with isoelectric points in both the acidic and basic range. This work was presented at the Photonics West Meeting (SPIE) in January 2003.

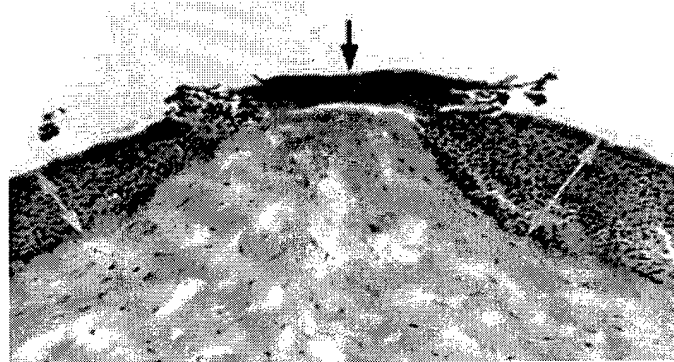
Low Fluence 1540 nm Laser Exposure Induces an Acute Proliferative Tissue Response

The engineered rabbit corneal tissues developed in the present study are appropriate models for the acute post-exposure response of the native tissue following 1540 nm laser exposure. The tissue reaction in the first 24 hrs post-exposure appeared to be restricted to the epithelial cells and was markedly different depending upon the energy applied to the tissue. Based on previous studies of the 1540 nm laser by our research group, we expected that high tissue energies (100 J/cm²) would produce a thermal coagulation of the native rabbit cornea. The histopathological response of the engineered corneal tissue was analogous to the response of the native rabbit corneas with essentially identical nuclear and cytoplasmic patterns of coagulative necrosis. The proliferative response of the native and engineered corneal tissues to low tissue energies was not expected. We believe this is the first report of acute post-exposure corneal cell proliferation with the 1540 nm laser. We are currently planning experiments to investigate underlying mechanisms of this proliferative effect using markers to identify matrix metalloproteinase activity and cyclin expression. In addition, we also plan to extend the post-exposure incubation of the native and engineered tissue from 48-96 hours to explore the extent of the proliferative response. It is compelling to hypothesize that low fluence laser application might be a

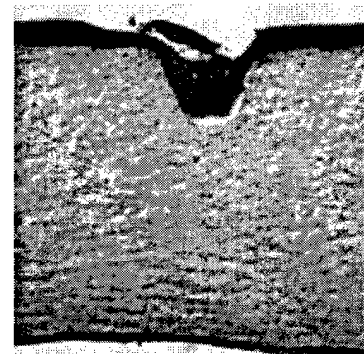
therapeutic approach to high fluence induced corneal damage. However, an excessive proliferative response might be detrimental to visual acuity. If specific mechanisms are discovered in both the native and engineered rabbit corneal tissue, we plan to extend these investigations using engineered human corneal tissue as a first approximation of the potential risk / benefit of the 1540 nm laser induced proliferative effect.

Figure 4. Comparison of Low Fluence Proliferative Response of Native Rabbit Cornea and an Engineered Rabbit Corneal Tissue Model.

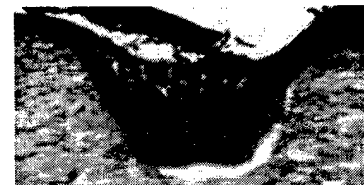
Frame A. Representative Image of Damage to a Rabbit Cornea 24 hours after Low Fluence 1540 nm Laser Exposure. The epithelium within the beam path was markedly damaged (black arrow); however, only minor damage was evident in the stroma. The epithelial cells adjacent to the damaged epithelium (yellow arrows) demonstrated a marked proliferation. Minimal cellular response was observed in the adjacent stroma



Frame B. Representative Image of Damage to an Engineered Rabbit Corneal Model 24 hours after Low Fluence 1540 nm Laser Exposure. The most superficial layer of the epithelium within the beam path was markedly damaged. However, the underlying epithelial cells showed a proliferative response that was analogous to the native rabbit cornea following low fluence tissue exposure (Frame A). The stromal matrix in the model tissue was affected more severely than the native rabbit cornea.



Frame C. Higher Magnification Image of the Corneal Model shown in Frame B. The marked epithelial proliferation is primarily restricted to the cells of the *stratum basale* in a similar manner as observed with the native tissue. This result indicates that the engineered tissues are an appropriate model for the rabbit cornea.



This finding is important to the AFOSR mission because it provides the first validated model to explore the currently anecdotal issue of tissue responses to low fluence radiation in the wavelength range from 700-1540 nm (“red light / infrared” zone).

Personnel Supported

Dr. Thomas Eurell-P.I.
Ms. Sharon Meachum-Research Technician

Presentations and Publications

Eurell, TE, Johnson, T, and Roach, P. A morphometric comparison of the acute rabbit and pig corneal response to 1540 nm laser light following ex vivo exposure. Proceeding of SPIE Vol: 4246; p 180-184, 2001.

