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Confocal laser scanning microscopic examination of transport pathways and barriers of some peptides across the cornea

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Summary

Recent advances in optical microscopy involving confocal imaging are now becoming available which dramatically improve resolution, contrast, and rejection of out-of-focus noise as compared to conventional imaging. With this technique, it is possible to optically section thick specimens, allowing interior structures of living specimens that are normally obscured in conventional imaging to be visualized. The unique transparency of the cornea makes microscopic examination by this technique particularly applicable. In the present study, the technique, performed in both light-reflected and fluorescence modes, was used to obtain information about cellular structures, transport pathways and barriers of two peptides, poly-L-lysine and insulin in the cornea. In the light-reflected mode, serial images showing detailed structures of various layers of the cornea including the epithelial surface, wing cells, basal cells, stroma, and endothelium were clearly demonstrated. The transport pathways of fluorescently labeled peptides were studied using the fluorescence mode. The results suggest preferential uptake of polylysine through epithelial surface defects caused by non-uniform shedding of superficial 'old' cells and through intercellular spaces of the cornea. In contrast, corneal uptake of insulin was found to occur predominantly via internalization of the peptide by the surface cells. In both cases, the penetration of these peptides was severely limited to the outermost layer of the corneal epithelium. The results also indicate charge discrimination effects to penetration of negatively charged insulin by the cornea.

Introduction

Confocal microscopy has recently emerged as a new and powerful microscopic technique, offering the possibility of good resolution without the usual artifacts attendant with sample preparation, required in conventional light and electron microscopy. Confocal imaging is a technique in which a specimen is illuminated and scanned point by point with a finely focused laser beam. The illuminated point is viewed with a spatially restricted optical system so that at any one time only signals emanating from this point are detected. This contrasts with conventional light microscopic methods, where a relatively large region of the specimen is normally illuminated by an array of excitatory light. The confocal imaging technique virtually eliminates out-of-focus interference and thus high contrast images with low,

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diffuse, backgrounds can be obtained (Sheppard and Wilson, 1982; Wilson and Sheppard, 1984). In addition, with this technique the resolution of standard light microscopy can be surpassed if optics of high numerical aperture are used (Brakenhoff et al., 1979). The confocal principle is also applicable in fluorescence microscopy where, due to the incoherence of the fluorescence light, an even higher resolution may result (Cox et al., 1982). With the integrated image signal processor which has been optimally adapted to the signal of the optical system, serial and 3-dimensional images of the specimens are now possible.

The study of transport pathways of peptides across epithelia throughout the body has been an area of intense interest due to its importance in drug delivery. However, most evidence to date, is based primarily on physicochemical properties and flux measurements, and thus are indirect and speculative. Direct microscopic examination of peptide absorption in the cornea has previously been attempted (Kaye et al., 1973, Tanjum, 1974; Hirsch et al., 1976), but observation of the transport process in living tissue specimens has not been reported.

In the present study, confocal scanning microscopy was used to provide direct information on corneal transport of two peptides, poly(L-lysine) and insulin. Information from this study may allow development of more efficient and perhaps safer methods of peptide delivery, i.e. the use of penetration enhancers that selectively and transiently modify membrane transport barriers.

Experimental

Animal and materials

Male albino New Zealand rabbits (New Franken, WI) weighing between 2.5 and 3.0 kg were used. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed. Lighting was maintained in the caging facilities for 24 h a day to provide a constant experimental environment. FITC-labeled poly(L-lysine) ($M_r = 15\,000$–$30\,000$) and insulin ($M_r \approx 6000$) were obtained commercially (Sigma, St. Louis, MO). Glutaraldehyde (Ladd, Burlington, VT), used in the scanning electron microscopic study, was from 70% stock stored under argon in sealed ampules. All other chemicals were either reagent or analytical grade and were used as received.

Methods

Confocal scanning microscopy The instrument used was the MRC/Lasersharp fluoroscan confocal microscope (Bio-Rad, Cambridge, Massachusetts), originally developed at the Medical Research Council's Laboratory of Molecular Biology (U.K.). The system consists of a computer controlled laser scanner assembly which attaches to the Nikon Optiphot microscope. A 25 mW argon ion laser (Ion Laser Technology, Salt Lake City, UT) operating at 488 nm wavelength was used as the excitation source. Reflected or fluorescent signals are de-scanned, spatially filtered and detected by a photomultiplier-S-20 photocathode system. Images are assembled in an integral image processor and displayed on a digital video monitor. Corneal specimens, freshy excised or taken after specific treatments from in vitro perfusion experiments, were directly mounted in a non-fluorescing mounting media (Polysciences, Warrington, PA) and were examined microscopically without further tissue processing.

