Identification of Insulin in the Tear Film and Insulin Receptor and IGF-I Receptor on the Human Ocular Surface

Eduardo M. Rocha,[1] *Daniel A. Cunha,*[2] *Everardo M. Carneiro,*[2] *Antonio C. Boschero,*[2] *Mário J. A. Saad,*[1] *and Lício A. Velloso*[1]

Purpose. Insulin produces pleiotropic effects on sensitive tissues, including the ocular surface, through the tyrosine kinase insulin receptor. Cerebrospinal fluid and secreted fluids, such as milk and saliva, have been reported to contain insulin. In the present study, the presence of insulin was examined in tear film, and the expression of insulin and insulin-like growth factor (IGF)-1 receptor was examined in the human cornea and conjunctiva.

Methods. Stimulated tear samples collected from 33 volunteers (17 men, 16 women), aged 23 to 51 years, who were fed or fasted for 12 hours, were assayed for total protein and insulin content by the biuret dye test and a radioimmunoassay, respectively. Frozen sections of human cornea ($n = 4$) and conjunctiva ($n = 3$) were incubated with anti-insulin receptor and anti-IGF-1 receptor antibodies and developed with a secondary antibody–peroxidase conjugate.

Results. Insulin was detected in all tear samples analyzed, the mean concentration being 0.404 ± 0.129 ng/mL. There were no gender-related differences. In fed subjects, tears tended toward a higher insulin content than those in fasted individuals. There was no linear correlation between insulin and total protein content (mean, 4.61 ± 0.79 mg/mL) in the tear film. Insulin and IGF-1 receptors were detected in the plasma membrane and cytoplasm of corneal and conjunctival epithelial cells.

Conclusions. To the best of the authors' knowledge, this study represents the first demonstration of insulin in human tear film and the presence of insulin and IGF-1 receptor on the human ocular surface. These results suggest that the pancreatic hormone may play a metabolic and/or mitogenic role on the ocular surface. (*Invest Ophthalmol Vis Sci.* 2002;43:963–967)

From the [1]Laboratory of Clinical Physiopathology, Department of Clinical Medicine, Faculty of Medical Sciences; and the [2]Department of Physiology, Institute of Biology, Campinas State University (UNICAMP), Campinas, São Paulo, Brazil.

Supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil; Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasilia; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq): Financiadora de Estudos e Projetos (FINEP) and Programa Nacional de Excelência (PRONEX), Brasilia, Brazil.

Submitted for publication July 25, 2001; revised November 14, 2001; accepted November 28, 2001.

Commercial relationships policy: N.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Mário J. A. Saad, Laboratório de Fisiopatologia Clínica, Departamento de Clínica Médica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil, CEP: 13081-970; emrocha@unicamp.br.

Insulin is a 7000-Da hormone that is produced and secreted by the pancreatic β-cells and that exerts metabolic and mitogenic effects in several target tissues.[1] The presence of insulin in the cerebrospinal fluid has been recognized for many years, and the role of insulin in the central nervous system is currently under intense investigation.[2,3] Secreted fluids, such as milk and saliva, are also known to contain insulin, which may play important roles in the metabolism and growth of those exocrine glands.[4,5] In addition, topical insulin therapy has been considered for promotion of corneal wound healing and treatment of diabetes mellitus.[6–8]

Previous studies have identified hormone and growth factor receptors in the ocular surface and lacrimal gland.[9,10] This information, in addition to the detection of growth factor secretion in tears and lacrimal glands, gives support to their direct role in ocular surface maintenance in healthy and unhealthy eyes.[11–13] As previously hypothesized, growth factors may be responsible for cell proliferation and differentiation, wound healing, and suppression of inflammation in response to local, environmental, and neural control.[11] It is interesting to note that some growth factors previously identified as epithelial growth promoters with clinical applications, such as epidermal growth factor (EGF), insulin-like growth factor (IGF)-1, and nerve growth factor (NGF), share, in part, similar mechanisms of signal transduction with insulin.[14–16]

Despite the critical influence of insulin on lacrimal gland and corneal tissue physiology and pathology, including cell culture maintenance, as indicated in previous studies,[17–21] there is no information about insulin secretion in human tears or its molecular mechanisms of action on the ocular surface. In the present study, tears of healthy volunteers were analyzed for the presence of insulin, and the expression of insulin receptor (IR) and IGF-1 receptor (IGF-1R) were investigated in the human cornea and conjunctiva.

