Diabetes Alters the Response to Bacteria by Enhancing Fibroblast Apoptosis

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Diabetics suffer from both more frequent bacterial infections and greater consequences of infection. However, bacteria-induced tissue destruction and the subsequent response in diabetics have received relatively little attention. To investigate this issue, we inoculated the scalp of control or db/db diabetic mice, with the pathogen Porphyromonas gingivalis, which causes connective tissue destruction in humans. Both bacteria-induced cytokine expression and tissue loss were similar in diabetic and control mice. However, there was a significantly higher rate of fibroblast-specific apoptosis in the diabetic group, which was measured as cells that were double positive for the terminal deoxynucleotidyltransferase-mediated deoxy-UTP nick end labeling assay and expression of vimentin. The higher rate of fibroblast apoptosis could be explained in the diabetic group by enhanced levels of activated caspase-3. Apoptosis was evident during the peak healing period and coincided with reduced numbers of fibroblasts, diminished collagen I and III expression, and significantly reduced formation of new connective tissue matrix in diabetic mice. Thus, diabetes may impair the healing response to bacteria-induced connective tissue loss by increasing the number of caspase-3-activated fibroblasts, leading to greater apoptosis and reduced numbers of fibroblastic cells. (Endocrinology 145: 2997–3003, 2004)

Bacteria can directly cause tissue injury. This can occur through the production of exotoxins and collagenolytic enzymes (1–3). In addition, bacteria can indirectly cause tissue loss by inducing inflammation that, in turn, stimulates the expression and activation of host-derived matrix metalloproteinase (4–7). Periodontal disease is a condition in which bacteria are thought to induce the expression of cytokines such as IL-1 and TNF, which subsequently stimulate a cascade of events culminating in loss of connective tissue (8, 9). Under normal conditions this bacteria-stimulated injury is healed by the action of fibroblasts. Although it is widely known that diabetes can affect fibroblast proliferation, other mechanisms may also come into play that have not been well studied. These include the loss of fibroblasts through apoptosis. In support of this, Darby et al. (10) suggested that there may be a higher degree of apoptosis during healing in diabetics after excisional skin wounds.

It has been well documented that there is delayed or incomplete healing of wounds in diabetic humans and in animal models of diabetes (11–14). The inflammatory aspects of wound healing appear to be particularly affected by diabetes. The initial infiltration of wounds by polymorphonuclear leukocytes and monocytes/macrophages is delayed and reduced in diabetic mice (12, 15). In contrast, there is greater cytokine expression and a more sustained infiltration of diabetic wounds by leukocytes and at the later stages of healing (16). The early deficit in monocytes/macrophages in diabetic wounds may lead to inadequate growth factor production (12, 17–19), and the sustained presence of inflammatory cells may lead to altered regulation of proteases (20–23), both of which could impair healing.

In contrast to traumatic wounds, the impact of diabetes on bacteria-induced tissue destruction and the subsequent healing response have received relatively little attention. We investigated the repair of bacteria-induced tissue loss in diabetic mice. 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. The enhanced fibroblast apoptosis coincided with decreased numbers of fibroblasts and a reduced capacity to produce matrix. This points to another mechanism by which diabetes may interfere with the capacity to repair tissue damage, particularly that caused by bacterial infection.

Materials and Methods

Animals

Genetically diabetic C57BL/KsJ-lepr-db/db mice and their nondiabetic littermates, C57BL/KsJ-lepr-db/+ mice, were purchased from The Jackson Laboratory (Bar Harbor, ME). The db/db mice develop diabetes at 6–8 wk of age and had been diabetic for a minimum of 20 d before the experiments. Mice were considered to be diabetic when the glucose level exceeded 250 mg/dl. The glucose levels during the experimental period were typically 400–450 mg/dl in db/db mice. All animal procedures were approved by the institutional animal care and use committee, Boston University Medical Center.

Inoculation of bacteria

Broth-grown Porphyromonas gingivalis strain 381 at log phase growth was fixed with 1% paraformaldehyde for 6 h. After mice were anesthetized with injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), 5 × 10<sup>8</sup> P. gingivalis were inoculated at the midline of the scalp between the ears. This produces an inflammatory response, bacteria-induced tissue loss, and a healing response that can be identified in histological
sections between the coronal and the occipital sutures. Mice were killed at 0, 1, 3, 5, 8, and 12 d after inoculation. There were six mice for each group at each data point (n = 6).

