Using the NOD mouse, a model for type 1 diabetes, we examined how reduced concentrations of epidermal growth factor (EGF) in the saliva, after onset of type 1 diabetes, affect oral wound healing. Diabetic NOD/LtJ mice on insulin therapy, prediabetic NOD/LtJ, and age- and sex-matched BALB/cJ mice were given a cutaneous tongue punch and allowed to undergo normal healing. With diabetes onset and a reduction in saliva-derived growth factor levels, the rate of tongue wound healing was reduced compared with nondiabetic NOD/LtJ and healthy BALB/cJ mice. Addition of exogenous EGF to the drinking water did not accelerate the rate of healing in BALB/cJ or prediabetic NOD/LtJ; however, diabetic NOD/LtJ mice exhibited accelerated wound healing similar to healthy mice. These results demonstrate that loss of growth factors from saliva is associated with profoundly reduced oral wound healing, suggesting that therapeutic treatment with topical delivery may be beneficial to patients with type 1 diabetes and oral wound complications. Diabetes 50:2100–2104, 2001

Saliva and its constituents carry out a number of biological functions that are key to maintaining oral health. The component proteins produced and released into saliva by the salivary glands are grouped according to their functions: lubrication of the oral cavity (mucins, proline-rich proteins), remineralization (statherin, proline-rich proteins), digestion (amylase, lipase, proteases), antimicrobial activity (lysozyme, peroxidases, histatins), and mucosal integrity (water, mucins, growth factors) (1–3). Biologically active growth factors include epidermal growth factor (EGF) and nerve growth factor (NGF), both of which are synthesized and secreted by the granular convoluted tubule cells of the salivary glands (4,5). Transforming growth factors α and β (6–8), insulin, IGF-I and -II (9–12), and fibroblast growth factor (13) have been localized to the ductal cells in salivary glands from which they are actively secreted into saliva.

By far, the most highly characterized of the growth factors for growth-promoting potential is EGF. EGF is produced in large quantities by the salivary glands and then is actively secreted in an exocrine manner into saliva (4). Injection of EGF, isolated from the submandibular gland, into neonatal mice or rats causes premature incisor eruption and precocious eyelid opening (14). In addition, members of this peptide growth factor family have been shown to be mitogenic for a variety of cell types, including those of the oral cavity (15–17).

In the adult mouse, salivary gland–derived EGF seems to play an important role in wound healing of skin and soft tissue as well as in maintaining organ homeostasis (18–23). Wound healing of the skin is enhanced by licking, most likely occurring by deposition of EGF onto the wound area. Another mechanism for EGF and other growth factors that promote wound healing include autocrine (local) production and secretion by cells at the site of a wound (24). It also has been reported that salivary gland–derived EGF enhances the healing of gastric ulcers and tongue lesions (17,19). In the partial hepatectomy model for liver regeneration (25), disruption of salivary EGF production through submandibular gland ablation results in delayed liver cell proliferation and a reduced capacity to regenerate the liver. Sialoadenectomy also has been shown to reduce sperm maturation and isoproterenol- and diet-induced parotid gland hyperplasia (20,21). Removal of maternal salivary glands prevents an increase in plasma and saliva EGF normally observed during pregnancy (23), a loss that results in the reduction in numbers of mothers who complete term pregnancies and overall pup size. Animal models of metabolic disease, such as diabetes, also have reported lower concentrations of growth factors in saliva (26–28).