Materials and Methods

Human Tissues and Tear Film Collection

Human corneas ($n = 4$) and conjunctivae ($n = 3$) were prospectively obtained from a donor (3 hours after death) and from sporadically obtained surgical specimens. Samples were immediately frozen in dry ice and stored at −80°C until the time for experimental use. Epidemiologic data are shown in Table 1.

Thirty-three healthy volunteers were recruited consecutively from coworkers in the Department of Ophthalmology for tear collection, between 8:00 and 8:30 AM on three consecutive Mondays. To verify the influence of food intake in tear insulin level, five male and five female volunteers were randomly requested to observe a 12-hour fast before tear collection and the remaining volunteers (12 men and 11 women) were allowed to have a regular breakfast 1 to 2 hours before tear collection (mean, 1680 kJ [400 kcal]). The mean age of the men was 33.2 years and of the women was 30.6 years, the mean tear volumes were 46.4 μL in the men and 53.3 μL in the women, and the mean protein content was 4.7 mg/mL in the men and 4.5 mg/mL in the

TABLE 1. Characteristics of Tissue Donors Used for Immunohistochemical Identification of IR and IGF-1R Proteins in Epithelial Cells of Human Cornea and Conjunctiva

Tissue	Sample	Age/Gender	Condition	Anti-IR	Anti-IGF-1R	Negative Controls
Cornea	1	36/M	Donor	+	+	−
Cornea	2	34/F	Keratoconus	+	+	−
Cornea	3	18/M	Keratoconus	+	+	−
Cornea	4	27/F	Stromal opacity	+	+	−
Conjunctiva	1	18/F	Cataract	+	+	−
Conjunctiva	2	51/M	Cataract	+	+	−
Conjunctiva	3	77/F	Cataract	+	+	−

women. During collection, inferior conjunctival fornix was gently dried with paper tissue and tear production was stimulated by a jet of pressurized air onto the cornea. Tears were collected with a micropipette and disposable tips for 1 to 3 minutes, transferred to tubes (Eppendorf, Fremont, CA) containing 30 μL NaCl 0.9% and frozen at −80°C until use in experiments. Informed consent was obtained from patients (or a family representative) and volunteers before collection.

The research project was approved by the Ethics Committee of Unicamp (Campinas State University) and conducted in accordance with the guidelines established by the Declaration of Helsinki.

Immunohistochemistry

Corneas and conjunctivae were excised, embedded in optimal cutting temperature compound (OCT; Miles, Elkhart, IN) and stored at −80°C. Tissue specimens were cut into 6-μm sections at −20°C and transferred to poly-L-lysine (Sigma, St. Louis, MO) precoated glass slides (Perfecta, São Paulo, Brazil). The slides were exposed to acetone for 5 minutes, incubated in 0.1% H_2O_2 for 5 minutes, washed in PBS (0.05 M sodium phosphate, 0.15 M sodium chloride, pH 7.3) and exposed to 2% normal goat serum solution (Vector Laboratories, Burlingame, CA) for 20 minutes at 4°C. The sections were then overlaid with an aliquot of purified rabbit polyclonal antibodies to the β subunit of IR, or to IGF-1Rα (Santa Cruz Biotechnology, Santa Cruz, CA), prepared using 10 μL antibody stock solution (200 μg/mL) diluted in 990 μL 0.3% bovine serum albumin (BSA; Gibco BRL, Grand Island, NY) in PBS, to give a final concentration of 2 μg/mL, or negative control solutions, which included BSA 0.1% in PBS, preimmune IgG (Sigma), and anti-IR, after overnight preincubation with IR peptide at 4°C (Santa Cruz Biotechnology).

