Tumor Necrosis Factor- α Mediates Diabetes-Enhanced Apoptosis of Matrix-Producing Cells and Impairs Diabetic Healing

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Diabetics suffer increased infection followed by increased apoptosis of fibroblasts and bone-lining cells during the healing process. To investigate a potential mechanism, we inoculated *Porphyromonas gingivalis* into the scalp of type 2 diabetic (db/db) or control mice and inhibited tumor necrosis factor α (TNF- α) with etanercept. Mice were euthanized at the early phase of infection (21 hours) or during the peak repair of the bacteria-induced wound (8 days). At 21 hours, TNF- α inhibition significantly reduced fibroblast apoptosis and caspase-3 activity in both diabetic and normoglycemic mice ($P < 0.05$). During healing etanercept reduced fibroblast apoptosis and caspase-3 activity by almost 50% in diabetic but not normoglycemic mice ($P < 0.05$). Concomitantly, etanercept significantly increased fibroblast number by 31% and new matrix formation by 72% in diabetic mice. When bone was examined during healing, administration of the TNF- α blocker reduced apoptosis of bone-lining cells by 53%, increased their number by 48%, and enhanced new bone formation by 140% in the diabetic group ($P < 0.05$). The degree of connective tissue and osseous healing stimulated in the diabetic mice by anti-TNF- α treatment was within the range that is physiologically relevant. This enhanced healing may in part be explained by blocking TNF- α -induced apoptosis of critical matrix-producing cells. (*Am J Pathol* 2006, 168:757–764; DOI: 10.2353/ajpath.2006.050907)

Tumor necrosis factor α (TNF- α) is a pleiotropic cytokine that plays an important role in immunity and inflammation. During chronic illness, markedly elevated TNF- α secretion can contribute to cachexia, hemorrhage, necrosis, and in severe cases, death. Overproduction of TNF- α is thought to play a role in a number of disease processes

including arthritis,^{1,2} psoriasis,³ periodontal disease,⁴ inflammatory bowel disease,⁵ and chronic obstructive pulmonary disease.⁶ In each case, TNF- α is associated with persistent inflammation and tissue destruction. The inflammatory events stimulated by TNF- α can lead to connective tissue destruction by the release of lytic enzymes produced by resident cells as well as by recruited inflammatory cells.

In addition to causing destruction, TNF- α can affect the repair process. Application of TNF- α causes a decrease in wound strength that may be due to decreased collagen type I and type III expression.^{7,8} In contrast, inhibition or deletion of TNF- α generally enhances repair processes. Genetic ablation of TNF receptor-1 improves wound healing by enhancing angiogenesis, collagen production, and re-epithelialization.⁹ Similarly, administration of anti-TNF- α antibody in mice significantly increases collagen deposition.¹⁰ Most of these studies have examined the impact of TNF- α on incisional wound healing in normal animals. In pathological conditions it is possible that TNF- α may impair wound healing through other mechanisms.

Diabetes is associated with excessive TNF- α expression. This may result from constitutive overproduction by adipose tissue in type 2 diabetes, the effects of hyperglycemia and advanced glycation end products and an exaggerated or more persistent response to stimuli such as bacteria or wound healing.^{11–13} TNF- α overexpression in diabetes is thought to contribute to several complications in diabetes, including retinopathy, nephropathy, neuropathy, and diabetes-enhanced periodontal disease.^{14–17} Delayed or incomplete healing of wounds has been well documented in diabetic humans and in animal models of diabetes.^{18,19} Whether diabetes-associated TNF- α overexpression contributes to impaired wound healing has not been established.

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Apoptotic programmed cell death is a critical mechanism for removing unwanted cells during development, preventing autoimmunity by removing autoreactive cells, and protecting the host from infected or tumorigenic cells. In pathological situations enhanced apoptosis may occur inadvertently, aggravating damage that occurs during infection or interfering with healing.^{20–22}

