

# Diabetes-enhanced inflammation and apoptosis – impact on periodontal pathosis

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Diabetes mellitus is a metabolic disease estimated to affect over 19 million people in the U.S.A. Based on an evaluation by the National Health and Nutrition Examination Survey (NHANES) 1999–2002, the prevalence of diagnosed and undiagnosed diabetes was estimated at 9.3% in U.S. adults, with an additional 26% having the pre-diabetic condition of impaired fasting glucose (14). Diabetes mellitus has a dramatic impact on health, causing a high degree of morbidity and mortality in affected individuals as well as placing an economic burden on the health-care system (68).

The two major forms of diabetes mellitus, types 1 and 2, share many co-morbidities, but are characterized by having distinct etiologies. Type 1 diabetes occurs when the beta cells of the pancreas are destroyed and insufficient amounts of insulin are produced. In most cases, type 1 diabetes is the result of autoimmune-induced inflammation with destruction and apoptosis of beta cells (22). Approximately 90% of patients with diabetes have type 2 diabetes (28). The development of type 2 diabetes is directly related to increased amounts of visceral adipose tissue (12, 46). Adipose tissue is viewed as an active hormone-regulating organ that releases metabolically active molecules that can inhibit the body's ability to respond to insulin, referred to as insulin resistance (26).

Early in the development of type 2 diabetes, insulin resistance requires the production of extra insulin, a hyperinsulinemic state, to maintain a normal blood glucose level. In the majority of obese individuals, hyperinsulinemia can occur with an increase in beta-cell mass which leads to a greater capacity to produce insulin (21). As a result these individuals do not develop hyperglycemia. However, in approximately one-third of obese individuals there is a decreased beta-cell mass caused by a marked increase in beta-

cell apoptosis, rendering these individuals incapable of compensating for the insulin-resistant state. It is at this point that hyperglycemia becomes evident, leading to a diagnosis of type 2 diabetes. Thus, type 2 diabetes is caused by a combination of insulin resistance coupled with insufficient production of insulin to overcome the insulin resistance (28). It is striking that both type 1 and type 2 diabetes are negatively affected by the death of beta cells in the pancreas resulting in inadequate insulin production. For this reason the term, non-insulin-dependent diabetes (or NIDDM) has been replaced by the term, type 2 diabetes.

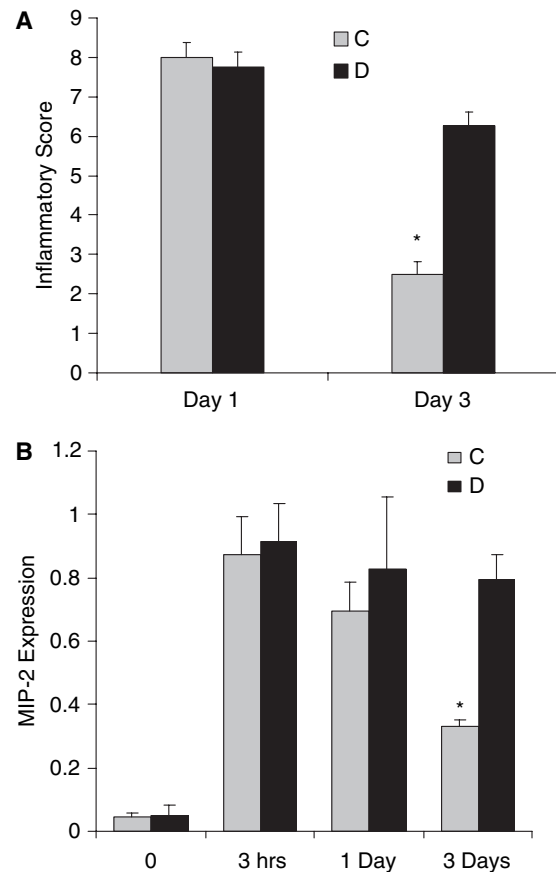
Long-term manifestations of diabetes include retinopathy, neuropathy, nephropathy, angiopathy, atherosclerosis, periodontitis, and other diabetic complications such as impaired wound healing (16, 24). These complications have been associated with inflammatory pathways that are enhanced in the diabetic state (1, 5, 15, 18, 61, 65). For example, high glucose levels can lead to the activation of the pathways that increase inflammation, oxidative stress, and apoptosis (8). In some cells hyperglycemia can lead to activation of the mitogen-activated protein kinase or protein kinase C pathways, both of which stimulate cytokine production and promote inflammation (20, 64). High glucose levels can indirectly affect tissue by the formation of advanced glycation end products, which accumulate during prolonged hyperglycemia and which are pro-inflammatory.

