
Corneal wound healing after laser surgery

Aptamers as couriers for agents that modulate corneal healing

Heather Baldwin BSc FRCOphth MD, who carried out her research at St Thomas' Hospital, UK, followed with a description of how an improved understanding of corneal healing may lead to ways to reduce the incidence of haze following surface ablation procedures.

She noted that neither epithelial debridement nor stromal injury alone would produce haze. Haze only occurs when both the epithelium and stroma are injured, she pointed out.

"We can hypothesise that proliferating epithelium stimulates migration of keratocytes into the wound. These keratocytes are activated and they secrete new types of extracellular tissue. This extracellular tissue can disrupt the regular arrangement of the collagen lamellae and result in what we call haze," she explained.

To test this theory, Dr Baldwin and her associates carried out a study in which rabbits underwent epithelial debridement together with microkeratome incisions of varying depths.

The study showed that the shallower the microkeratome cut, the greater was the recruitment of keratocytes and the secretion of abnormal collagen. Conversely, the deeper the microkeratome cut the less this reaction occurred until, at a depth of 160 microns, it did not occur at all, she said.

"We can use this understanding in corneal refractive surgery. If we just debride the epithelium there is a signal put out by the regenerating epithelium. But there is no signal from the stroma, which is undamaged, so there is no haze. Likewise in LASIK, although there is a signal from the intrastromal wound there is none from the epithelium, so once again, no haze. But in PRK there is a signal from both the epithelium and the stroma which results in an interaction leading to haze," Dr Baldwin said.

LASEK barrier hypothesis

The absence of haze after LASEK, where alcohol is used to create an epithelial flap, suggests that there is also a time element involved in the epithelial-stromal interaction occurring after surface ablation procedures, she continued.

As the cells of the epithelial flap have largely been killed they will not release cytokines. The flap therefore serves as a temporary barrier to epithelial migration and by the time the epithelial cells have begun to cover the stroma the keratocytes will no longer be releasing cytokines and there will be no interaction between the healing responses of the two tissues. As a result there will also be no haze, she said.

"As we improve our understanding of corneal wound healing's physical characteristics in terms of distance and time and also its chemical characteristics in terms of growth factors it is possible we will discover more opportunities for modulating the epithelial stromal interaction in corneal wound healing and obtain better results in our patients," Dr Baldwin added.

In vitro eye model

Madhavan Rajan MD FRCS ophth, FRCS Moorfields Eye hospital, London, told the clinical research symposium that a new in-vitro model, developed at St Thomas’ Hospital, UK, may provide further insights into the wound healing process after refractive surgery without the shortcomings of animal trials or the ethical and legal concerns of clinical studies.

He noted that most animal studies have involved species with eyes that are different in several important results from those of humans. For example, rabbit eyes lack Bowman’s membranes and their stromas completely regenerate after trauma. Moreover, it can be difficult to perform slit-lamp or confocal microscopy in animal subjects.

Dr Rajan and his associates therefore designed an in vitro corneal model to evaluate stromal and epithelial interactions following corneal refractive surgical procedures. The in vitro setup enables corneas from donor eyes to retain their curvature and transparency in an air-interface organ culture environment for up to four weeks, he noted.

The device consists of two PMMA plates which secure the donor corneas in culture. An agar gel fills the interior of the cornea to maintain its curvature and the two plates are placed into a Petri dish containing a culture medium and stored in a humid environment

Corneal transparency and biomechanical integrity these cells cause.

To identify candidate aptamers, Dr Angunawela and his team use special culturing techniques to separate the different types of cells in corneal tissue. They then exposed the separated cultures to different aptamers to determine affinities between the aptamers and the different cell types.

"Aptamer selection is extremely time consuming and we were able to automate this using a liquid handling robot capable of PCR. We conjugated the final aptamer to a green fluorophore, which allowed detection by laser confocal microscopy. We used a scrambled aptamer as a control."

To date they have identified one candidate aptamer, which demonstrated a significantly stronger affinity for the activated keratocyte phenotype than for skin fibroblasts, which are very similar. However, the molecules’ affinity for activated keratocytes was not significantly greater than its affinity for passive keratocytes.

"Our initial results have been very encouraging and have shown that aptamers can be used to distinguish between close cellular phenotypes. Potential sources of error include to difficulty in growing a purely passive culture of keratocytes and the choice of a green fluorophore for detection as there is competing green background fluorescence," he added.
in a sterile incubator at 37° C.