In our previous studies we injected fixed bacteria into the scalp of mice to cause apoptosis and to promote destruction of connective tissue and resorption of underlying calvarial bone. Inoculation of bacteria causes the formation of an inflammatory infiltrate consisting primarily of polymorphonuclear leukocytes (PMNs), which are thought to contribute to injury of the connective tissue and resorption of bone.^{23–25} This model has the advantage that the repair process occurs in a closed environment and is not susceptible to infection from exogenous bacteria. After inoculation resorbed bone is repaired by osteoblasts that differentiate from precursors in the periosteum that lines the calvarial bone, while fibroblasts migrate in from the wound edges to repair the damaged connective tissue.^{23,24} We previously reported that diabetes increases apoptosis of fibroblasts and osteoblasts and their precursors found in the periosteal layer lining the bone surface. However, the mechanism of diabetes-enhanced apoptosis of these critical matrix-producing cells was not established. The purpose of the studies presented here was to investigate whether TNF- α played a significant role in diabetes-enhanced apoptosis in connective tissue and bone. This was accomplished by applying the TNF- α inhibitor etanercept to mice with a bacteria-induced wound. This inhibitor, which contains the extracellular domain of the human TNF receptor 2, is effective in blocking murine TNF- α and is unlikely to be affected by the formation of neutralizing antibodies during the time course of the experiments.²⁵

Materials and Methods

Animals

Genetically diabetic C57BL/KsJ-lepr^{-/-} (db/db) mice and their nondiabetic littermates, C57BL/KsJ-lepr^{-/+} (db/+), were purchased from the Jackson Laboratory (Bar Harbor, ME). db/db mice were diabetic for a minimum of 3 weeks before the experiments were started. Mice were considered to be diabetic when glucose levels exceeded 250 mg/dl. During the experiments the serum glucose levels in the db/db mice were typically 400 to 450 mg/dl and 100 to 150 mg/dl for normoglycemic controls. All animal procedures were approved by the Institutional Animal Care and Use Committee, Boston University Medical Center.

Bacterial Inoculation and TNF- α Inhibitor Application

Both diabetic and normoglycemic littermates were inoculated with bacteria as previously described.^{24,26} To measure fibroblast apoptosis associated with the bacte-

ria-induced injury, live *Porphyromonas gingivalis* strain 381 (1×10^8) was inoculated at the midline of the scalp in short-term experiments. To study the healing phase, a higher dose of bacteria was inoculated (5×10^8), using formalin-killed bacteria to create an equivalent injury in diabetic and normal mice.²⁴ By using fixed bacteria the results obtained reflect differences in the repair process between diabetic and normoglycemic mice rather than a diminished capacity of diabetic mice to kill the bacteria. The TNF- α -specific inhibitor etanercept was generously provided by Amgen (Thousand Oaks, CA). Apoptosis of resident fibroblasts was measured during the injury phase shortly after inoculation of bacteria. In these experiments etanercept was administered by intraperitoneal injection (3 mg/kg body weight) 24 hours before the experiment started, and 15 μ g of etanercept was injected subcutaneously into the scalp 30 minutes before bacterial challenge. Experiments were also performed to examine apoptosis during the healing phase. To avoid interfering with the early inflammatory events and restricting the impact of TNF- α inhibition to the period of healing, etanercept was administered only on days 3 and 6 after bacterial inoculation, and mice were euthanized on day 8. There were six mice for each group for each data point ($n = 6$).

Preparation of Histological Specimens

The scalp, still attached to calvarial bone, was fixed in 4% paraformaldehyde at 4°C for 3 days, decalcified in Immunocal (Decal Chemical Corp., Congers, NY) at 4°C for 12 days and washed with Cal-arrest (Decal Chemical Corp.). Then the specimens were embedded in paraffin and 5- μ m sagittal sections were prepared. The area of interest was at the midline between the occipital and coronal sutures.

Detection of Apoptotic Fibroblasts and Bone-Lining Cells

Apoptotic fibroblasts and apoptotic bone-lining cells were detected by an *in situ* terminal dUTP nick-end labeling (TUNEL) assay by means of a TACS 2 TdT-Blue Label kit purchased from Trevigen (Gaithersburg, MD), following the manufacturer's instructions. The number of fibroblastic apoptotic cells and nonapoptotic fibroblasts in the same field was counted at $\times 1000$ magnification. Apoptotic fibroblastic cells had a fusiform appearance in the TUNEL assay, distinguishing them from inflammatory cells, which have either a rounded (mononuclear cells) or multilobed appearance (PMNs). The number of apoptotic bone-lining cells was counted in the same specimens and identified by their appearance in the periosteal layer adjacent to calvarial bone. Counts and measurements were made by one examiner and confirmed by an independent examiner. One-way analysis of variance was used to determine significance between groups at the $P < 0.05$ level.