## Diabetes and inflammation

Both type 1 and type 2 diabetes are associated with elevated levels of systemic markers of inflammation. Increased serum levels of both tumor necrosis factor- $\alpha$

and interleukin-6 have been demonstrated in diabetes (6, 57). The tendency of diabetic individuals to have higher levels of inflammation has serious consequences that contribute to microvascular and macrovascular diabetic complications (49). Macrovascular complications, namely, cardiovascular disease, have critical consequences for patients with diabetes. Eighty per cent of individuals with type 2 diabetes die from coronary artery disease (11).

Given that diabetes affects oral health and many oral health problems involve bacteria-induced inflammation, there has been considerable interest in determining whether diabetes alters the inflammatory response to oral pathogens. For example, human gingival crevicular fluid from patients with type 1 diabetes with periodontal disease has higher levels of both prostaglandin E<sub>2</sub> and interleukin-1 $\beta$  compared with fluid from nondiabetic patients with similar levels of periodontal disease (54). Furthermore, monocytes isolated from periodontal patients with type 1 diabetes produce significantly greater amounts of tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , and prostaglandin E<sub>2</sub> in response to lipopolysaccharide when compared with nondiabetic patients (53, 54). The impact of diabetes on the inflammatory response to *Porphyromonas gingivalis* in a connective tissue setting is shown in Fig. 1 (25). When *P. gingivalis* is inoculated into connective tissue the inflammatory response after 1 day is similar in diabetic and control mice. However, at 3 days the inflammatory infiltrate consisting largely of polymorphonuclear leukocytes was reduced in the control group, whereas it remained high in the diabetic mice (Fig. 1A). Moreover, when the polymorphonuclear leukocyte chemoattractant macrophage inflammatory protein 2 was measured, the messenger ribonucleic acid (mRNA) levels were also initially similar but were more persistently high in the diabetic group (Fig. 1B). These results are not limited to type 1 diabetic mice because similar data were obtained with a type 2 diabetes model (48). Moreover, this difference was not the result of a deficit in bacterial killing in the diabetic group because inoculation of fixed bacteria induced a similar persistent inflammation. That tumor necrosis factor played a central role in this process was established by the reversal of prolonged chemokine expression by specifically inhibiting tumor necrosis factor with etanercept, a recombinant human soluble tumor necrosis factor- $\alpha$  receptor (48). Thus, cytokine dysregulation associated with prolonged tumor necrosis factor expression may represent an important mechanism through which diabetes alters the host response to bacterial challenge.



**Fig. 1.** *Porphyromonas gingivalis* stimulates formation of a more persistent inflammatory infiltrate in diabetic mice. Diabetes was induced by multiple low-dose streptozotocin treatment, while control mice were treated with citrate buffer alone. (A) *P. gingivalis* was inoculated subcutaneously into the scalp of diabetic (D) or control (C) mice and the degree of polymorphonuclear leukocyte infiltration was measured in hematoxylin & eosin-stained sections at the center of the infiltrate. A scale was used ranging from no inflammation to severe inflammation with necrosis (0–9). (B) mRNA levels of the polymorphonuclear leukocyte chemokine macrophage inflammatory protein 2 (MIP-2) were measured by RNase protection assay. Adapted from Graves et al. (25).

Diabetes may also render individuals more susceptible to the systemic consequences of local infection and thereby aggravate conditions where systemic inflammation contributes to pathologic processes. To address this issue we examined the inflammatory response of the heart/aorta of diabetic *db/db* mice that develop type 2 diabetes (41). Subcutaneous inoculation of lipopolysaccharide stimulated a local inflammatory response. More surprising was the observation that it also induced a systemic inflammatory response that was significantly more rapid and more pronounced in the cardiovascular tissue of diabetic animals. Thus, diabetes may enhance the inflammatory response to bacteria at both

the site of infection and also systemically through a greater response to endotoxemia or bacteremia.