Scanning electron microscopy Immediately after the rabbits were killed, the corneas were carefully removed and placed in a fixative solution containing 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 2 h. After washing in the same buffer, the tissues were post-fixed in 2% OsO_4-0.1 M phosphate buffer solution, pH 7.3, and were dehydrated using a graded series of ethanol solutions at concentrations of 35, 50, 70, 90, 95, and 100%. Prior to viewing, the specimens were critical-point dried in a pressure chamber using liquid carbon dioxide. Subsequently, a uniform 100 Å layer of gold was applied on the specimen surfaces by means of Sputter coating (Balzers-SCD 030). The specimens were finally viewed in a scanning electron microscope (Jeol JSM-35C).

In vitro perfusion studies Rabbits were killed with an intravenous injection of an overdose of sodium pentobarbital given via the marginal ear vein. The corneas were removed by cutting the sclera 2 mm laterally to the limbus and the crystal-

line lens and iris were removed. Care was taken to avoid contamination and physical trauma to the tissue. The corneas were then immediately mounted on pin-typed corneal rings and clamped in the perfusion chamber (Machine Shop, University of Wisconsin-Madison). In contrast to the standard corneal mounting procedure utilizing a suction device, previously described (Schoenwald and Haung, 1983), the procedure presented here provides better preservation of tissue integrity and viability as indicated by higher and longer membrane resistance and potential respectively (Rojanasakul and Robinson, 1989). The two faces of the cornea were bathed with glutathione-bicarbonate Ringer's solution, pH 7.4. Addition of specific labeled peptides was made either in the donor side alone or in both sides as specified in Results and Discussion. The buffer was reported to preserve integrity of the excised cornea for up to 6 h (O'Brien and Edelhauser, 1977). Aeration with a mixture of 95% O_2 and 5% CO_2 was initiated to provide oxygen and circulation in the tissue bath. Electrophysiology studies based on active potential measurements, conducted in this laboratory, showed that tissue viability of the cornea in this buffer was maintained for at least 6 h. All experiments were carried out at 37°C using a constant temperature bath (American Scientific Products) with external circulator connected through a water jacket of the perfusion cell. A 0.67 cm^2 area of tissue was exposed to the donor and receptor compartments, each compartment having a volume of 7 ml.

Solution preparation The glutathione-bicarbonate Ringer's solution was prepared in two parts; the first part contained NaCl (12.4 g/l), KCl (0.716 g/l), $Na_2HPO_4 \cdot H_2O$ (0.206 g/l), and Na_2CO_3 (4.908 g/l); the second part contained $CaCl_2 \cdot 2H_2O$ (0.23 g/l), $MgCl_2 \cdot 6H_2O$ (0.318 g/l), glucose (1.80 g/l), and oxidized glutathione (0.184 g/l). Equal parts of both solutions were mixed prior to use. Solutions were stored in the refrigerator and used within 2 weeks. All labeled peptides were dissolved in the buffer solution at a fixed concentration of 0.01 mg/ml and were used immediately after preparation. The amount of peptide added did not appreciably alter the total solute concentration and pH of the final solutions.

Results and Discussion

Confocal reflectance imaging of the cornea

Confocal scanning imaging of the cornea was performed in both the light reflected and fluorescence modes. The former was used to visualize superficial and interior structures of the cornea, while the latter was used to visualize fluorescent peptide probes and their transport pathways in the cornea. In Fig. 1, panels a–f show serial images of a freshly excised cornea in the light reflected mode. Surface imaging of the epithelium (Fig. 1a) reveals the presence of both light and dark cells, similar to that observed by scanning electron microscopy (Burns et al., 1971; Pfister, 1973; Grass and Robinson, 1988). The surface of the epithelium is fairly flat and composed of polygonal shaped cells. The intercellular junctions appear tight and the cells are attached to each other along relatively straight cell boundaries. Previous electrophysiology studies of the cornea, conducted by Holt and Cogan (1946), and confirmed in this laboratory, indicated that the epithelium, due to the limited dimension of the intercellular junctions, contributed to most of the membrane electrical resistance and presented the major transport barrier for passive permeation of charged molecules.