Dr Rajan and his team have also modified their confocal microscopy system in such a way as to enable scanning of the in vitro cornea model in real time and also enable viewing of the keratocytes in tandem with epithelial healing process.

**LASIK vs PRK**

In a study involving 56 human donor corneas placed in their specially designed in vitro setting, the researchers compared the epithelial and keratocyte cell kinetics following PRK and LASIK.

They found that in corneas that underwent PRK, the epithelial flap regenerated within 92 hours, compared to 120 hours in eyes that underwent LASIK. In both procedures the magnitude of keratocyte loss corresponded to ablation depth, and keratocyte regeneration was dependent on epithelial closure. However, eyes that underwent LASIK lost a lower percentage of keratocytes, although the keratocyte regeneration was significantly delayed in the LASIK flap, Dr Rajan noted.

“It was very clear that there was a difference in wound healing response of surface and intrastromal procedures,” he said.

Another study carried out with the in vitro corneal holder indicated that mitomycin-C may not be harmful to corneal tissues if applied for only one minute but the epithelial and keratocyte kinetics was significantly altered if it was applied for two minutes.

The study involved 24 corneas that underwent PRK alone or PRK with mitomycin-C (0.2 ug/ml) for one or two minutes. The researchers found that epithelial migration was significantly delayed in the mitomycin-C groups. They also observed that the epithelium was poorly differentiated and significantly thiner in the group that received the two-minute application.

In addition, although there was no significant difference between the groups in terms of keratocyte loss there was a significant delay in keratocyte regeneration in the mitomycin-C groups. Furthermore, the anterior stromal cell density was significantly lower in the two-minute group than it was in the one-minute group.

“Mitomycin-C application did not result in increased loss of keratocytes, but it significantly delayed keratocyte repopulation in the anterior stroma,” Dr Rajan said.

**LASEK vs epi-LASIK**

Clinical evidence is sparse with regard to whether procedures like LASEK, where the epithelial cells are killed, is better than epi-LASIK, where the flap remains alive, in terms of corneal wound healing or haze. said Christoph W. inkl. von Mohrenfels MD, Augenklinik Klinikum Rechts der Isar, Technische Universität München.

“It is important to do the laboratory research but you also have to do the clinical studies. It’s no good having a good wound healing model if it is not corroborated by clinical findings and vice versa,” he pointed out.

He noted that numerous studies have indicated that LASIK significantly reduces both the extent and duration of pain compared to PRK and that the incidence and degree of haze is also significantly lower with LASIK. However, there is currently very little in the published literature to indicate how LASIK compares with epi-LASIK, a mechanical procedure that does not expose the epithelium to the toxic effects of alcohol, Dr von Mohrenfels said.

Proponents of LASIK maintain that the presence of a dead flap will delay epithelial healing and therefore uncope the epithelial-stromal dialogue that gives rise to keratocyte proliferation and haze. Proponents of epi-LASIK argue that as the epithelial keratocytes are largely undamaged they will not initiate the dialogue in the first place.

In a study by Vikentina Katsanevaki MD and her associates (J Refract Surg 2006) histological examination of mechanically separated epithelial flaps from four eyes showed that the 87 per cent to 99 per cent of all epithelial cells were morphologically normal after 24 hours.

“But you still have a few morphologically degraded cells which are dying and you will have some cytokines. There is only a partial presence of the basal membrane which means that these cells are also dying, leading to a dying epithelium,” Dr von Mohrenfels noted.

Moreover, the results of an organ culture study support the epithelial barrier hypothesis of LASIK. Dr von Mohrenfels collaborated in the study with its principal author Romesh Angunawela FRCOphth.

The researchers examined human corneas that underwent either epi-LASIK or LASEK with 20 per cent or 100 per cent alcohol. They found that at one week there was a positive correlation between the percentage of living cells in the epithelium and the degree of central stromal keratocyte proliferation.

In corneas that underwent epi-LASIK, 98.7 per cent of epithelial cells were alive and the rate of keratocyte proliferation was 42 per cent, while the values for LASEK with 20 per cent alcohol were 12.3 per cent and 15 per cent, respectively and those for LASEK with 100 per cent alcohol were 100 per cent and five per cent, respectively.