Preparation of specimens

The scalp of each calvarium was split along the sagittal suture. One half was immediately frozen in liquid nitrogen for later RNA extraction. The remaining half scalp together with calvarial bone was left intact and fixed in 4% paraformaldehyde at 4 C for 3 d. After fixation the specimens were decalcified in Immunocal (Decal Chemical Corp., Congers, NY) at 4 C for 12 d and washed with Cal-Arrest (Decal Chemical Corp.). Then the specimens were embedded in paraffin, and 5-μm sagittal sections were prepared.

Histomorphometry

The area of intense neutrophil infiltration on d 1 or the area of tissue necrosis on d 3 was measured at ×100 magnification in sections stained with hematoxylin and eosin using computer-assisted image analysis. Van Gieson-stained sections were used to assess the area of new collagen formation at ×100 magnification on d 5, 8, and 12 as described previously (24). The total fibroblast number in 10 randomly selected fields in the area of healing was counted at ×1000 magnification. Fibroblasts were identified by their characteristic cytoplasmic and nuclear appearance in hematoxylin- and eosin-stained sections. Fibroblast density was expressed as fibroblast number per square millimeter.

Detection of apoptotic fibroblasts

Specific apoptosis of fibroblasts was detected by the terminal deoxyribonucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling (TUNEL) assay, in situ Tdt-mediated deoxy-UTP-biotin nick end labeling (Trevigen, Gaithersburg, MD) combined with immunohistochemistry for vimentin-positive cells using a specific antibody (Cortex Biochem, Inc., San Leandro, CA). For the latter, a kit from Vector Laboratories, Inc. (Burlingame, CA), with tyramide signal amplification was used (PerkinElmer, Boston, MA). The double-labeled TUNEL and vimentin-positive cells were counted at ×1000 magnification. The negative control consisted of an equal amount of nonimmune goat serum and the labeling reaction mix without the TdT enzyme for immunohistochemistry and the TUNEL assay, respectively.

Detection of activated caspase-3

The number of fibroblastic cells positive for activated caspase-3 was measured by immunohistochemistry using a specific antibody (Trevigen), which only detects cleaved caspase-3, and a detection kit from Vector Laboratories, Inc., with tyramide signal amplification. Sections were counterstained with hematoxylin. Immunopositive fibroblastic cells with the characteristic nuclear appearance of fibroblasts were counted at ×1000 magnification. The negative control consisted of an equal amount of nonimmune purified rabbit IgG.

Cytokine and procollagen expression

Total RNA was extracted from pulverized scalps frozen in liquid nitrogen with TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer’s instructions. The concentration and integrity of the extracted RNA were verified by denaturing agarose gel electrophoresis. RNA from six animals was pooled, and gene expression was measured by the ribonuclease (RNase) protection assay. Twelve micrograms of total RNA from the specimens at 0, 1, 3, and 5 d were incubated with 43P-labeled riboprobes specific for murine IL-6, IL-1, macrophage inflammatory protein-2 (MIP-2), and monocytic chemotactic protein-1 (MCP-1). 32P-Labeled riboprobes specific for murine procollagen I and procollagen III were incubated with 4 μg total RNA. After hybridization, specimens were subjected to RNase digestion using a kit from BD PharMingen (Franklin Lakes, NJ) following the manufacturer’s instructions. After electrophoresis on a 6% polyacrylamide gel, radiolabeled bands were visualized using a PhosphoImager (Bio-Rad Laboratories, Hercules, CA). The OD of the protected bands was measured with Image ProPlus software (Media Cybernetics, Silver Spring, MD), which was then normalized by the value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same line. The experiments were performed twice with similar results.

Histological analysis

Cell counts and histomorphometric data were obtained by one examiner, and more than half of the slides were assessed by a second examiner, who confirmed the results. The statistical significance of differences between diabetic and control mice was determined by t test at the P < 0.05 level.

Results

Inflammation and destruction of soft tissue induced by bacteria

To investigate host-bacteria interactions that lead to tissue destruction, we used a murine model. The scalp was chosen as the site of inoculation because it provides a convenient connective tissue environment that provides a reproducible response to bacteria (25, 26). P. gingivalis was inoculated because it leads to connective tissue destruction, particularly in diabetics (27–30). In addition, P. gingivalis is a pathogen that is a source of focal infection that can disseminate and cause tissue destruction in many different organs (31, 32). After the inoculation of bacteria, intense infiltration of leukocytes was observed on d 1, and necrosis was observed on
d 3 (Fig. 1). On d 5, inflammation decreased, and tissue healing started. On d 8, new matrix formation was clearly evident.