Due to the unique transparency of the cornea which allows a greater degree of light penetration as compared to other tissues, examination of detailed structures in the deeper layers of the cornea can generally be obtained without significant deterioration in image quality. Serial images of the deeper layers including the wing cells, basal cells, stroma, and endothelial cells are shown in Fig. 1c–f. Cell borders and intercellular spaces between the wing and basal cells of the epithelium are evident (Fig. 1c and d). Note that under the same magnification the intercellular spaces of the epithelium become wider and wider with increasing depth. In contrast, cell diameter becomes increasingly smaller with depth, being largest in the flattened superficial cells and smallest in the columnar basal cells.

Confocal microscopy also provides information about structure of the stroma. Corneal nerves, which can not normally be visualized except by

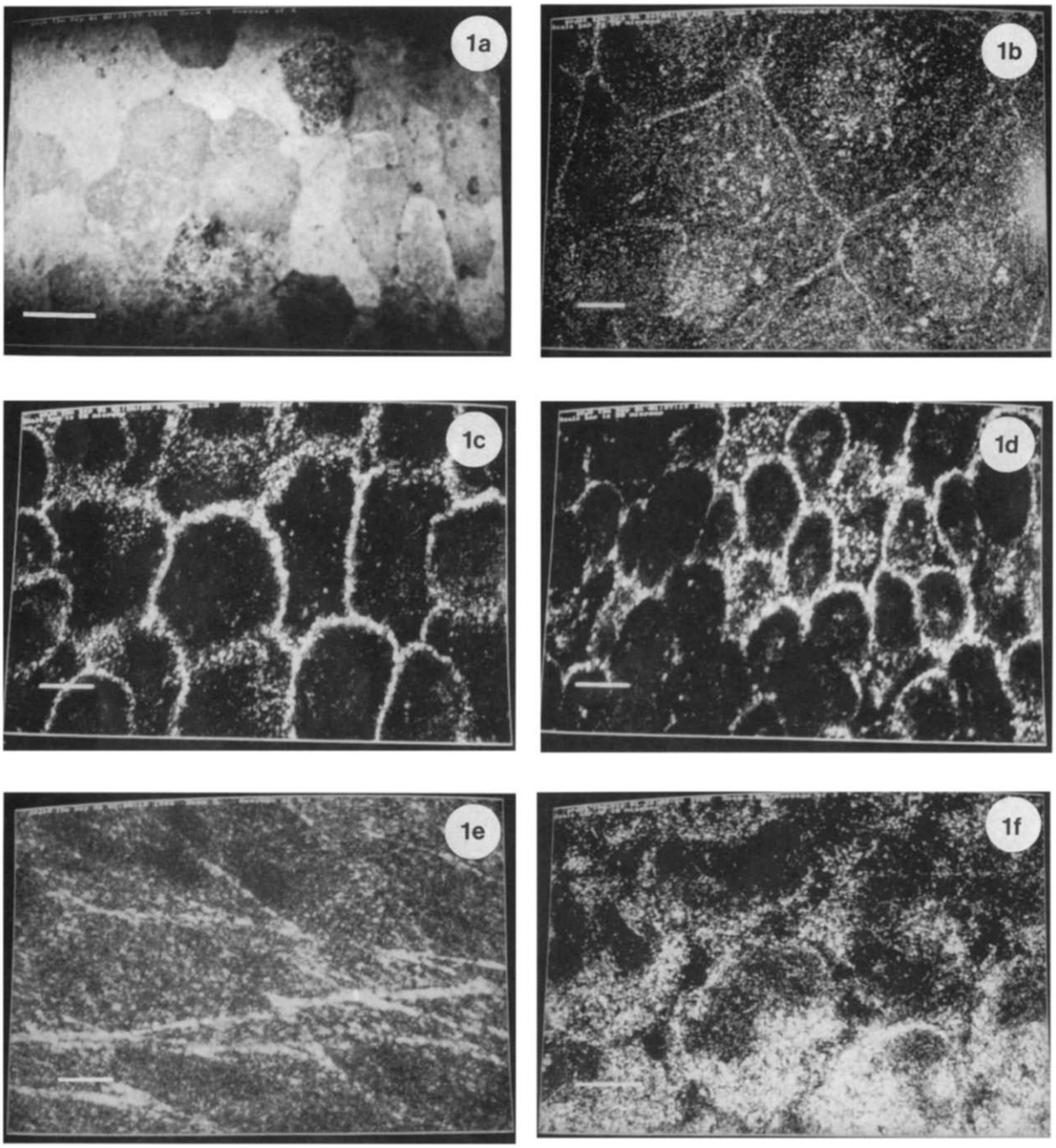
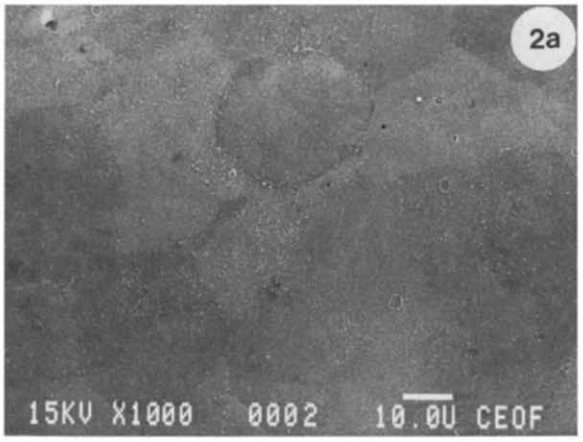
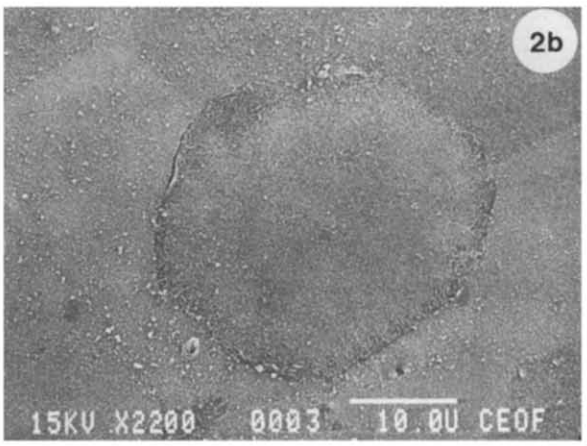
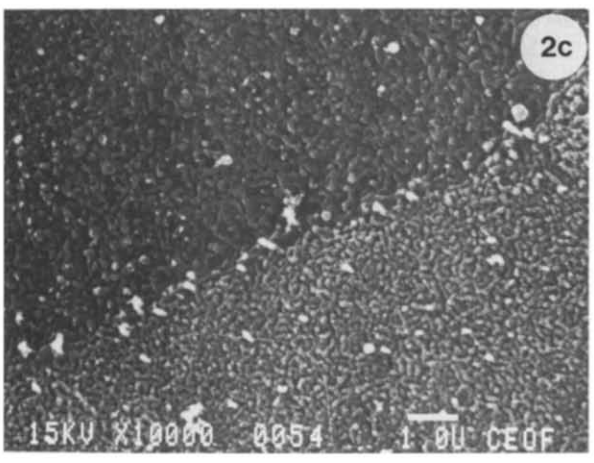