“Therefore you can hypothesise that if you have a living epithelium you probably have more growth factors and this would make more problems for the keratocytes. But we will have to wait for more studies to prove whether you will have more growth factors if you keep the epithelium dead or alive.”

**Biomechanical integrity and refractive stability**

In a joint presentation at the clinical research symposium, Philip Jaycock MD, MR Coll Orth and Nathaniel Knox Cartwright MRC Ophth and Nathaniel Knox Cartwright MRC Ophth of St Thomas’ Hospital, London UK described how the impact of LASIK on corneal biomechanical integrity may raise the risk not only of ectasia, but also of regression over the long term.

Dr Knox Cartwright noted that while LASIK was originally heralded as a panacea to all the problems of PRK such as pain and haze, it has recently begun to emerge that the short-term benefits of LASIK may be offset by biomechanical instability in the longer term.

However, many continue to maintain that the risk of biomechanical instability following LASIK is negligible provided surgeons avoid performing the procedure on keratoconus suspects and leave a residual stromal depth of at least 250 microns.

“These claims are based on the erroneous assumption that biomechanical instability equates to ectasia. In fact, biomechanical instability is a spectrum with ectasia at the very worst end of it, representing biomechanical failure,” he said.

Several studies published over recent years provide evidence for LASIK’s long-term refractive instability compared to PRK, he noted. One study (Rajan et al, Ophthalmology, 2004;111:1813-1824) showed that refraction following myopic PRK remained stable throughout 12 years of follow-up. Similarly, another study (O’Brart et al, J Cataract Refract Surg 2005;31:1104-1113) showed that refraction after hyperopic PRK remained stable for 7.5 years of follow-up.

In contrast, two more studies have shown that following LASEK there was a trend towards regression for hyperopic LASIK at five years (Jaycock et al, Ophthalmology 2005;112:191-199) and for myopic LASIK at six years (Sekundo et al, J Cataract Refract Surg 2003;29:1152-1158). Moreover, the hyperopic LASIK study a third of patients regressed by more than one dioptre, Dr Knox Cartwright added.

**Increased severing of corneal lamellae**

Dr Jaycock noted that the likely cause of the increased biomechanical instability following LASIK compared to PRK was the increased number of collagen lamellae that are severed in the intrastromal procedure. Collagen lamellae are more densely interwoven in the superficial third of the stroma than in the deeper two thirds. In addition, X-ray diffraction studies indicate that collagen fibres cross perpendicularly in the centre and cross increasingly obliquely towards the periphery of the cornea. (Meek et al, Exp Eye Res 2004;78:503-512). Thus the cornea is stronger anteriorly than posteriorly and stronger peripherally than centrally. In a 6.0 dioptre PRK correction, approximately five million collagen fibres are severed, whereas for a corresponding 2.0 procedure, 230 million fibres are severed, Dr Jaycock pointed out.

“It is somewhat unfortunate that the standard microkeratome flap incision severs the cornea at the strongest point in both the anterior-posterior and radial planes. PRK and LASIK clearly have a very different impact on biomechanical properties,” Dr Jaycock added.

**Interferometry studies**

Dr Jaycock noted that studies using recently developed types of interferometric devices indicate that microkeratome flap formation alone will significantly alter corneal biomechanics. For example, an in vitro study using ovine corneas that had undergone microkeratome incisions, testing with electronic speckle pattern interferometry showed a 27 per cent decrease in structural integrity in the peripheral region of the cut (Jaycock et al, J Cataract Refract Surg 2005;31:175-181).

Dr Knox Cartwright reported that experiments using radial shearing speckle interferometry have shown similar results in human eye bank corneas and demonstrated that, at least in the short term, corneal strength does not recover significantly during wound healing.

Using finite element modelling it is possible to extrapolate from these findings that there is a progressive weakening of the cornea as the microkeratome depth increases, he said. So, for example, a microkeratome incision depth of 150 microns would reduce corneal integrity by 17 per cent, and an incision with a depth of 300 microns would reduce it by 77 per cent.

Dr Jaycock concluded the joint presentation by suggesting that the ideal refractive procedure would combine the biomechanical advantages of PRK with the functional and cellular advantages of LASIK. This might be possible by creating an intrastromal flap just below Bowman’s membrane using a femtosecond laser.

“The idea behind a sub-Bowman’s approach is that in preserving the corneal epithelium and not activating stromal keratocytes we should have no pain, no haze and maintain maximal biomechanical stability.”