## Diabetes and advanced glycation end products

Advanced glycation end products are nonenzymatic additions of glucose molecules to proteins. The number of glucose molecules that attaches to proteins is exposure-dependent, meaning that both the time and concentration of glucose contribute to this condition. Therefore, advanced glycation end products form under normal conditions, accumulating as individuals age and under hyperglycemic conditions such as diabetes. There are several mechanisms through which advanced glycation end products appear to affect wound healing: by enhancing or prolonging inflammation, stimulating apoptosis or affecting production or remodeling of extracellular matrix (51, 56, 62). Insight into the effects of advanced glycation end products on the healing process comes from studies with diabetic mice (23). When advanced glycation end products are blocked with an inhibitor the rate of wound closure and the production of collagen are improved in diabetic mice. Blocking advanced glycation end products also down-regulates matrix metalloproteinase activity at later time points, which may contribute to improvements in matrix formation. Advanced glycation end-product-enhanced inflammation may also aggravate periodontal disease. When the same inhibitor is applied to diabetic mice with experimentally induced periodontitis the degree of bone loss is reduced (33). Moreover, inhibition of advanced glycation end products decreases the levels of tumor necrosis factor- $\alpha$  and interleukin-6 in the gingival tissue of diabetic mice. These findings link advanced glycation end products with an exaggerated inflammatory response to oral pathogens in diabetes-enhanced periodontal disease.

Mineralized tissues are affected by diabetes. Decreased expression of genes that induce osteoblast differentiation, decreased growth factor production and diminished extracellular matrix production have been demonstrated in diabetic conditions (7, 31, 40). Consistent with these findings, the effects of advanced glycation end products extend to include alterations in bone metabolism and are most evident in bone formation (55). It has been reported that application of advanced glycation end products to calvarial defects in nondiabetic animals mimics diabetes-impaired bone healing. Advanced glycation

end products have also been shown to lead to diminished extracellular matrix production and to interfere with osteoblast differentiation (13, 42, 55).

## Diabetes and apoptosis

Advanced glycation end products can affect connective tissue and bone by promoting apoptosis of matrix-producing cells. By inducing apoptosis the number of cells capable of producing matrix would be reduced. Apoptosis is programmed cell death that can be triggered by various signals and is characterized by well-defined morphologic changes (34, 47). Apoptosis is important as a critical mechanism for removing unwanted cells during development, as a means of preventing autoimmunity, and as part of a response to protect the host from cells that have been infected or have become tumorigenic. Although the percentage of cells that are apoptotic at any given time may seem low, the cumulative effect over a 24-hour period can be quite high.

Apoptosis is mediated by a family of proteases called caspase that are activated by processing from its inactive precursor (zymogen). Apoptosis occurs rapidly, within an hour of effector caspase activation. Thirteen members of the human caspase family have been identified. Some of the family members are involved in apoptosis, and these can be divided into two subgroups. The first group consists of caspase-8, -9, and -10, which function in the final signaling of cell death. The second group contains caspase-3, -6, and -7, and work as effectors to carry out the processes associated with cell death.

A body of evidence is emerging that apoptosis plays an important role in several diabetic complications. These include apoptosis of neuronal cells in diabetic neuropathy (35), diabetes-enhanced myocardial apoptosis, which plays a role in cardiac pathogenesis (9), and apoptosis of mesangial cells, which occurs in diabetic nephropathy (43, 66). Diabetes is associated with the production of pro-apoptotic factors, the formation of reactive oxygen species, tumor necrosis factor, and advanced glycation end products. Reactive oxygen species are potent inducers of apoptosis (44). Tumor necrosis factor can induce apoptosis by binding to the tumor necrosis factor receptor-1, which has a 'death domain' that triggers the initial events in apoptosis, and by stimulating expression of pro-apoptotic genes (3, 60). Advanced glycation end products may also promote apoptosis of critical matrix-producing cells through several mechanisms; the direct activation of caspase activity, as well as