Fig. 1. Confocal reflectance images at different levels of the cornea. (a and b) Surface view of the epithelium showing light and dark cells at different magnifications (bars = 50 and 10 μm, respectively). (c and d) Epithelial wing and basal cells showing intercellular spaces, (bars = 10 μm). (e) Corneal nerve of the stroma (bar = 10 μm). (f) Corneal endothelium (bar = 10 μm).

special histological staining, can clearly be seen (Fig. 1e). In the deepest layer, i.e. the endothelium, image quality partially deteriorated (Fig. 1f). Generally, the limit to depth of optical sectioning for thick specimens in the confocal system is set by a fall-off of light intensity rather than by







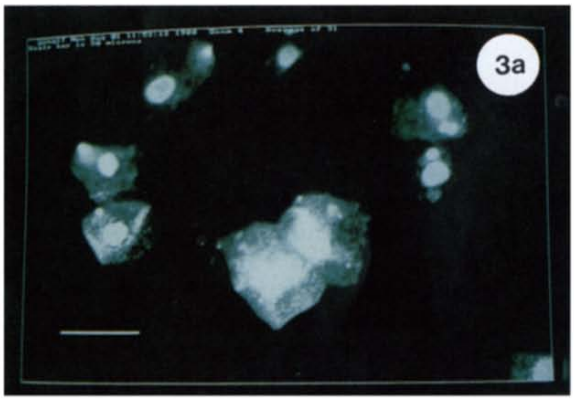
degradation of image quality. The present fall-off is presumably due to attenuation of the incident exciting and emergent beams as they pass through the interior structures.