Roach, P, Eurell, TE, and Johnson, T. Corneal exposures from 1540 nm laser pulses. Proceedings of SPIE Vol.: 4246; p 89-96, 2001.

Eurell, TE, Johnson, T, and Roach, P. Morphometric comparison of the acute rabbit corneal response to 1540 nm laser light following in vitro exposure to millisecond or nanosecond pulse widths. Proceeding of SPIE Vol.: 4617; p26-29, 2002.

Eurell, TE, Johnson, T, and Roach, P. Histomorphometric and proteomic analysis of the acute rabbit corneal tissue response following in vitro exposure to 1540 nm laser light. Proceedings of SPIE, Vol.:4953; p113-116, 2003.

Eurell, TE, Johnson, T, and Roach P. Proteomic analyses of the acute tissue response for explant rabbit corneas and engineered corneal tissue models following in vitro exposure to 1540-nm laser light. SPIE Vol.: 5319; p341-343, 2004.

Fahrner, LJ, Tan, W, Vinegoni, C, Eurell, TE, and Boppart, SA. Structural and functional imaging of engineered tissue development using an integrated OCT and multiphoton microscope SPIE Vol.: 5319; p1-10, 2004.

Invited Presentation-Eurell, TE "Silicon Nanoparticles: A New Generation of Biomedical Markers." Presented at The Nanotechnology Industry Workshop, University of Illinois at Urbana-Champaign, Friday May 9, 2003. I presented new information regarding the use of nanoparticles to follow cell maturation in organotypic corneal equivalents.

Eurell, T.E., Brown, D.R., Gerding, P.A. and Hamor, R.E. Alginate as a new biomaterial for the growth of porcine retinal pigment epithelium. *Vet. Ophthalmology*, 6(3): 237-243, 2003. The idea for this study stemmed from a discussion with Dr. Ben Rockwell last year and should prove useful to the ongoing AFOSR ocular research mission.

Proposals Submitted Stemming From This Research

An NIH proposal (NIH Assignment # 1 RO1 EY13398-01) was developed and submitted to the National Eye Institute using data obtained through collaboration with Drs. Roach and Johnson. The proposal was entitled "Effects of Infrared Laser Exposure on the Cornea".

Interactions and Transitions

Interactions

January 25, 2001- I attended the Ultrashort Laser Bioeffects Workshop for AFOSR Investigators in San Jose, CA. We reviewed the status of research projects among several AFOSR investigators working on laser-tissue interaction.

March 28-29, 2002- 48 rabbit corneal equivalents and 24 human corneal equivalents were brought to the Uniformed Services University of the Health Sciences laser lab for millisecond and nanosecond exposures to 1540 nm laser light. Project progress was reviewed with Dr. Thomas Johnson-Department of Preventive Medicine and Biometrics, Uniformed Services University of the Health Sciences.

June 11-12, 2002- 12 rabbit corneal equivalents, 12 human corneal equivalents and 12 human skin equivalents were brought to the Uniformed Services University of the Health Sciences laser lab for millisecond and nanosecond exposures to 1540 and 1318 nm laser light. Project progress was reviewed with Dr. Thomas Johnson.

August 6-8, 2002- Presented project progress at the AFOSR Bioeffects Laser Workshop, Kennybunkport, ME.

November 19-21, 2002- Traveled to Brooks AFB with engineered tissues for laser exposure and reviewed current research findings and approaches with Drs. Pat Roach and Ben Rockwell at the AFRL/HEDO facility.

March 31-April 1, 2003- Traveled to Bethesda with engineered tissues for laser exposure and reviewed current research findings and approaches with Dr. Tom Johnson at USUHS.

August 25-29, 2003- Performed histomorphometric analysis of 290 histologic sections from an *in vivo* skin exposure conducted by Brooks AFRL/HEDO scientists.

Transitions

Customer: Mission Research Corporation (MRC), Advanced Technologies Division, 8560 Cinderbed Road, Suite 700, Newington, VA 22122-8560. Contact Person: Dr. W. Michael Bollen, Project Manager, phone (703) 339-6500.

Result: The scientific product for the technology transfer was custom engineered human skin equivalents produced to evaluate the force vectors within a tissue generated by infrared laser exposure.

Application: MRC has a custom designed laser and purpose built pressure transducers to evaluate laser induced pressure waves within tissue; however, they could not find an appropriate target tissue to meet their custom size requirements. We designed and constructed engineered human skin equivalents that allowed MRC scientists to accomplish their goals. Feedback from MRC scientists indicated that the homogeneity of the engineered tissues we produced was particularly important to the success of the mission. The methods we developed for the construction of human corneal equivalents supported by the present AFOSR grant provided the basis for this technology transfer.