Both patients with type 1 and type 2 diabetes, as well as animal models of diabetes, have reduced levels of EGF in their saliva (29,30). In the present investigation, we examined the impact of diabetes on wound healing by measuring the rate of oral wound healing after tongue-punch biopsies (25). With the onset of type 1 diabetes, NOD mice exhibit a decrease in the concentration of EGF in saliva with a concomitant decrease in oral wound healing rate that could be reversed with the addition of EGF to the drinking water.
RESEARCH DESIGN AND METHODS

Materials. Female NOD/LtJ and BALB/cJ mice between 7 and 10 weeks of age were purchased from the Department of Pathology Mouse Colony (University of Florida, Gainesville, FL). Onset of diabetes in NOD/LtJ mice was determined by measuring urine and blood glucose levels as described previously (26,27). Mice with blood glucose levels $>$280 mg/dl for 2 consecutive weeks were considered diabetic (28). Mice in the Department of Pathology Mouse Colony become overtly diabetic starting at 12 ± 2 weeks of age. For the current experiments, mice 10–12 weeks of age were used to minimize xerostomic conditions related to the development of Sjögren’s syndrome-like exocrine tissue pathology, which does not develop before 12–16 weeks of age (31,32). Diabetic mice were maintained on daily intramuscular insulin injections (1 unit of Humulin/animal/24 h; Novo Nordisk, Bagsvaerd, Denmark) for 2 weeks before the initiation of the wound-healing experiments. All of the experiment procedures were approved by the University of Florida IACUC committee for animal welfare. Care was taken to minimize pain and suffering of the mice.

Treatment and wounding of the tongue in mice. Healthy age-matched BALB/c mice, prediabetic NOD/LtJ mice, and insulin-maintained diabetic NOD/LtJ mice were divided randomly into three groups ($n = 4–6$ mice) and sedated with an i.p. injection of 0.3 ml of pentobarbital (1% in phosphate-buffered saline). Superficial circular punch biopsy wounds measuring $\sim 2.0$ mm were made in the middle of the tongue using a 1.0-mm Biopsy Punch (Ace-punch, Ft. Lauderdale, FL) by ablatting the epithelial layer without damage to the underlying muscle. During sedation, three photographs of the wound areas were taken for each animal. For 4 days thereafter, animals were anesthetized every 24 h and new photographic documentation of the wound site healing was recorded. Animals that received EGF were provided 20 ml of fresh drinking water that contained 2.5 $\mu$g of EGF/ml or regular tap water. Epidermal growth factor (rhEGF) was obtained from Intergen (Purchase, NY). All other reagents were purchased from commercial sources and were of ultrapure quality. Wounds healed typically within 4 days. After examination of wound healing on day 4, animals were killed, and the submandibular gland, tongue, and total body weight were obtained.

Measurement of wound area. While the animals were anesthetized, the maximum length and width of the wounds were measured using a stereomicroscope equipped with a calibrated eyepiece. The wound size was confirmed using a Hewlett-Packard Scanjet IIcx and NIH Image Shareware (7). The wound size was calculated by the method (1) of Josefson’s syndrome-like exocrine tissue pathology, which does not develop before 12–16 weeks of age (31,32). Diabetic mice were maintained on daily intramuscular insulin injections (1 unit of Humulin/animal/24 h; Novo Nordisk, Bagsvaerd, Denmark) for 2 weeks before the initiation of the wound-healing experiments. All of the experiment procedures were approved by the University of Florida IACUC committee for animal welfare. Care was taken to minimize pain and suffering of the mice.

RNA isolation and RT-PCR detection of EGF mRNA. After the animals were killed, the submandibular glands were explanted, minced in phosphate-buffered saline, and placed in lysis buffer, and mRNA was isolated using a Micro-FastTrack Kit (Invitrogen). Isolated mRNA was stored at $\sim 70°C$ in ethanol until all samples were collected. After isolation, mRNA was pelleted by centrifugation, and cDNA was prepared by reverse transcription using Superscript II Reverse Transcriptase. Copy DNA was synthesized in a standard 20-ml reverse transcriptase reaction and subsequent amplification of the desired mRNA by primer addition using the Perkin-Elmer-Cetus reverse transcriptase–polymerase chain reaction (RT-PCR) kit (San Francisco, CA). The amplification conditions were 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min in a Biometra thermocycler for 25 cycles. All nucleotide primers and probes were synthesized in the ICBR DNA Synthesis Core Laboratory at the University of Florida (Gainesville, FL) and are described elsewhere (7,16). The primer sets used were as follows: $\beta$-actin, forward 5' TGAAAGTCTGGTGATGAACGGAATTTGCGC 3', reverse 5' CATGGACCATGAGGTCACGAC 3'; and EGF, forward 5'TAAGCCCGAGCCGAGAAGTCTA 3', reverse 5'AGTCGCTTCATCATAAATGCAGA 3'.