Scanning electron microscopy

Due to its dominant role in determining transport properties of the cornea, the apical cell layer of the epithelium was examined in greater detail using high-resolution scanning electron microscopy (SEM). Panels a–c of Fig 2 show SEM micrographs of a typical epithelial surface at increasing magnification. At low magnification, individual epithelial cells can be separated into light and dark cells on the basis of their brightness under the scanning microscope. Dark cells were usually found in clumps distributed in a random fashion. The relative density of surface elaborations accounts for the variable texture of these cells. Light cells have more microvilli per unit area than dark cells (Fig. 2c). Previous studies by Burns et al. (1971) and Pfister (1973) indicated that this difference was related to cell age. The younger cells contain more elaborate reticulation, whereas the older, desquamating cells have less. Fig. 2a and b also shows one of the typical older cells that is about to slough off from the superficial layer. Under normal physiological conditions, the epithelial surface undergoes relatively rapid turnover. The shedding by the old cells and replacement by the younger underlying cells occur continuously with an average turnover of approx. 1 day. The entire process from inception to shedding of an epithelial cell takes approx. 4–8 days (Davson, 1980).

It is interesting to note that the intercellular junctions along the border of the degenerating old cells are disrupted while those between the normal cells appear very tight (Fig. 2b). The surface cracks