After incubation for 4 hours with primary antibody in a humidified chamber at 4°C, the sections were washed in PBS and incubated with a biotinylated goat anti-rabbit IgG antibody (Vector Laboratories). After incubation with the secondary antibody, sections were again washed in PBS and incubated with an avidin-biotin complex (Vector Laboratories) for 30 minutes at 25°C, before being developed with a 3,3′-diaminobenzidine (DAB) substrate kit (Vector Laboratories).

For histologic correlation, conventional hematoxylin (Sigma) counterstaining was performed on tissue sections, the slides were mounted (Entellan; Merck, Darmstadt, Germany) and coverslipped. Photographic documentation (ASA 100 film; Eastman Kodak, Rochester, NY) was performed with an optical microscope (DMLS; Leica; Heidelberg, Germany) at ×100 and ×400 magnification.

Insulin and Protein Quantification

The insulin content in the tears was measured by radioimmunoassay. To ensure sensitivity, specificity, and reproducibility of the method, the following procedures were performed: Titration curves with duplicate samples of commercially available insulin (Amersham, Aylesbury, UK) were run in parallel, samples with similar dilutions of IGF-1 (Sigma) or containing only buffer were analyzed, and tear samples were run in duplicate. The sensitivity range was between 0.1 ng/mL and 10 μg/mL, and interassay and intra-assay coefficients of variation were estimated as 0.12 and 0.075, respectively.

Protein quantification was performed by applying aliquots of 20 μL tears incubated with 1.0 mL biuret dye buffer (Labtest, Lagoa Santa, Brazil) for 15 minutes and the absorption read in a spectrophotometer (model 432; Femto, São Paulo, Brazil) at 545 λ, using human serum albumin as a standard (Labtest).

Statistical Analysis

The data are expressed as the mean ± SEM. Comparisons of the insulin concentration in tears between female and male, and fed and fasted, were performed with ANOVA and the Mann-Whitney test. In three subjects who had insulin detection in tears collected in both fed and fasted situations, Student's *t*-test for paired data was used. The level of significance was set at $P < 0.05$. The correlation between insulin and protein levels in the tear film was analyzed by linear regression (Statview software; SAS Cary, NC).

RESULTS

IR and IGF-1R Expression in Cornea and Conjunctiva

Corneal epithelial cells expressed IR predominantly in the cytoplasm in all levels of the epithelial layer (Fig. 1A). Expression was also seen at the cell surface in plasma membrane, but the substantial amounts present in the cytoplasm probably represented newly synthesized or recycled pools of the β subunit of IR. In addition, IGF-1Rα protein was identified, predominantly in the plasma membrane, which is in accordance with the described location of the α subunit of the receptor of this growth factor (Fig. 1B).[22]

Conjunctiva epithelial cells also expressed IR predominantly in the cytoplasm of all levels of the epithelium of the bulbar conjunctiva (Fig. 2A). As shown in Figure 2B, IGF-1R protein was also identified, predominantly in the plasma membrane of all levels of epithelial cells in the bulbar conjunctiva.

In control sections, replacing the first antibody with 0.3% BSA-PBS, rabbit preimmune IgG, or even anti-IR previously inactivated by the incubation with IR peptide produced no specific staining (Figs. 1C, 1D, 2C, and 2D).

Insulin and Protein Quantification

The mean level of insulin detected in all tear samples analyzed was 0.404 ± 0.129 ng/mL ($n = 33$). Gender was not a condition influencing insulin values in tears. Thus, in fed subjects insulin concentration in tears was 0.652 ± 0.170 ng/mL versus 0.556 ± 0.219 ng/mL ($P = 0.24$, Mann-Whitney) in women ($n = 12$) and men ($n = 11$), respectively, whereas in fasted subjects insulin concentration was 0.30 ± 0.087 ng/mL versus 0.108 ± 0.038 ng/mL ($P = 0.076$, Mann-Whitney) in women ($n = 5$) and men ($n = 5$), respectively. However, when levels were compared in fed (0.601 ± 0.138 ng/mL; $n = 23$) and fasted (0.204 ± 0.055 ng/mL; $n = 10$) subjects at the same time point, a significant difference was encountered ($P = 0.04$, Mann-Whitney; Fig. 3).