Figure 1. TNF- α inhibition reduces fibroblast apoptosis and caspase-3 activity in both diabetic and normoglycemic mice during the early response to bacterial stimulus. Live *P. gingivalis* was inoculated into the scalp of diabetic (db/db) and normoglycemic (db/+) littermates. TNF- α was inhibited by local and systemic injection of etanercept while control mice received an injection of vehicle alone. Mice were euthanized 21 hours after bacterial inoculation. Fibroblast apoptosis was detected with the TUNEL assay, and caspase-3 activity was measured using a fluorimetric kit. An **asterisk** indicates a significant difference in mice treated with etanercept compared to vehicle alone ($P < 0.05$).

Histomorphometry

New bone and newly formed connective tissue matrix were identified in Van Gieson-stained histological sections by its characteristic blue color. The area of new bone and new connective tissue matrix between the coronal and occipital sutures was measured at $\times 100$ magnification using computer-assisted image analysis in Van Gieson-stained sections as previously reported.^{23,24} The number of normal fibroblasts and normal bone-lining cells was counted at $\times 1000$ magnification in hematoxylin and eosin (H&E)-stained sections. Bone-lining cells consisted of periosteal cells and mature osteoblasts. Approximately 20 fields were counted per section to measure fibroblast numbers and 10 fields per section to assess bone-lining cells. Statistical differences between groups were determined by one-way analysis of variance at the $P < 0.05$ level.

Detection of Caspase-3 Activity

After euthanasia the scalp was immediately dissected from the calvaria and frozen in liquid nitrogen. Tissue was placed in lysis buffer (R&D Systems, Minneapolis, MN) and disrupted using FastPrep (Q-Biogene, Solon, OH). Total protein was determined using the BCA protein assay kit (Pierce, Rockford, IL) and 300 μ g was assayed per data point. Caspase-3 activity was measured by a fluorometric kit purchased from R&D Systems. The results are the mean of three independent assays and are expressed as the percent maximum. Significance was established with one-way analysis of variance at the $P < 0.05$ level.

Real-Time Polymerase Chain Reaction

Eight days after bacterial inoculation, total RNA was extracted from the scalp, and real-time polymerase chain reaction was performed as previously described.²⁷ Taq-Man primer and probe sets for murine TNF- α and caspase-3 were purchased from Applied Biosystems (Foster City, CA). Results were normalized with an 18S ribosomal primer and probe set purchased from Qiagen (Valencia, CA). The experiment was performed twice with duplicate specimens, and the results were pooled to establish statistical significance with one-way analysis of variance.

Results

We have previously reported that when bacteria are inoculated into the scalp, there is an initial period of fibroblast apoptosis observed within 24 hours.²⁴ When peak healing occurs on day 8, there is a high level of apoptosis of fibroblastic and osteoblastic cells in diabetic mice.^{23,24} Therefore, experiments were performed to assess the role of TNF- α in diabetes-enhanced apoptosis at these two time points. At 21 hours fibroblast apoptosis was relatively high in both diabetic and normoglycemic mice, with the level of apoptosis being 2.3-fold higher in the diabetic group (Figure 1A). In contrast, there were virtually no apoptotic fibroblasts before inoculation in the diabetic or normoglycemic groups and no difference between them (data not shown). Specific inhibition of TNF- α reduced fibroblast apoptosis by 50% in diabetic mice and by 36% in nondiabetic mice, both of which were significant ($P < 0.05$).

Table 1. Diabetes Enhances TNF- α and Caspase-3 mRNA Levels during Peak Healing

	TNF- α	Caspase-3
Normoglycemic	1	1
Diabetic	4.5 $\pm$ 0.1	2.9 $\pm$ 0.05

Real-time PCR was performed from RNA isolated from the scalps of normoglycemic and diabetic (db/db) mice 8 days after bacterial inoculation. The diabetic values for TNF- α and caspase-3 were significantly higher than for normoglycemics ($P < 0.05$).

Caspase-3, an important effector caspase downstream of TNF receptor signaling, was also evaluated (Figure 1B). The highest level of caspase-3 activity was in the diabetic group. After treatment with the TNF- α inhibitor, caspase-3 activity was significantly reduced by 41% and 35% in the diabetic and nondiabetic groups, respectively ($P < 0.05$) (Figure 1B). In fact, the level of caspase-3 in the etanercept-treated diabetic mice was reduced to that of the normoglycemic mice ($P > 0.05$).