Densitometric analyses were performed to determine changes in the steady-state levels of mRNA for EGF relative to the concentration detected for the housekeeping gene $\beta$-actin. Photographs of agarose gels were scanned using the Hewlett-Packard Scanjet I1x and NIH Image Shareware (7).

Statistical analysis. All measurements of variance are given as standard errors of the mean. The distribution of rates for wound healing was found to be normal (P > 0.05) and was analyzed by a parametric analysis of variance (ANOVA). Tests of ANOVA between independent means were performed using SAS computer software programs (SAS Institute, Cary, NC). Values were considered significant at P < 0.05.

RESULTS

Detection of EGF steady-state mRNA levels. With the onset of diabetes in NOD/LtJ mice, there was a concomitant decline in the concentration of salivary EGF (28). Consistent with this observation, RT-PCR analysis of mRNA from the submandibular gland of healthy control female BALB/c mice and prediabetic and diabetic NOD/LtJ mice revealed a reduction in the steady-state concentration of EGF mRNA with diabetes onset (Fig. 1). Based on densitometric analysis of the amplicons in agarose gels, diabetic NOD/LtJ mice had a 25–30% (P < 0.05) reduction in EGF mRNA, as compared with control mice. Prediabetic NOD/LtJ mice, when compared with BALB/cJ mice, showed a reduction of $\sim 5%$. Prediabetic NOD/LtJ mice, however, reportedly have a substantially higher concentration of EGF protein in their saliva than female BALB/cJ mice (34 vs. 54 ng EGF/ml) (28).

Measurement of lingual wound-healing rates in diabetic mice. With the use of a punch-biopsy apparatus, BALB/cJ, prediabetic NOD/LtJ, and diabetic NOD/LtJ mice received a lingual surface wound. The rates of healing of these wounds then were followed over a 4-day period. As shown in Fig. 2, prediabetic NOD/LtJ mice healed within 3 days of wounding, whereas both BALB/cJ and diabetic NOD/LtJ mice still had measurable wound sites (P < 0.01). Although the same procedures and apparatus were used for the wounding of mice within each group, the initial wound size in diabetic NOD/LtJ mice was $\sim 67\%$ of the nondiabetic mice (21 mm$^2$ for prediabetic vs. 14 mm$^2$ for diabetic NOD/LtJ). With respect to normal wound-healing rates, prediabetic NOD/LtJ mice had a 100% reduction in the wound area (Fig. 3; P < 0.001) over the 4-day observation time. BALB/cJ mice had an 83% reduction in wound area, whereas diabetic NOD/LtJ mice had wound sites that were reduced in size only by 58% (Fig. 3; P < 0.05), even accounting for the smaller initial wound size. Taking this into account, the order of healing rates among the groups of mice were prediabetic NOD/LtJ > BALB/cJ > diabetic NOD/LtJ.

Introduction of EGF into the drinking water of BALB/cJ and prediabetic NOD/LtJ mice yielded a slightly more rapid wound-healing process when compared with syngeneic mice that were not provided exogenous EGF (Fig. 3; P > 0.05). This was true for each of the 3 days on which measurements of the wound area were taken. Similarly, diabetic NOD/LtJ mice that received exogenous EGF also showed markedly increased rates of healing, nearly approaching those of the prediabetic NOD/LtJ and BALB/cJ
mice (Figs. 2 and 3). At each of the three 24-h time measurements, the wound areas decreased by 25, 38, and 77%, respectively ($P < 0.01$). Despite the smaller wound size of the diabetic NOD mice at the time of the punch, the size of the wound areas at 24–72 h were in general healing more rapidly than those of the mice that had not received EGF in the drinking water (Figs. 2 and 3; $P < 0.05$).