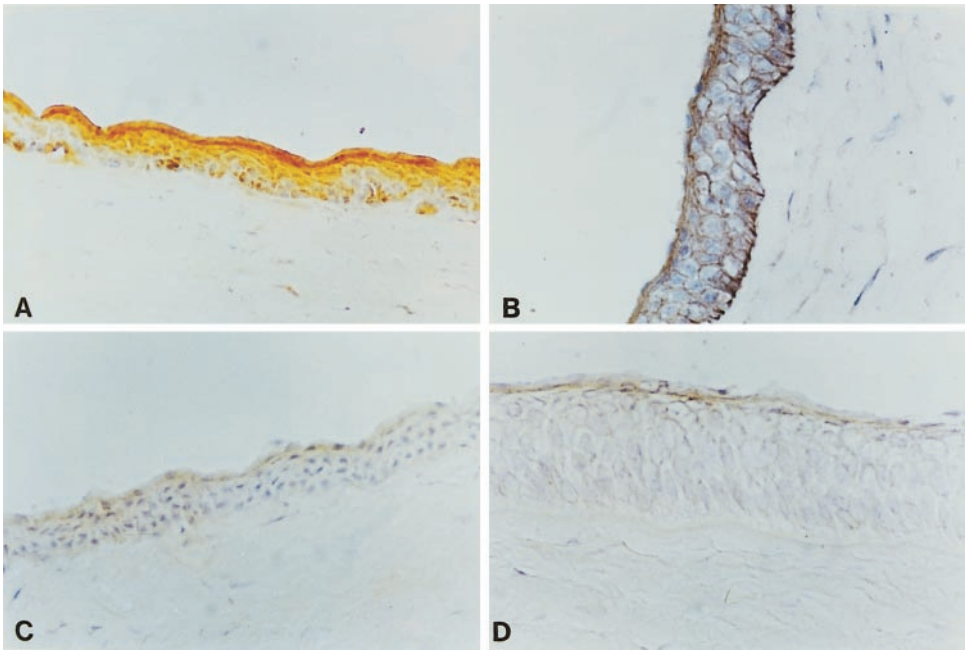

FIGURE 1. Presence of IR (**A**) and IGF-RI (**B**) proteins in the corneal epithelial cells. (**C**, **D**) Negative controls of the same respective samples run in parallel. Magnification, ×400.

From three subjects, two women and one man, we collected tears in both situations, fasted and fed, and the insulin levels were 0.44 and 0.30 in the women and 0.06 ng/mL in the man, after a 12-hour fast. A few weeks later, levels were 0.85 and 0.65 in the women and 0.23 ng/mL in the man in the fed state. Thus, food intake increased the insulin levels in tears more than two times ($P = 0.049$, paired Student's *t*-test).

The mean levels of protein in tear film were 4.61 ± 0.79 mg/mL. No differences were found between the groups ($P = 0.851$, ANOVA). To estimate whether insulin in tears is a function of total protein concentration, an analysis was performed, with no significance detected ($r = 0.083$, $P = 0.2282$).

DISCUSSION

Its presence in tear film raises the question of insulin's role in ocular surface physiology and pathology. Clinical states, such as diabetes or insulin resistance, are known to be related to dry eye and ocular wound healing. Besides sharing similar mechanisms of signal transduction with various growth factors, such as EGF and IGF-1,[15,16] insulin is a well-known growth factor and life-supporting agent for corneal epithelial cells in vitro.[19,20] This information suggests that insulin may have relevant actions on the ocular surface in physiologic situations in vivo and may exert a mitogenic stimulus similar to that exerted by other growth factors.

In addition, in previous studies the glucose levels have been measured in ocular surface tissues, and patients with diabetes have had higher glucose levels in tears than have healthy controls.[23,24] Also, corneal hydration control is affected by glucose serum levels,[25] which may influence glucose levels in the aqueous humor and tear film. Despite that, the mechanisms of local action of insulin on glucose metabolism in the ocular surface are unknown.