We previously reported that TNF- α mRNA levels are elevated to a similar extent in normoglycemic and diabetic mice 1 day after bacterial inoculation in this model.²⁸ Real-time polymerase chain reaction was performed to determine whether the levels were equivalent at day 8 (Table 1). Both TNF- α and caspase-3 mRNA levels were approximately three- to fourfold higher in the diabetic compared to the normoglycemic mice. Experiments were performed to determine whether TNF- α played a unique role in fibroblast apoptosis and in caspase-3 activity in the diabetic mice. TUNEL-positive apoptotic fibroblasts were identified by their characteristic appearance (Figure 2A). The number of apoptotic fibroblasts was 2.4-fold higher in the diabetic compared to the normoglycemic group (Figure 2B). After inhibition of TNF- α , fibroblast apoptosis in diabetic mice was decreased 44% ($P < 0.05$). However, there was no significant change in nondiabetic mice ($P > 0.05$). When caspase activity was measured, caspase-3 was 1.4-fold higher in the diabetic mice compared to normoglycemic controls. For diabetic mice the TNF- α inhibitor reduced caspase-3 activity by 45% (Figure 2C), which was statistically significant ($P < 0.05$). TNF- α inhibition did not change caspase-3 activity for nondiabetic mice ($P > 0.05$). As was noted for the number of apoptotic fibroblasts, the TNF- α blocker reduced caspase-3 activity in the diabetic mice to a level equivalent to that of normal mice, eliminating the diabetes enhancement ($P > 0.05$).

To assess the potential impact of fibroblast apoptosis on healing, fibroblast density on day 8 was measured (Figure 3). The density of fibroblasts was 1.4-fold higher in the normal compared to the diabetic mice. When the normoglycemic mice were treated with TNF- α blocker, there was no change in density ($P > 0.05$). In contrast there was a significant increase (31%) in fibroblasts in the diabetic mice after application of the TNF- α inhibitor ($P < 0.05$). Fibroblast density in TNF- α inhibitor-treated diabetic mice increased to a level similar to that of normal control mice ($P > 0.05$).

The formation of new connective tissue matrix is another critical factor in the healing response. The formation

Figure 2. TNF- α inhibition reduces fibroblast apoptosis and caspase-3 activity in diabetic but not normoglycemic mice during the healing response to bacteria-induced injury. Formalin-killed *P. gingivalis* was inoculated into the scalp of diabetic (db/db) and normoglycemic (db/+) littermates. Mice were treated with systemic and local injection of etanercept to inhibit TNF- α or with equivalent vehicle alone. Mice were euthanized on day 8. The TUNEL assay was used to detect fibroblast apoptosis, and a fluorimetric kit was used to measure caspase-3 activity. In **A** the arrows point to TUNEL-positive fibroblastic cells in a section that was counterstained with nuclear fast red. For **B** and **C** an asterisk indicates a significant difference in diabetic mice treated with etanercept compared to vehicle alone ($P < 0.05$).

of new matrix was 3.2-fold higher in the normoglycemic compared to the diabetic group (Figure 4A). The TNF- α inhibitor increased the formation of new connective tissue matrix by 72% in the diabetic mice ($P < 0.01$) but had no effect on the nondiabetic mice ($P > 0.05$) (Figure 4B). The effect of TNF- α inhibition on the amount of matrix

Figure 3. Inhibition of TNF- α increases fibroblast density in diabetic but not normoglycemic mice during healing. Formalin-killed *P. gingivalis* was inoculated into diabetic and normoglycemic mice, which were treated with etanercept as described in Figure 2. Mice were euthanized 8 days after bacterial inoculation. Fibroblast number was counted at $\times 1000$ magnification in H&E-stained sections. An **asterisk** indicates a significant difference in diabetic mice treated with etanercept compared to vehicle alone ($P < 0.05$).

produced per fibroblast was calculated from the fibroblast number counted at $\times 1000$ magnification in H&E-stained sections and new connective tissue matrix assessed by image analysis of Van Gieson-stained sections as described in Materials and Methods. By calculating from data in Figures 3 and 4 etanercept caused a 28% increase in the amount of new connective tissue

Figure 5. Inhibition of TNF- α reduces apoptosis of bone-lining cells in diabetic mice but not normoglycemic mice. Formalin-killed bacteria were inoculated into diabetic and normoglycemic mice, which were treated with etanercept or vehicle alone as described in Figure 2. Apoptosis of bone-lining cells on day 8 was measured by the TUNEL assay. An **asterisk** indicates a significant difference in diabetic mice treated with etanercept compared to diabetic mice treated with vehicle alone ($P < 0.05$).

matrix produced per fibroblast in the diabetic group, which was significant ($P < 0.05$), but had no effect on the amount in the nondiabetic group ($P > 0.05$).