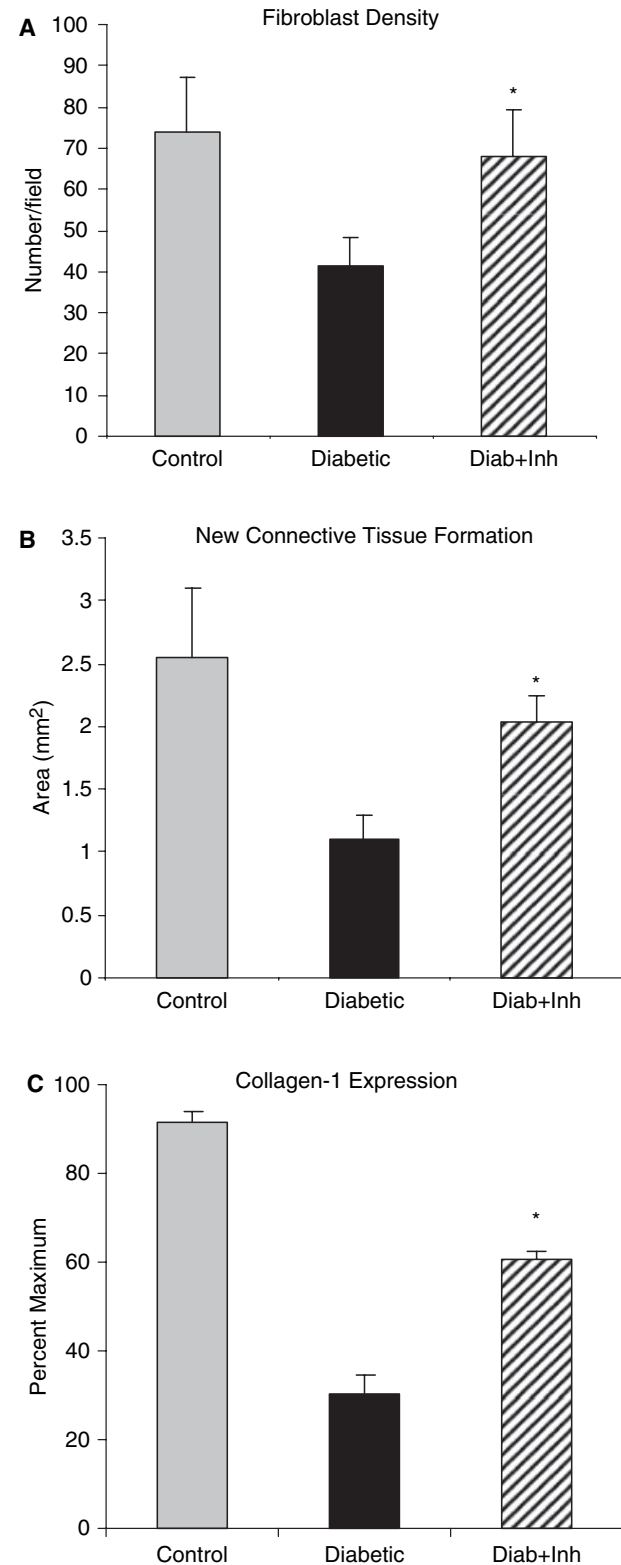
indirect pathways that increase oxidative stress or the expression of pro-apoptotic genes that regulate apoptosis (3, 29, 30, 67).

*In vivo* experiments have established that advanced glycation end products induce fibroblast apoptosis (3). *In vitro*, advanced glycation end products have a global effect of enhancing the mRNA levels of pro-apoptotic genes, including several classes of molecules such as ligands, receptors, adaptor molecules, mitochondrial proteins, and others (3). Interestingly, advanced glycation end products stimulate nuclear factor- $\kappa$ B activation, which is anti-apoptotic in most cell types (63). Thus, advanced glycation end products and other pro-apoptotic molecules, such as tumor necrosis factor, stimulate both anti- and pro-apoptotic factors and the net result reflects the overall balance between them (4). This balance may be influenced by members of the forkhead transcription factors, such as winged-helix-forkhead box class O1 (FOXO1) transcription factor, that globally induce expression of pro-apoptotic genes and may represent a mechanism to overcome nuclear factor- $\kappa$ B-associated anti-apoptosis (3).