Fig. 2. Scanning electron micrographs of the corneal epithelium. (a) Low-magnification micrograph of a typical epithelial surface showing light, dark, and one of the degenerating dark cells (bar = 10 μm). (b) Degenerating cell showing disrupted intercellular junctions at higher magnification (bar = 10 μm). (c) Intercellular junction between the degenerating and light cells at higher magnification. Note the difference in density of the microvilli between the two cells (bar = 1 μm).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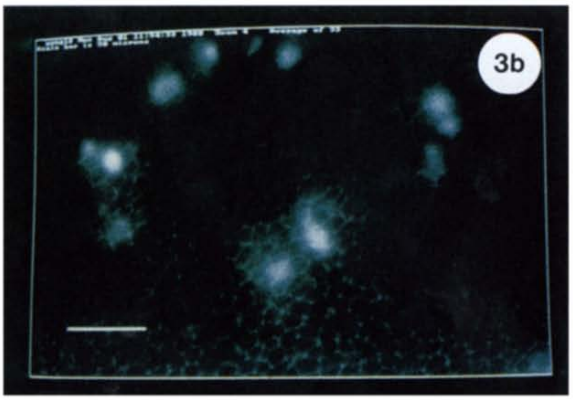
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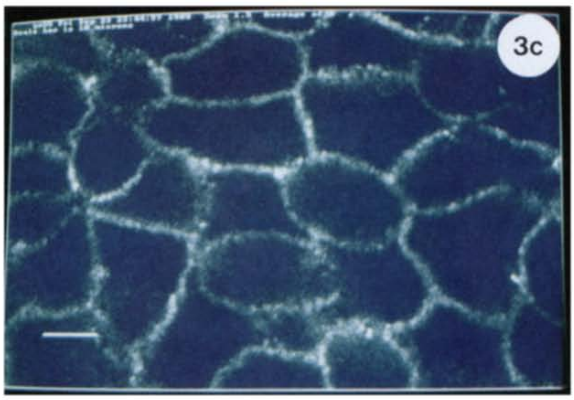
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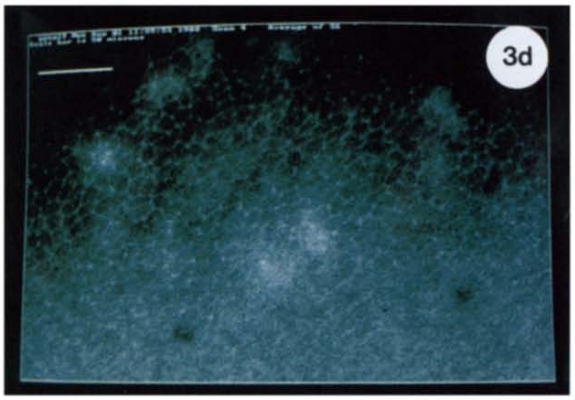
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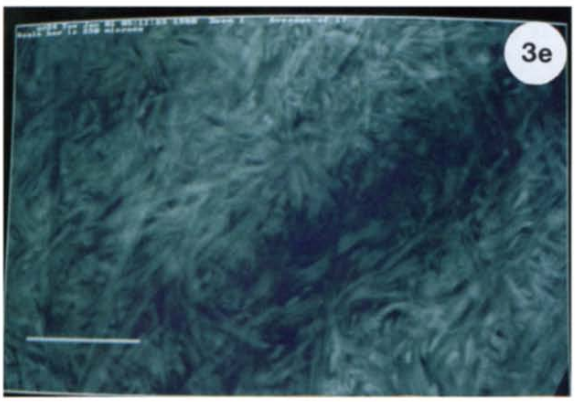
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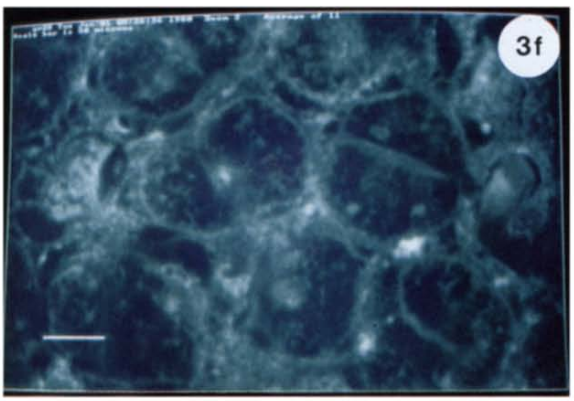
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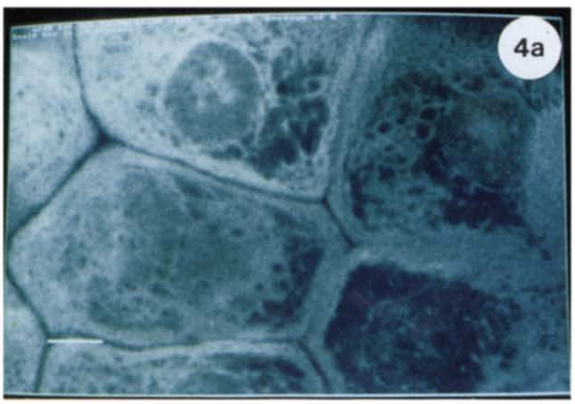
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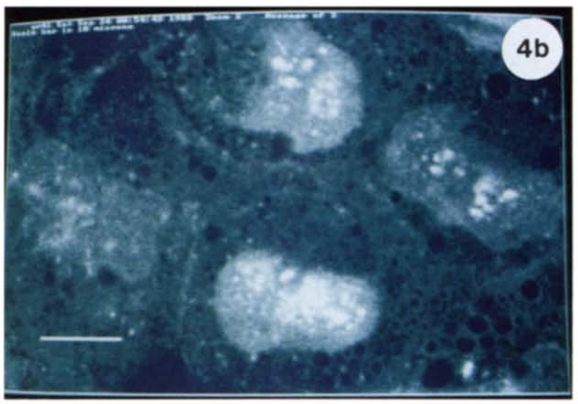
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and defects resulting from continuous shedding of superficial cells were later found to be the major pathway for movement into the cornea by the peptide, polylysine, and presumably for most large hydrophilic compounds.

Confocal fluorescence microscopy

Transport pathway of FITC-labeled poly(L-lysine)

In an early attempt to visualize the transport pathway of the peptide, conventional epifluorescence microscopy was used. Intense fluorescence of the peptide was observed on the corneal surfaces but the image quality was poor and the cell structures obscured. At deeper focal levels, no signals could be detected. In contrast, by minimizing the background glow due to out-of-focus fluorescence and optical sectioning through the structures, the confocal system makes it possible to visualize sharp fluorescent images at various levels throughout the cornea.