**Comparison of body and tissue weights.** The total body weight and that of the tongue and submandibular glands were obtained to determine the influence of type 1 diabetes and insulin treatment on the health and hydration state of tissues from the diabetic mice. As shown in Table 1, despite slight differences in the size of BALB/cJ and NOD/LtJ mice, there were no significant differences in the body weight or wet weight of tissues from the NOD/LtJ mice, whether they were diabetic or not.

**DISCUSSION**

Studies by Cohen and Levi-Montalcini (14,33,34) demonstrated that the submandibular glands of mice are one of the richest sources of EGF in the body. More recently, research reported from various laboratories has focused on the roles of saliva-derived growth factors in the control of oral and systemic wound healing (19–23). In these paradigms of wound repair, removal of the submandibular gland from mice and rats resulted in retarded rates of healing, which could be reversed by EGF supplementation. Not surprising, then, systemic disease, such as type 1 diabetes, which can negatively impact salivary gland function, dramatically alters the concentration of such factors as NGF and EGF in saliva (26–28). However, the present study is the first to specifically examine the impact of diabetes on the salivary-EGF wound-healing model. Although there may be strain-specific differences in wound-healing rates as evidenced by the ability of nondiabetic mice to heal wounds more quickly, the effect of diabetes on wound healing was consistent across all strains studied.

**FIG. 2.** Histogram of the time course for healing of the punch-biopsy wound area on the lingual surface of healthy BALB/cJ and NOD/LtJ mice. After introduction of the wound, sets of mice were supplied with rhEGF in the drinking water as described previously (24,35). Results represent the mean ± SE for three separate sets of mice. The right half of each histogram represents mice that were provided with drinking water with EGF; the left half represents pair-fed mice that received regular water supply. PD, prediabetic; D, diabetic.

**FIG. 3.** Surface view of the tongue wound-healing site at 1 and 4 days after tissue injury. A: Wound site immediately after punch-biopsy wound in BALB/cJ mice. B: Wound site 72 h after punch-biopsy wound in BALB/cJ mice. C: Wound site immediately after punch-biopsy wound in prediabetic NOD/LtJ mice. D: Wound site 72 h after punch-biopsy wound in nondiabetic NOD/LtJ mice. E: Wound site immediately after punch-biopsy wound in diabetic NOD/LtJ mice. F: Wound site 72 h after punch-biopsy wound in diabetic NOD/LtJ mice. Photographs are representative of the wound in each group of animals. Magnification ×20. Arrows highlight the wound area on the lingual surface epithelium. PD, prediabetic; D, diabetic.
NOD/LtJ mice to heal more rapidly than the BALB/cJ mice, with or without the addition of exogenous EGF, diabetes and the decline in saliva concentration of EGF negatively impacted wound-healing rates of oral tissue.

Previous reports of reduced EGF levels resulting from diabetes have come from chemically induced insulin-dependent animals or models for type 2 diabetes (26,27). Here, we provide evidence that naturally occurring autoimmune type 1 diabetes also has a reduced expression of EGF levels. Messenger RNA analysis of the submandibular gland confirmed previous protein analyses that showed a reduction in saliva and serum concentrations of growth factors in diabetic animal models (25–28). The reduced levels of growth factors in saliva seem to decrease the wound-healing capacity of oral epithelium after superficial wounds to the tongue. However, consistent with previous reports (25), addition of EGF to the drinking water of normal healthy mice or prediabetic NOD/LtJ mice did not accelerate wound healing. Only in the diabetic mice, with reduced concentrations of salivary EGF, did supplementation with EGF improve the kinetics of healing to a similar level as that detected in the healthy controls. It is not known what other growth factors that are synthesized by the salivary glands (5–13) may be sensitive to disruption by changes in blood glucose and insulin levels or whether they additionally influence wound healing as saliva-derived EGF. Transforming growth factor-α (TGF-α), a member of the EGF growth factor family and capable of binding to the EGF receptor (24), is also produced by the salivary glands and is present in saliva (7). Unlike EGF, however, TGF-α is produced by the parotid gland in addition to the submandibular gland. What role TGF-α has in oral wound healing or in the impact of diabetes on the concentration in saliva has not been examined. We have observed that although the saliva concentration of TGF-α is lower in NOD/LtJ than in BALB/cJ mice, unlike EGF, the expression of this growth factor does not seem to be influenced by type 1 diabetes in this animal model (M.G. Humphreys-Beher, S. Cha, H. Nagashima, A.B. Reck, unpublished data). Brown et al. (35) demonstrated enhanced cutaneous wound closure after application of platelet-derived growth factor and TGF-α to db/db diabetic mice but not with EGF. Like EGF, NGF and IGF-II are reduced in saliva, whereas IGF-I concentrations remain high in diabetic mice (10,26).