As the area of inflammation and tissue destruction induced by bacteria could influence the rate of healing, these parameters were measured in diabetic and control mice. The mRNA expression of proinflammatory cytokines, including IL-1, IL-6, MIP-2, and MCP-1 was measured (Fig. 2). The mRNA expression of these cytokines was very low at time zero, increased rapidly to a peak at 1 d, and decreased on d 3 and 5. For most time points, there was relatively little difference in cytokine gene expression between diabetic and control mice.

When the area of the inflammatory infiltrate and tissue loss was measured, results consistent with the level of cytokine expression in the experimental and control groups was obtained. On d 1, the area of inflammation was similar in diabetic and control groups (Fig. 3). The area of tissue necrosis on d 3 closely matched the area of inflammation on d 1 and was not different between diabetic and normoglycemic mice.

**Matrix repair**

Newly formed connective tissue matrix was identified in Van Gieson-stained histological sections by its characteristic blue color (Fig. 4A). The area of new connective tissue matrix on d 5, 8, and 12 was measured and is shown in Fig. 4B. The new matrix formation started on d 5 in both groups, with no significant difference. On d 8 there was peak formation of new matrix in both groups. The amount of new matrix in control mice at this time was five times higher than that in diabetic mice.

**FIG. 2.** Cytokine mRNA expression after inoculation of bacteria. IL-1, IL-6, MIP-2, and MCP-1 mRNA expression was measured by RNase protection assay from total RNA obtained from the scalp after *P. gingivalis* inoculation. A, Autoradiograms of cytokines and GAPDH expression in diabetic *db/db* (+) and control *db/+* (−) mice. B, Densitometric analysis of cytokine expression normalized by relative GAPDH levels in the same lane. ■, Diabetic *db/db* mice; □, normoglycemic littermate control *db/+* mice.

**FIG. 3.** The inflammatory infiltrate and area of tissue necrosis resulting from inoculation of bacteria are similar in normoglycemic and diabetic mice. Histological sections, described in Fig. 1, were examined by computer-assisted image analysis. The area of inflammation on d 1 and that of tissue necrosis on d 3 were measured. There was no difference between diabetic and normoglycemic control mice (*P* > 0.05). ■, Diabetic *db/db* mice; □, normoglycemic littermate control *db/+* mice.
diabetic mice. The difference was statistically significant ($P < 0.01$). The area of newly formed matrix decreased on d 12 in both groups and was three times higher at this time point in normoglycemic compared with diabetic mice ($P < 0.05$).

The expression of matrix proteins was assessed by measuring the mRNA levels of procollagen I and procollagen III mRNA in both groups. On d 5 there was a similar expression of procollagen I and procollagen III mRNA in both groups. On d 8 when the expression of procollagen I and procollagen III mRNA in the control group continued to increase to the peak, the expression of procollagen I and procollagen III mRNA in diabetic group decreased. On d 12, the expression of procollagen I and procollagen III mRNA in both groups decreased. On d 8 and 12, the mRNA levels for procollagen I in diabetic mice were one half and procollagen III mRNA levels were one fourth to one third those in normoglycemic mice.

**Apoptosis of fibroblasts**

The TUNEL assay combined with immunohistochemistry for vimentin expression was used to specifically detect apoptotic fibroblasts (Fig. 6). There were virtually no apoptotic fibroblasts at baseline in the diabetic and nondiabetic mice and no difference between these two groups (data not shown). Fibroblast apoptosis was first detected at low rates at the same time point when new matrix production was observed on d 5. Fibroblast apoptosis peaked on d 8, when the healing response was also at its highest level. On d 8 apoptosis of fibroblasts in diabetic mice was 2.5 times higher than that in normoglycemic mice. The difference was statistically significant ($P < 0.01$). On d 12 the apoptosis of fibroblasts in both groups decreased greatly. There was no significant difference between the two groups.

To better understand the apoptotic pathways that may be involved, the number of fibroblastic cells with activated caspase-3 was measured on d 8 when apoptosis was at its highest level (Fig. 6C). The percentage of caspase-3-positive fibroblastic cells in the diabetic mice was 2.6-fold higher than the controls, agreeing well with the difference between the two groups when apoptotic fibroblasts were examined. The difference was statistically significant ($P < 0.05$).
To assess the potential impact of fibroblast apoptosis, fibroblast density was measured on d 8 and 12 (Fig. 7). The fibroblast density in diabetic mice at both time points was approximately 40% lower than that in the control mice ($P < 0.01$).