## Diabetes, apoptosis, and wound healing

Wound healing is a complex process including inflammation, formation of granulation tissue and production of new structures, and tissue remodeling. These processes are regulated by cytokines and growth factors and may be modulated by systemic conditions such as diabetes. Apoptosis also plays an important role in the healing process. A critical component for healing is the generation of a sufficient number of matrix-producing cells early in the healing process associated with formation of granu-

lation tissue. Apoptosis of matrix-producing cells at this stage may interfere with the ability to produce enough matrix and limit repair (17, 52). In contrast, the elimination of inflammatory cells at the late stage of inflammation and excess fibroblasts during tissue remodeling by apoptosis is very important for healing



**Fig. 2.** Inhibition of fibroblast apoptosis enhances the healing response in diabetic animals. A high dose of *Porphyromonas gingivalis* was inoculated into the scalp of diabetic (*db/db*) mice and their normoglycemic control littermates to create a bacterially induced injury. One group of diabetic mice was treated with the pan-caspase inhibitor Z-VAD-fmk to block apoptosis and mice were sacrificed 8 days later during the peak healing period. (A) Fibroblast density was measured in hematoxylin & eosin-stained sections. (B) The area of newly formed connective tissue matrix was measured in van Gieson-stained sections. (C) Collagen 1 mRNA levels were measured in healing tissue by RNase protection assay. Each value represents the mean  $\pm$  SEM. \* $P < 0.05$ ; i.e. statistical significance compared to diabetic mice. Adapted from Al-Mashat et al. (2).

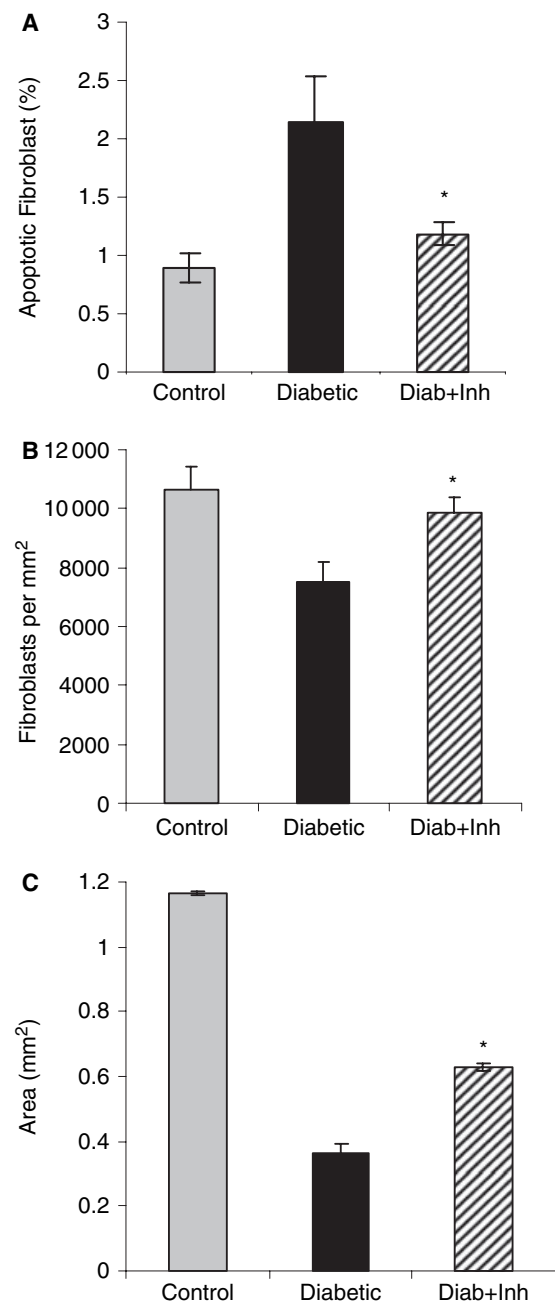
and for preventing excessive scar formation. For bacteria-induced injury, healing may follow a pattern that requires fibroblasts to infiltrate the destroyed matrix and initiate repair by producing a collagen-rich matrix. Any factor that influences apoptosis of cells in the wound environment, especially for repopulating fibroblasts in this process, can affect healing.

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 polymorphonuclear leukocytes and macrophages, sustained cytokine expression and infiltration by inflammatory cells, decreased production of growth factors, reduced cellular proliferation and extracellular matrix synthesis, and increased production of proteolytic enzymes. Studies suggest that increased apoptosis in a diabetic state causes delayed healing of incisional wounds (10, 17, 19, 58). We investigated the healing response of diabetic and normal mice following *P. gingivalis*-induced tissue destruction. Killed *P. gingivalis* was inoculated into the scalp of mice to induce an inflammatory injury (27, 38). 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. The results indicate that diabetes modifies the response to bacteria by significantly enhancing the number of fibroblasts positive for activated caspase-3 and the level of fibroblast apoptosis at the peak time of healing (38). The enhanced

fibroblast apoptosis coincided with decreased numbers of fibroblasts and a reduced capacity to produce matrix. Diabetes also caused a global induction of pro-apoptotic genes during the repair process (2). Out of a total of 276 apoptotic genes examined, 71 genes increased two-fold or more; 63 of these genes were pro-apoptotic and eight were anti-apoptotic genes. This points to another mechanism by which diabetes may interfere with the capacity to repair tissue damage by enhancing the death of matrix-producing cells.