Confocal images of polylysine-treated corneas taken at 4 h after perfusion are shown in Fig. 3a–f. When polylysine was applied to the epithelial surface, fluorescent peptide could be detected only in some of the superficial cells (Fig. 3a). No peptide was detected in any of the underlying layers, indicating that this superficial layer behaves as a rate-limiting barrier for penetration of the polylysine. Interestingly, polylysine did not uniformly fill up the entire superficial cell surface or selectively locate at the intercellular junctions as expected. Instead it filled up some of the surface cells in a random fashion. Evidence from confocal reflectance and electron scanning microscopies of the corneal surface reveal that these filled areas are the cellular spaces resulting from the degenerating old cells. When polylysine was perfused from both sides of the cornea, a similar result was obtained on the epithelial surface. However, polylysine can penetrate the endothelium, enter the stroma, and readily fills up the intercellular spaces of the epithelium (Fig. 3b–f). The penetration of polylysine in the intercellular spaces was found to be limited to the lower layers of the epithelium, confirming the occluding role of the surface intercellular junctions. This finding is in agreement with results obtained in a previous study (Tonjum, 1974) which demonstrated the ability of a protein, horseradish peroxidase, to penetrate the cornea up to the surface layer. However, in that study, due to limitations of transmission electron microscopy, the transport phenomenon on the surface could not be examined.

With regard to the possibility of enzymatic degradation in the cornea which might influence the transport behavior of peptides, it was anticipated that an enzymatic conversion might result in smaller subunits of polylysine and thus a higher rate of peptide penetration in the tissue. However, since polylysine is a homologous biopolymer, each fraction should possess the same chemical property and thus its transport pathway should remain unchanged, assuming that charge and not size is the critical structural feature. Furthermore, since polylysine was preferentially taken up into the intercellular spaces rather than inside the cells, where major proteolytic activity is believed to be located, degradation of the peptide is expected to be limited. An understanding of extracellular enzymatic activity and its significance to the overall transport process are of great importance and further research is needed.

Finally, for the purpose of improved peptide absorption, results from this study imply that ef-

Fig. 3. Confocal fluorescence images at different levels of the polylysine-treated cornea. (a) Typical surface image of the corneal epithelium showing the presence of FITC-labeled polylysine in some of the cellular regions (bar = 50 μm). (b) Wing cells of the epithelium showing preferential localization of polylysine in the intercellular spaces. Note the fluorescence glow resulting from out-of-focus signals in the superficial layer (bar = 50 μm). (c) High-magnification image of the epithelial wing cells showing intercellular polylysine (bar = 10 μm). (d) Basal epithelial layer-basement membrane junction showing intercellular and uniformly distributed polylysines in the two layers (bar = 50 μm). (e) Corneal stroma showing distribution of polylysine along the collagen fibers (bar = 250 μm). (f) Endothelium (bar = 50 μm).

Fig 4. Confocal fluorescence images of the insulin-treated cornea. (a) Surface view of the epithelium showing intracellular uptake of insulin. Note the lack of insulin in the intercellular junction (bar = 10 μm). (b) Endothelium showing intracellular uptake and nuclear accumulation of insulin (bar = 10 μm).

fective improvement in transport of peptides, that are selectively absorbed through intercellular spaces, can be obtained by the use of compounds or methods that modify superficial junctions, preferably in a reversible manner.

Transport pathway of FITC-labeled insulin In recent years, interest in non-parenteral delivery of insulin has increased dramatically as evident from the large number of publications on the subject. Advances in chemical synthesis and recombinant technology have contributed to such progress. However, significant improvements in peptide delivery by noninvasive means have been limited due to problems involving penetration, as well as enzymatic, and immunologic barriers presented by the membranes. At present, our knowledge on the subject is insufficient and a better understanding of peptide processing by membranes is required. While the majority of work on insulin delivery has been focused on methods of improving absorption, relatively little is known about the basic mechanism of insulin transport across epithelia. In fact, the understanding of this aspect of membrane transport has been, to date, largely speculative. Undoubtedly, a better understanding of this transport process will help development of more effective delivery systems.