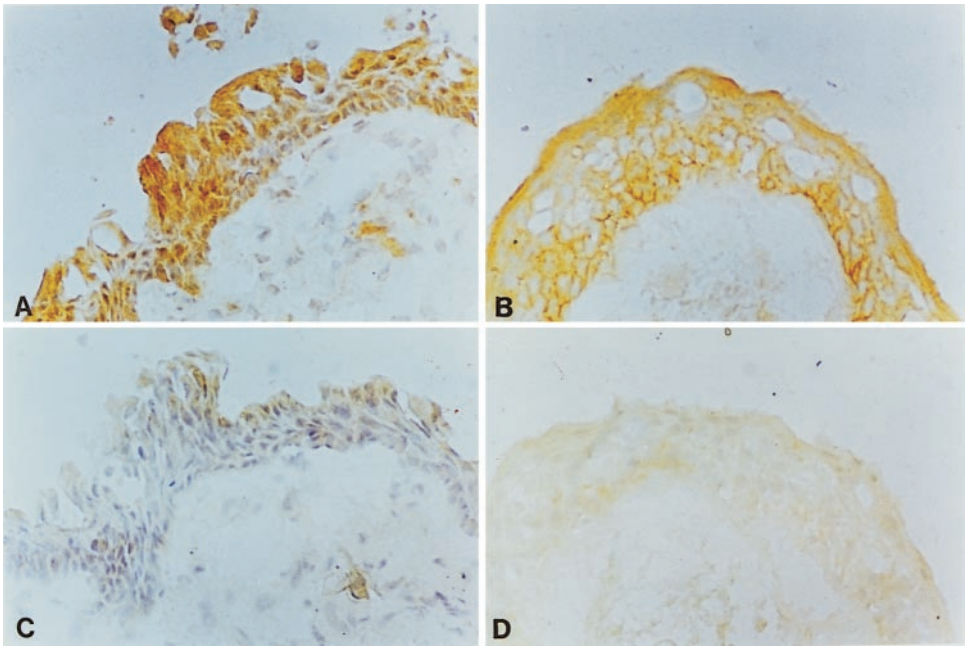

FIGURE 2. Presence of IR (**A**) and IGF-RI (**B**) proteins in the conjunctiva epithelial cells. (**C**, **D**) Negative controls of the same respective samples run in parallel. Magnification, ×400.

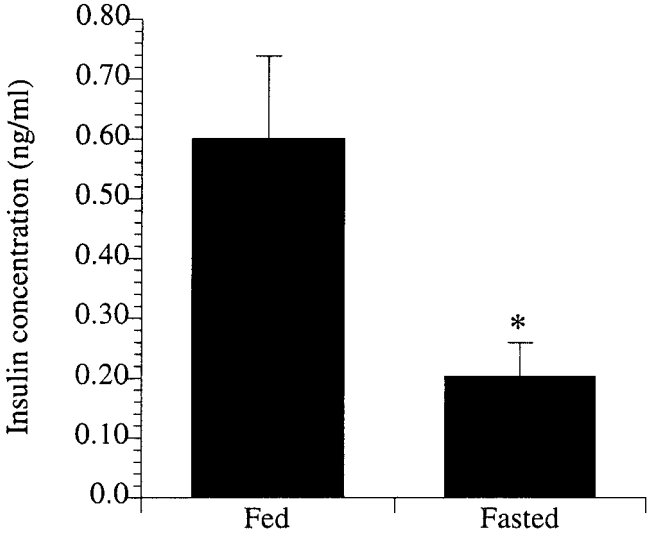

FIGURE 3. Comparison of mean insulin content in tears of fasted (n = 10) or fed (n = 23) subjects, measured by radioimmunoassay. $^*P < 0.05$.