Diabetes, a disease that affects vascular function, disrupts normal salivary gland function, including a reduced saliva flow rate and volume (36,37). Therefore, the possibility that xerostomia contributes to the reduced wound healing in diabetic NOD/LtJ tissue cannot be discounted (25). However, no differences were noted in diabetic mice maintained on insulin treatment and nondiabetic NOD/LtJ or BALB/cJ mice. Restoration of normal rates of healing in diabetic mice by EGF treatment supports the concept that reduced saliva-derived EGF is a major contributor in the pathogenic complication of decreased wound healing associated with diabetes. A direct action for EGF in the healing processes of the lingual surface is supported further by the presence of the EGF receptor in the epithelial cells that form the taste buds as well as the need for saliva-derived EGF to maintain the presence and morphology of this tissue (38).

The punch wounds generated on the lingual surface of diabetic mice were consistently smaller in size than prediabetic mice or healthy controls. Although diabetic mice received daily insulin injections, it is possible that microvascular constriction, as well as neuropathy and tissue desiccation, affected the physiological state of the tongue. No changes in salivary gland or total body weight were observed for the diabetic group compared with the controls. However, the tongue, being exposed to the external environment, may be more prone to desiccation. This potential complication in generating equal-sized punch wounds between diabetic and prediabetic mice probably needs to be factored into preventing the full restoration of the accelerated wound-healing rate observed after the addition of EGF to the drinking water.

Although a positive impact of EGF on lingual wound healing was noted in the diabetic mice, EGF had little effect in the healing rates of healthy or prediabetic mice. A number of factors could account for this observation. As indicated, saliva contains a number of biologically active peptides besides EGF (5–10), some of which may not be affected by hyperglycemia and insulin fluctuations observed with diabetes. Locally infiltrating cells and platelets at the site of wounds produce a variety of growth factors (39), and these have been shown to play important roles in the wound healing of skin, suggesting that several saliva-derived growth factors might play an important role in oral wound healing. Despite this autocrine contribution, with a longer period of diabetes onset than the 2 weeks used in these studies, a more accelerated pattern of healing may have been determined with EGF supplementation in the drinking water. In addition, we used hrEGF in a mouse receptor system, a combination that may be suboptimal for receptor activation. Thus, with slightly different binding kinetics, a longer period of lingual confinement/contact may be necessary to promote optimal wound healing than could be accomplished through the transient exposure provided by EGF in the drinking water.

In conclusion, we provided further evidence that saliva-derived EGF is an important protagonist in oral wound healing. Furthermore, metabolic disorders, such as diabetes, reduce the level of EGF in saliva with the consequence that the normal wound-repair process is negatively affected. The introduction of growth factor supplementation may be beneficial to patients with identified complications in situations that require tissue repair associated with dysregulation of salivary sources of EGF.
ACKNOWLEDGMENTS
This work was supported by NIH/NIDCR Grant DE-13290.

The authors acknowledge the technical contributions of Joy Nanni and Stephanie Diggs.

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