Studies were also performed to assess osseous healing after bacterial stimulation, which also peaks on day 8 in both diabetic and normoglycemic mice.²³ The number of apoptotic bone-lining cells was 1.4-fold higher in the diabetic compared to normal control mice. After admin-

Figure 4. Inhibition of TNF increases new matrix formation in diabetic but not normoglycemic mice. Formalin-killed bacteria were inoculated and diabetic and normoglycemic mice were treated with etanercept or vehicle alone as described in Figure 2. **A:** Newly formed connective tissue matrix was identified in Van Gieson-stained histological sections. **B:** The area of newly formed matrix on day 8 was measured with computer-assisted image analysis. An **asterisk** indicates a significant difference in diabetic mice treated with etanercept compared to diabetic mice treated with vehicle alone ($P < 0.01$). Original magnifications, $\times 400$.

Figure 6. Inhibition of TNF- α increases the number of normal bone-lining cells in diabetic but not normoglycemic mice. Bacteria were inoculated into diabetic and normoglycemic mice, which were treated with etanercept as described in Figure 2. The number of normal bone-lining cells per mm bone length on day 8 was counted at $\times 1000$ magnification in H&E-stained sections. An **asterisk** indicates a significant difference in diabetic mice treated with etanercept ($P < 0.05$).

istration of TNF- α blocker, apoptosis in diabetic mice was reduced 53% ($P < 0.05$) (Figure 5). For the nondiabetic group TNF- α inhibitor did not significantly change the number of apoptotic bone-lining cells ($P > 0.05$). However, TNF- α inhibition reduced the level of apoptosis in the diabetic group so that it matched that of the normoglycemic controls ($P > 0.05$).

To assess the potential impact of apoptosis, the number of normal bone-lining cells per mm bone length was counted and found to be 1.6-fold higher in the normoglycemic mice than in the diabetic mice (Figure 6). Treatment with etanercept increased the number by 48% in diabetic mice ($P < 0.05$). For nondiabetic mice there was

no change ($P > 0.05$). The percent increase in bone-lining cells was consistent with the change in the number of apoptotic cells. In addition, inhibition of TNF- α raised the number of osteoblastic cells to a level that was similar to the normoglycemic group.

New bone formation was measured in Van Gieson-stained sections and was much higher in the normoglycemic compared to diabetic animals ($P < 0.05$) (Figure 7A). For diabetic mice TNF- α inhibitor improved the amount of new bone formation by 140% (Figure 7B). For nondiabetic mice there was no difference between groups with or without TNF- α inhibitor application ($P > 0.05$).

Discussion

In the model used here, bacteria that induce an inflammatory injury to soft tissue and bone are inoculated into mouse scalp.^{23,24} This model has the advantage that soft and hard tissue healing after bacteria-induced injury occurs in a similar time frame and the wound is not exposed to the outside environment where healing can be altered by external factors. *P. gingivalis* was used because it induces a host response that leads to destruction of connective tissue and bone in a process that involves induction of cytokines, recruitment of inflammatory cells, activation of matrix metalloproteinases, and initiation of osteoclastogenesis.^{29,30} During wound healing or in response to bacterial products, TNF- α expression is induced in the skin by a variety of cell types including keratinocytes, monocytes, macrophages, PMNs, and Langerhans cells.^{31–34} The experiments reported here indicate that an early response to *P. gingivalis* involves

Figure 7. Inhibition of TNF- α enhances new bone formation in diabetic but not normoglycemic mice. Formalin-killed bacteria were inoculated into diabetic and normoglycemic mice, which were treated with etanercept or vehicle alone as described in Figure 2. **A:** Newly formed bone matrix on day 8 was identified in Van Gieson-stained sections by its characteristic blue color. **B:** The area of newly formed bone was measured with computer-assisted image analysis. An **asterisk** indicates a significant difference in diabetic mice treated with etanercept compared to diabetic mice treated with vehicle alone ($P < 0.05$). Original magnifications, 400.

apoptosis of matrix-producing cells, an event that is elevated in diabetic mice. When TNF- α is inhibited, both normal and diabetic mice exhibit significantly reduced apoptosis at the same time point. The results with normoglycemic mice agree well with our previous report that bacteria-induced apoptosis is attenuated in TNF receptor-deficient mice compared to wild-type controls.³⁵ However, at a later time point during the healing phase, only diabetes-enhanced apoptosis of fibroblasts and bone-lining cells can be accounted for by TNF- α activity. Interestingly, TNF- α mRNA levels are similar in normoglycemic and diabetic mice at early time points,²⁸ whereas on day 8 mRNA levels of TNF- α are almost four times higher in the diabetic compared to normoglycemic mice (Table 1). This may explain why inhibition of TNF- α with etanercept reduces fibroblast apoptosis in both diabetic and normoglycemic mice during the early injury phase but only in diabetic mice during the later healing phase.