Ocular surface and corneal structure alterations related to diabetes mellitus in humans, such as dry eye, epithelial defects, and corneal edema, have been described.[26–28] Previous studies of the influence of hyperglycemia or sensory innervation damage respond in part, but not for all the various events involved in those alterations.[25,28,29] Previous work involving streptozotocin diabetic animal models failed to demonstrate that neurotransmitter depletion is an initial event related to neurotrophic diabetic corneas, suggesting the possibility that a deficiency of other trophic factors may be involved.[30] Considering that insulin is a powerful trophic element,[1] that its secretion is severely impaired by streptozotocin treatment,[31] and that insulin is necessary for corneal epithelial cell proliferation or culture maintenance,[19,20] it can be hypothesized that reduced levels of insulin or insulin resistance may play a role in the pathogenesis of those corneal abnormalities. Our present findings of insulin secretion in the tear film and IR in the cornea and conjunctiva may give support for further understanding of the actions of this hormone on the ocular surface. From the same perspective, IGF-1 has been considered useful for corneal reepithelialization, both in experimental and clinical studies.[14,32]

Binding assays have demonstrated the presence of IGF-1R, but not of IR, in pigmented rabbit bulbar conjunctiva. Moreover, the expression of mRNA of IGF-1, IGF-1R, and IGF-binding proteins (IGFBPs) was detected in various rat tissues, including cornea and conjunctiva.[33,34]

Our findings of IGF-1R in human cornea and conjunctiva epithelial cells suggest that this growth factor works directly in the cornea cells and/or promotes proliferation and migration of corneal epithelia in a paracrine mode trough conjunctiva cells, which, based on this input, secrete other elements necessary for corneal wound healing.

Another question raised by our study is the source of the insulin present in the tear film. The similarity with the insulin levels found in the serum,[35] in addition to the elevation in fed compared with 12-hour fasted individuals, suggests that insulin present in the tear film is delivered to the ocular surface after being produced by pancreatic β-cells, carried through the blood stream, and transported to the tear film from one or probably more sources of lacrimal secretion (i.e., lacrimal glands and conjunctiva). A closer control of the caloric intake with glucose tolerance tests and monitoring of insulin serum levels would help to confirm this hypothesis. Another possibility suggested by previous studies indicating that insulin is synthesized by neoplastic cells,[36] salivary glands,[4] and other organs, such as the liver,[37] is that lacrimal glands and conjunctiva produce insulin, the same as other hormones (e.g., melatonin, prolactin, and thyroxine), as has been previously reported (see Ref. 18 for review), but support for this is far from consensual.[38]

Considering the relevance of insulin secretion on the ocular surface and the short half-life of this hormone (10 minutes, in one study),[35] it could be predicted that a constant and regular flux of tears would be necessary to keep satisfactory levels and functionality, which may be not be possible in adverse situations such as dry eye and/or diabetes mellitus.

The marked gender-related differences in the tear film and lacrimal glands, which involve a complex relationship between various hormones and are thought to offer crucial elements for the higher prevalence of dry eye in women,[18,39] do not extend to the insulin levels in stimulated tears of healthy adult individuals, as presented in this report. Different findings might appear if a wide age range population were evaluated as reported by a study evaluating EGF in the tear film, in which significant gender differences were found, with higher levels in male nonstimulated tears.[40] Also, different protein and hormone profiles might appear if nonstimulated tears were collected, as previously reported.[41]

Similarly, the absence of linear correlation between insulin and total protein content in the tear film in healthy fed individuals, male or female, may indicate that the insulin variation range is below the levels that affect total protein secretion. Larger samples involving extended age range, nonstimulated tears, or comparisons with specific proteins secreted in the tears, as previously documented, may be helpful in confirming this finding.[42]

In the future we hope to determine the underlying mechanisms involved in insulin secretion in the tear film and signal transduction on the ocular surface. Such analysis may help to understand the pathogenesis of ocular surface alterations in diabetes mellitus.

Acknowledgments

The authors thank Luis Janeri, Inara Ignacchitti, and Renata D. Pithon for technical assistance.