To prove that enhanced apoptosis actually contributes to diminished wound healing functional

**Fig. 3. Tumor necrosis factor inhibition reduces fibroblast apoptosis and increases fibroblast density and new matrix formation in diabetic mice but not in normoglycemic mice during the healing response to bacterially induced injury.** *Porphyromonas gingivalis* was inoculated into the scalp of diabetic (leptin-receptor-deficient; *db/db*) and normoglycemic (*db/+*) littermates. Mice were treated with systemic and local injection of etanercept to inhibit tumor necrosis factor or with equivalent vehicle alone and were sacrificed on day 8 during the peak healing period. (A) The terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was used to detect fibroblast apoptosis. (B) Fibroblast number was counted at 1,000 $\times$  magnification in hematoxylin & eosin-stained sections. (C) Newly formed connective tissue matrix was identified in van Gieson-stained histologic sections by its characteristic blue color (original magnification 400 $\times$ ). \*Significant difference in diabetic mice treated with etanercept compared to vehicle alone ( $P < 0.05$ ). Adapted from Liu et al. (36).

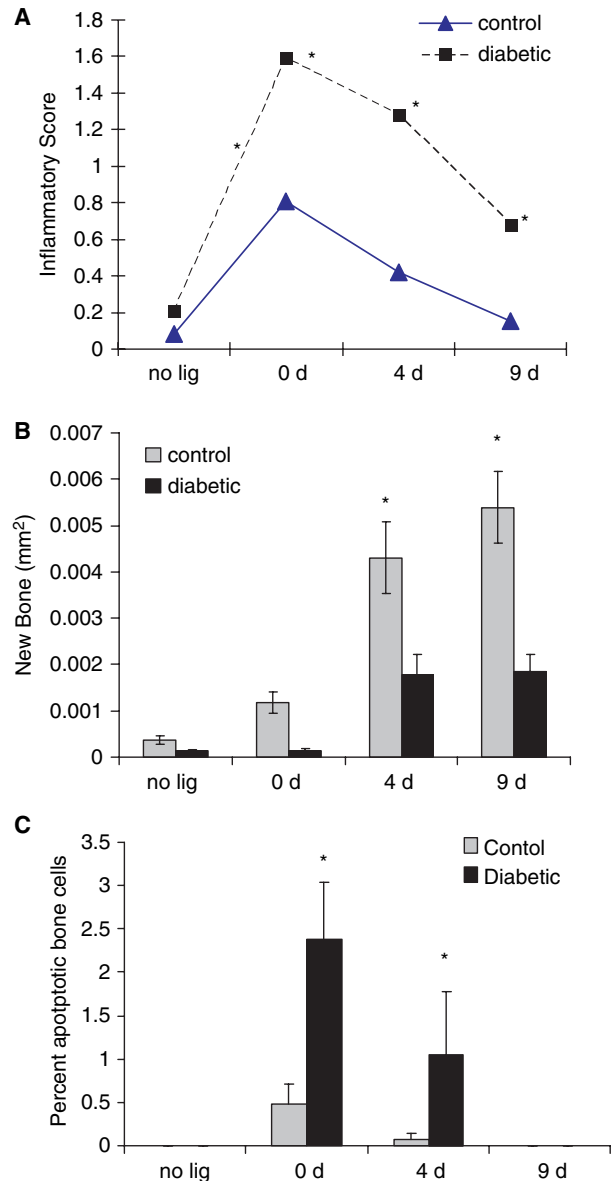


studies are necessary (2). Fig. 2 demonstrates that diabetes-enhanced apoptosis contributes to deficits in diabetic healing following a bacterially induced wound. Diabetes caused a decrease in the number of fibroblasts during the peak healing period, which was significantly reversed when apoptosis was inhibited, demonstrating that apoptosis had the important physiologic effect by decreasing the number of fibroblasts that could participate in wound healing. That this was significant was shown by an increase in matrix production, consistent with an increase in fibroblasts. In addition, blocking apoptosis significantly improved the level of collagen-1 expression.