In the present study, confocal fluorescence was used to examine the transport mechanism and pathway of insulin across the cornea. Insulin, a peptide hormone containing 51 amino acid residues, has a molecular mass of approx. 6000 Da and is a zwitterion. At physiological pH and the pH of the experiment (pH 7.4), it is negatively charged (isoelectric point, p*I*, of 5.4) is expected to be poorly absorbed and probably via the aqueous paracellular pathway. However, results from this study indicate otherwise. In the study where insulin was applied to the epithelial side of the cornea, it was found that insulin was taken up intracellularly by the surface epithelial cells (Fig. 4a). Relatively speaking, no insulin was detected in the intercellular junctions or any other layers of the cornea except for this outermost layer. The basis for intracellular uptake of insulin by the cornea is not yet clear, but presumably due to a process of endocytosis since passive uptake via a partitioning process of a large, hydrophilic, and charged molecule is unlikely. The lack of insulin in the underlying epithelial layers is of interest and is believed to be due to the lack of specific insulin receptors or immaturity of the cells. Alternatively, the charged insulin is unable to penetrate the intercellular space of the cornea to reach deeper tissues. Although information on endocytosis of peptides in the cornea has not been well-documented, there is evidence showing that endocytosis of insulin occurs in many other cell types in the body including fibroblasts (Schlessinger et al., 1978), hepatocytes (Gorden et al., 1978), and lymphocytes (Carpantier et al., 1978). Functionally, it is conceivable that the presence of a specialized transport system for insulin in the cornea may be of physiological importance, since the cornea is an avascular tissue and its total energy supply is provided by the surrounding tissues, the aqueous humor and tears. Insulin is known to promote glucose uptake into target cells and glucose is the major substrate for energy production in the cornea.

In other experiments where insulin was applied to both the epithelial and endothelial sides of the cornea, intracellular uptake of insulin is observed on both surfaces with pronounced accumulation of insulin in the endothelial nuclei (Fig. 4b). However, unlike the polylysine case, the presence of insulin in the stroma was minimal and barely detectable in the intercellular spaces of the epithelium. Nuclear accumulation of insulin was previously observed by Goldfine et al. (1977). In their study, specific cellular uptake via receptor-mediated endocytosis and subsequent nuclear receptor binding of insulin in human cultured lymphocytes was reported. Based on physicochemical properties of the peptide, it is of interest to observe a low degree of penetration of the peptide into the aqueous diffusional route of the cornea. Since the concentration of the fluorescent insulin used was far greater than the detection limit and, since this phenomenon was not observed in other peptides, i.e. polylysine and horseradish peroxidase, compounds with much higher molecular mass, it is conceivable that the cornea may present additional specific barriers for transport of insulin. In a previous study (Rojanasakul and Robinson, 1989), it was shown that the cornea exhibits a

permselective property allowing preferential passage of positively but not negatively charged compounds. The cornea, due to the presence of ionizable charged groups lining the aqueous diffusion pathway, was found to possess an amphoteric property and exhibits an apparent isoelectric point of 3.2. It was also found that the magnitude and polarity of this selectivity can be regulated by changes in pH and ionic strength of the bathing solution. Since at physiological pH, insulin is fully charged (negative), it is anticipated that an additional barrier created by charges on the membrane may result in a decrease in penetration. In contrast, polylysine, due to its excess free ϵ-NH_2 groups, which are fully protonated (positive) at this pH, would have an opposite experience to this charge barrier and would be expected to readily penetrate into any accessible spaces in the membrane. Earlier results confirm this point.

In this study, initial evidence on intracellular uptake of insulin by the cornea was presented. The significance of this finding either from a physiological or drug delivery standpoint requires further understanding of the basic mechanism of corneal insulin transport. Utilization of this specialized transport system for the purpose of enhanced peptide absorption, i.e. by encouraging endocytotic uptake, may be of potential benefit. In addition, modification of membrane permselectivity to promote absorption of negatively charged peptides like insulin may be achieved by a number of approaches including (a) neutralization or reversed polarity of the peptide by changing pH of the medium, (b) increased ionic strength of the medium, and (c) incorporation of high charge density cations, i.e. Ca^{2+} and Mg^{2+}, in the medium. The last two approaches are primarily intended to minimize effective charge density of the membrane through a process of electrostatic shielding (Rojanasakul and Robinson, 1990).

Conclusions

Confocal imaging has been demonstrated to give improved visibility and resolution of optical sections of the cornea. The technique is particularly applicable to fluorescence microscopy where dramatic improvements of image quality are possible with thick, living specimens because out-of-focus interference and tissue processing prior to microscopic observation are eliminated. This powerful new technique was successfully used to give direct microscopic evidence on transport pathways of polylysine and insulin in the cornea. Results obtained indicate that polylysine was selectively taken up through epithelial surface defects and utilizing the intercellular pathway of the cornea while insulin was predominantly taken up by the corneal surface cells with limited penetration in the aqueous diffusional pathway. In either case, the outermost epithelial layer appears to present the rate-limiting barrier for penetration of these peptides. Potential permselective barrier may have a significant influence on transcorneal penetration of negatively charged peptides.

Acknowledgments

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