References

1. Myers MG, White MF. Insulin signal transduction and the IRS proteins. *Annu Rev Pharmacol Toxicol.* 1996;36:615–658.
2. Margolis RU, Altszuler N. Insulin in the cerebrospinal fluid. *Nature.* 1967;215:1375–1376.
3. Fernandes MLLA, Saad MJA, Velloso LA. Insulin induces tyrosine phosphorylation of the insulin receptor and Shc, and Shc/Grb2 association in cerebellum but not in forebrain cortex of rats. *Brain Res.* 1999;826:74–82.
4. Carter DA, Wobken JD, Dixit PK, Bauer GA. Immunoreactive insulin in rat salivary glands and its dependence on age and serum insulin levels. *Proc Soc Exp Biol Med.* 1995;209:245–250.
5. Weström BR, Ekman R, Svendsen L, Svendsen J, Karlsson BW. Levels of immunoreactive insulin, neurotensin and bombesin in porcine colostrum and milk. *J Pediatr Gastroenterol Nutr.* 1987; 6:460–465.
6. Saragas S, Arffa R, Rabin B, Kronish J, Miller D, Mayman C. Reversal of wound strength retardation by addition of insulin to corticosteroid therapy. *Ann Ophthalmol.* 1985;17:428–430.
7. Woost PG, Brightwell J, Eiferman RA, Schultz GS. Effect of growth factors with dexamethasone on healing of rabbit corneal stromal incisions. *Exp Eye Res.* 1985;40:47–60.
8. Pillion DJ, Bartlett JD, Meezan E, Yanf M, Crain J, Grizzle WE. Systemic absorption of insulin delivered topically to the rat eye. *Invest Ophthalmol Vis Sci.* 1991;32:3021–3027.