There are many potential reasons for enhanced apoptosis in the diabetic animals. One explanation may be the effects of tumor necrosis factor- $\alpha$ , which is a potent inducer of apoptosis in fibroblasts and osteoblasts. Diabetes is associated with excessive tumor necrosis factor- $\alpha$  expression. This may be the result of 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. The role played by tumor necrosis factor- $\alpha$  in diabetes-enhanced apoptosis in connective tissue and bone was investigated using the bacterially induced wound model with or without the application of the tumor necrosis factor inhibitor etanercept (Fig. 3) (37). When the level of fibroblast apoptosis was measured during the peak healing period, 8 days after inoculation of bacteria, the level was more than twice that of normoglycemic mice. However, when diabetic mice were treated with the tumor necrosis factor inhibitor, etanercept, fibroblast apoptosis was reduced by almost 50% (Fig. 3A). One of the ways that apoptosis could interfere with wound healing is by limiting the number of cells available to produce matrix. This was evident by a significant decrease in fibroblast numbers in the diabetic group and an increase when diabetic mice were treated with tumor necrosis factor inhibitor (Fig. 3B). Just as importantly, the inhibition of tumor necrosis factor resulted in a significant increase in the formation of connective tissue matrix in the diabetic mice (Fig. 3C), which is probably the result of an increase in fibroblast density associated with decreased apoptosis.

## Apoptosis, bone, and periodontitis

Diabetic patients exhibit an increased susceptibility to infection. This may be evidenced by a twofold to



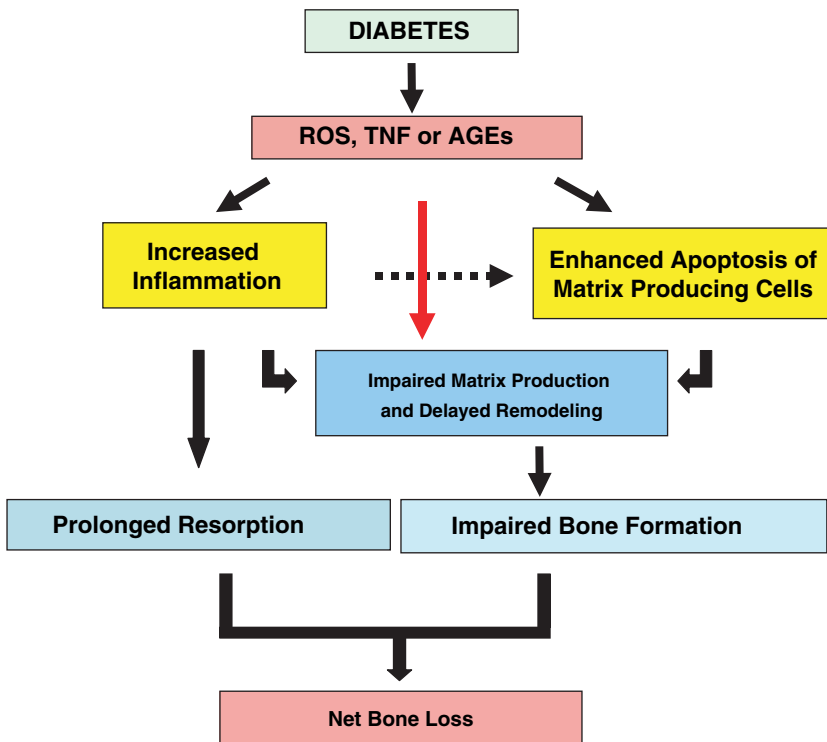
**Fig. 4.** Diabetes prolongs inflammatory infiltrate and the osteoclast formation, inhibits new bone formation, and increases osteoblast apoptosis. Ligatures were placed around the molar teeth of Zucker diabetic fatty (ZDF) type 2 diabetic and normoglycemic control rats and left in place for 7 days. Rats were sacrificed before placement of ligatures for baseline measurements, immediately after ligatures were removed (0 day) or 4 or 9 days later. (A) The interproximal gingiva between the first and second and second and third molars was examined for the presence of inflammatory cells (polymorphonuclear leukocytes and mononuclear cells) in hematoxylin & eosin-stained sections using the following scale: 0 (no inflammation), 1 (slight), 2 (moderate), 3 (severe). (B) The area of new bone formation was measured using the reversal line as a guide. (C) The per cent apoptotic bone lining cells was assessed by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Each value is the mean of five to seven rats. \*Significant difference between diabetics and normoglycemic control rats,  $P < 0.05$ . Adapted from Liu et al. (37).