The degree of connective tissue production and bone healing found with anti-TNF- α treatment is likely to be physiologically relevant. For example, the improvement in new matrix formation observed by inhibition of TNF- α in the diabetic mice, 72%, is similar to the increase reported when advanced glycation end-products are blocked by treatment of diabetic mice with soluble receptor to AGE (sRAGE).³⁶ It is also similar to the increase in matrix formation that is considered significant in aged individuals.³⁷ Similarly, the 48% improvement in bone healing, reflected by an increase in the number of bone-lining cells per mm bone length, and the 140% increase in new bone formation are within the range considered to be of therapeutic benefit. This is based on findings that a 50% improvement in the number of bone-lining cells or bone matrix production in animals and humans represents a therapeutically important outcome.^{38–40}

Caspase-3 is the primary effector caspase through which TNF- α induces apoptosis.⁴¹ During the early destructive phase after bacterial inoculation, caspase-3 activity was induced in the diabetic animals as well as in the normoglycemic controls. TNF- α inhibition reduced caspase-3 in both groups, although to a greater extent in the diabetic mice. In general, there was a close relationship between caspase-3 activity and the level of fibroblast apoptosis, suggesting that caspase-3 is an important effector caspase through which infection by *P. gingivalis* results in programmed cell death. The result at this time point is consistent with the previous observation that lipopolysaccharide-induced apoptosis in fibroblasts *in vivo* is mediated by TNF receptor-1 and involves TNF- α -dependent caspase-3 activation.^{42,43} During the healing phase, however, caspase-3 activity was TNF- α -dependent only in the diabetic group, agreeing well with results obtained by the TUNEL assay.

It has been well documented that there is delayed or incomplete healing of wounds in diabetic humans and in animal models of diabetes. Several cellular mechanisms have been proposed, including depletion or dysfunction of PMNs and macrophages, sustained cytokine expression and infiltration by inflammatory cells, decreased production of growth factors, reduced cellular proliferation and extracellular matrix synthesis, and increased pro-

duction of proteolytic enzymes.^{18,44–46} In addition to the above, enhanced rates of apoptosis could have a detrimental effect on the healing process.²⁰ Our previous study showed that the healing response to a bacteria-induced injury is associated with increased apoptosis of fibroblasts and osteoblasts at the peak time of healing.^{23,24} Data presented here indicate that this is likely to be physiologically significant because TNF- α inhibition reduced apoptosis and resulted in significantly higher numbers of fibroblasts and bone-lining cells in the diabetic group. The change in cell number agrees well with the increase in matrix production, indicating that anti-TNF- α treatment increased the number of cells producing new connective tissue matrix. However, this is unlikely to be the only reason for the increase because TNF- α inhibition also stimulated a small but significant increase in the amount of matrix produced per fibroblast in the diabetic group. It is striking that in the normoglycemic mice, in which apoptosis was not TNF- α -dependent, there was no change in cell numbers and no change in matrix production per cell when mice were treated with etanercept.

There is emerging evidence that apoptosis plays an important role in several diabetic complications. These include apoptosis of neuronal cells, which has been reported for diabetic neuropathy,^{47,48} diabetes-enhanced myocardial apoptosis, which contributes to cardiomyopathy,⁴⁹ and apoptosis of mesangial cells, which occurs in diabetic nephropathy.^{50,51} However, the signaling mechanisms responsible for enhanced apoptosis in these pathologies have not been conclusively established. That apoptosis of matrix-producing cells reported here was significantly enhanced in diabetic mice through a process that involves TNF- α is consistent with reports that TNF- α is dysregulated in diabetes in general and in the scalp model in particular.^{26,52,53} Thus, it is possible that TNF- α dysregulation may contribute to enhanced apoptosis observed in other diabetes-associated complications and that short-term inhibition of TNF- α may be beneficial under conditions in which infection is not problematic.

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