9. Rocha EM, Wickham LA, Silveira LA, Krenzer KL, Toda I, Sullivan DA. Identification of androgen receptor protein and 5α-reductase mRNA in human ocular tissues. *Br J Ophthalmol.* 2000;84:76–84.
10. Wilson SE, Lloyd SA, Kennedy RH. Basic fibroblast growth factor (FGFb) and epidermal growth factor (EGF) receptor messengers RNA production in human lacrimal gland. *Invest Ophthalmol Vis Sci.* 1991;32:2816–2820.
11. Plugfelder SC. Tear fluid influence on the ocular surface. *Adv Exp Med Biol.* 1998;438:611–617.
12. Tsubota K, Goto E, Fujita H, Ono M, Inoue H, Saito I, Shimmura S. Treatment of dry eye by autologous serum application in Sjögren's syndrome. *Br J Ophthalmol.* 1999;83:390–395.
13. van Setten G-B, Tervo T, Tervo K, Tarkkanen A. Epidermal growth factor (EGF) in ocular fluids: presence, origin and therapeutical considerations. *Acta Ophthalmol.* 1992;202:54–59.
14. Brown SM, Lamberts DW, Reid TW, Nishida T, Murphy CJ. Neurotrophic and anhidrotic keratopathy treated with substance P and insulin like growth factor 1. *Arch Ophthalmol.* 1997;115:926–927.
15. Sasaoka T, Ishiki M, Sawa T, et al. Comparison of the insulin and insulin-like growth factor 1 mitogenic intracellular signaling pathways. *Endocrinology.* 1996;137:4427–4434.
16. Habib T, Hejna JA, Moses RE, Decker SJ. Growth factors and insulin stimulate tyrosine phosphorylation of the 51C/Ship2 protein. *J Biol Chem.* 1998;273:18605–18609.
17. Sullivan DA, Hann LE. Hormonal influence on the secretory immune system of the eye. *J Steroid Biochem.* 1989;34:253–262.
18. Sullivan DA, Wickham LA, Rocha EM, Kelleher RS, Silveira LA, Toda I. Influence of gender, sex steroids hormones and the hypothalamic-pituitary axis on the structure and function of the lacrimal gland. *Adv Exp Med Biol.* 1998;438:11–42.
19. Lindberg K, Brown ME, Chaves HV, Kenyon KR, Rheinwald JG. In vitro propagation of human ocular surface cells for transplantation. *Invest Ophthalmol Vis Sci.* 1993;34:2672–2679.
20. Pellegrini G, Traverso CE, Franzi AT, Zingirian MZ, Canceda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet.* 1997;349:990–993.
21. Rocha EM, Lima MHM, Carvalho CRO, Velloso LA, Saad MJA. Characterization of insulin receptor and early elements involved in insulin signalling system in lacrimal and salivary glands of rat. *Curr Eye Res.* 2000;21:833–842.
22. Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. *Endocr Rev.* 1995;16:3–34.
23. Aragona P, Giufrida S, Di Stefano G, et al. Ocular surface changes in type 1 diabetic patients (Abstract). *Cornea.* 2000;19:S72.
24. Barcaroli MP, Del Beato P, Tanzilli P, De Mattia G, Vingolo EM. Diabetes mellitus and dry eye syndrome: tear film glucose level in diabetic patients [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 1997;38(4):**S**150. Abstract nr 734.
25. McNamara NA, Brand RJ, Polse KA, Bourne WM. Corneal function during normal and high serum glucose levels in diabetes. *Invest Ophthalmol Vis Sci.* 1998;39:3–17.
26. Goebbels M. Tear secretion and tear film function in insulin dependent diabetics. *Br J Ophthalmol.* 2000;84:19–21.
27. Azar DT, Spurr-Michaud SJ, Tisdale AS, Gipson IK. Altered epithelial-basement membrane interactions in diabetic corneas. *Arch Ophthalmol.* 1992;110:537–540.
28. Dogru M, Katakami C, Inoue M. Tear function and ocular surface changes in noninsulin-dependent diabetes mellitus. *Ophthalmology.* 2001;108:586–592.
29. Ishida N, Rao G, del Cerro M, Aquavella JV. Corneal nerve alterations in diabetes mellitus. *Arch Ophthalmol.* 1984;102:1380–1384.
30. Marfurt CF, Echtenkamp SF. The effect of diabetes on neuropeptide content in the rat cornea and iris. *Invest Ophthalmol Vis Sci.* 1995;36:1100–1106.
31. Junod A, Lambert E, Orci L, Pictet R, Gonet AE, Renold AE. Studies of the diabetogenic action of streptozotocin. *Proc Soc Exp Biol Med.* 1967;126:201–205.
32. Nishida T, Nakamura M, Ofuji K, Reid TW, Mannis MJ, Murphy CJ. Synergistic effects of substance P with insulin-like growth factor-1 on epithelial migration of the cornea. *J Cell Physiol.* 1996;169:159–166.
33. Narawane MA, Lee VHL. IGF-1 and EGF receptors in the pigmented rabbit bulbar conjunctiva. *Curr Eye Res.* 1995;14:905–910.
34. Burren CP, Berka JL, Edmondson SR, Werther GA, Batch JA. Localization of mRNAs for insulin-like growth factor-I (IGF-1), IGF-1 receptor, and IGF binding proteins in rat eye. *Invest Ophthalmol Vis Sci.* 1996;37:1459–1468.
35. Grodsky GM. Kinetics of insulin secretion: current implications. In: Le Roith D, Taylor SI, Olefsky JM, eds. *Diabetes Mellitus: A Fundamental and Clinical Text*. Philadelphia: Lippincott-Raven; 1996:12–20.
36. Seckl MJ, Mulholland PJ, Bishop AE, et al. Hypoglycemia due to an insulin-secreting small-cell carcinoma of the cervix. *N Engl J Med.* 1999;341:733–736.
37. Rosenweig JL, Havrankova J, Lesniak MA, Brownstein M, Roth J. Insulin is ubiquitous in extrapancreatic tissues of rats and humans. *Proc Natl Acad Sci USA.* 1980;77:572–576.
38. Vallejo G, Mean PM, Gaynor DH, Devlin JT, Robbins DC. Characterization of immunoreactive insulin in human saliva: evidence against production in situ. *Diabetologia*. 1984;27:438–440.
39. Sullivan DA, Wickham LA, Rocha EM, et al. Androgens and dry eye in Sjögren's syndrome. *Ann NY Acad Sci.* 1999;876:312–324.
40. Nava A, Barton K, Monroy DC, Plufgfelder SC. The effect of age, gender, and fluid dynamics on the concentration of tear film epidermal growth factor. *Cornea.* 1997;16:430–438.
41. Fullard RJ, Tucker DL. Changes in human tear protein levels with progressively increasing stimulus. *Invest Ophthalmol Vis Sci.* 1991;32:2290–2301.
42. McGill JI, Liakos GM, Goulding N, Seal DV. Normal tear protein profiles and age-related changes. *Br J Ophthalmol.* 1984;68:316–320.