fivefold higher risk for periodontal disease, which is reduced by effective control of hyperglycemia (39, 50, 59). It has been reported that periodontal treatment helps in the stabilization of serum glucose levels further implicating inflammation in diabetes although a meta-analysis has questioned whether the decrease is significant (45, 59).

Several mechanisms have been proposed to explain the greater severity of periodontal disease in diabetics (32). In general, these mechanisms would be expected to lead to enhanced formation of osteoclasts and increased bone loss. An alternative mechanism is that diabetes increased the death of bone lining cells that include osteoblast precursors and osteoblasts (27). When bone coupling was examined following *P. gingivalis* inoculation diabetes suppressed the amount of reparative bone formation that occurs. These findings demonstrated that diabetes contributes to the net loss of bone, in part, because it suppresses the coupled new bone formation that follows resorption. Interestingly, when apoptosis was inhibited the deficit in bone formation in the diabetic group was partially suppressed (2).

We have also examined the impact of diabetes on bone resorption and formation in the ligature-induced periodontitis model in diabetic rats (37). In this model, ligatures were placed around the second molars with inflammation, loss of attachment and

bone developing over a 7-day period (Fig. 4). Observations after 7 days of ligature placement showed that the numbers and activity levels of osteoclasts were similar in both diabetic and non-diabetic animals. Following the 7-day period, ligatures were removed and the coupling of bone formation and resorption was examined over the next 9 days. The diabetic condition was associated with an increase in the intensity of the inflammatory infiltrate as well as a more prolonged inflammation (Fig. 4A). In fact, while the inflammatory response returned to baseline levels in the non-diabetic animals after 9 days, it was still elevated in the diabetic animals 9 days after ligatures were removed. When the coupling of bone formation and resorption was examined the diabetic animals exhibited a significantly impaired ability to repair the resorbed bone (Fig. 4B). In fact, new bone formation was two to three times greater in the normal animals compared to the diabetic animals 9 days after the ligatures were removed. Most interestingly, the diabetic condition also significantly increased apoptosis (Fig. 4C), and decreased the number of bone-lining cells, osteoblasts, and periodontal ligament fibroblasts (data not shown) (37). Together, the findings from this study support a mechanistic role for apoptosis in association with the exaggerated inflammatory response in a diabetic condition. It appears from these findings that the higher rate of apoptosis leads to a reduced number of osteoblastic



**Fig. 5.** Mechanisms through which diabetes may affect periodontal disease progression. Diabetes-induced production of reactive oxygen species, tumor necrosis factor or advanced glycation end products can have direct effects on bone including increased osteoclast formation and resorption as well as inhibition of collagen production by osteoblasts. However, they could also have indirect effects by enhancing apoptosis of osteoblasts or their precursors. Taken together, direct and indirect effects of advanced glycation end products could contribute to enhanced resorption and impaired coupling, leading to diminished bone formation that occurs after resorption. AGE, advanced glycation end products; ROS, reactive oxygen species; TNF, tumor necrosis factor.

precursors and osteoblasts, thereby decreasing new bone formation.

It is becoming increasingly clear that inflammation such as tumor necrosis factor dysregulation plays an important role in the development of a number of diabetic complications. Furthermore, the structural and inflammatory alterations associated with advanced glycation end products have an impact on many of the clinical complications of diabetes. The exaggerated levels of periodontal disease in diabetic patients are consistent with a greater inflammatory response to infection. A previously unrecognized mechanism may be the impact of diabetes on bone coupling. Since bone mass is maintained by the coupling of bone formation and resorption, factors that impair bone formation lead to diminished bone mass. It is our view that one of the mechanisms contributing to the exaggerated bone loss associated with periodontal disease in patients with diabetes is decreased matrix formation as the result of enhanced apoptosis of matrix-forming cells. Thus the net effect of diabetes on periodontal disease progression may enhance net bone loss by enhancing resorption as well as interfering with coupling as summarized in Fig. 5.

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