**Discussion**

In the results presented above we injected fixed *P. gingivalis* into diabetic and normoglycemic animals. *P. gingivalis* was used because it is a Gram-negative anaerobic bacteria that causes loss of connective tissue, particularly in diabetics (28–30, 33). The choice of an anaerobic bacteria is consistent with their common association with diabetic skin infections or ulcers, particularly in more severe infections that significantly impair healing (34, 35). Like other bacteria, *P. gingivalis* induces a host response this includes induction of cytokines, recruitment of inflammatory cells, and activation of matrix metalloproteinases (33, 36). As the bacteria were fixed, the tissue destruction that occurs results from the host response to bacterial challenge. This is consistent with findings that lipopolysaccharides mediates apoptosis through induction of TNF (37). By using fixed bacteria, differences in the response to bacterial injury reflect changes in the healing response, rather than a diminished capacity of diabetic mice to kill bacteria.

Although the production of cytokines in diabetes has been thoroughly investigated, there is still no consensus as to whether diabetes ultimately causes enhanced, diminished, or no change in production compared with that in normoglycemic cohorts. The explanation may reside in the nature of the stimulus and the specific experimental conditions. In the study presented here, mice were challenged with a relatively large dose of bacteria, sufficient to stimulate abscess formation and induce destruction of connective tissue matrix. Thus, under a large bacterial stimulus in vivo, we found little difference between diabetics and normal animals in the up-regulation of proinflammatory cytokines. Given that proinflammatory cytokine expression was similar, it is not surprising that the amount of tissue destruction in the two groups was equivalent. However, under milder conditions diabetics may have a more persistent inflammatory response than their normal cohorts.
Despite having similar bacteria-induced tissue destruction, the healing response in the two groups did differ significantly. There was 2-fold higher expression of collagen I and 3- to 4-fold higher expression of collagen III in the control compared with diabetic group. When the amount of new matrix formed was examined, 3- to 5-fold higher levels were noted in the control compared with the diabetic mice. This result is consistent with many reports indicating a deficit in healing caused by a number of different etiologies, including trauma (10, 13, 14), burns (38), and radiation (39). Thus, healing after bacteria-induced abscess formation follows a similar pattern.

After bacteria-induced injury, fibroblasts infiltrate the destroyed matrix and initiate repair by producing a collagen-rich matrix. During the later stages of healing, fibroblasts are removed by apoptosis (40, 41). Therefore, delayed or impaired apoptosis of fibroblasts may lead to excessive scar formation. Conversely, excessive apoptosis of repopulating fibroblasts may inhibit the repair process. Weringer et al. (42) reported that the fibroblasts in healing traumatic wounds from diabetic hamsters underwent internal degeneration and speculated that this would impair healing. We determined whether there was enhanced fibroblast apoptosis after bacteria-induced tissue destruction. Fibroblast apoptosis was noted during all phases of the healing response and was particularly prominent during the peak healing phase in diabetic mice. This coincided with maximum expression of collagen genes and newly formed matrix. Thus, under normal physiological conditions, fibroblast apoptosis may rid the repaired tissue of excess fibroblasts, whereas under the influence of a pathological condition such as diabetes, apoptosis may contribute to the insufficient number of fibroblasts and diminished capacity for healing.

Increased apoptosis has been found in various organs affected by diabetes, including the eye, heart, vascular system, and bone (43–46). However, the mechanism of diabetes-enhanced apoptosis is not well understood. One factor might be a prolonged inflammatory response to bacteria in diabetes (16). A persistent infiltration of inflammatory cells coupled with advanced glycation end product (RAGE) axis caused by hyperglycemia or through other mechanisms, thereby in-duce apoptosis. 

Previous studies have supported the concept that repopulation of wounds by fibroblasts is due to a deficit in growth factor production and proliferation (55, 56). Thus, the failure to achieve a sufficient number of fibroblasts could potentially come from two different mechanisms: a failure to stimulate sufficient proliferation or a significantly enhanced rate of programmed cell death. Therefore, we suggest that increased apoptosis of fibroblasts contributes to the mechanisms of impaired healing after bacteria-induced abscess formation in diabetes. This is supported by a significantly decreased fibroblast density in the diabetic mice after periods of enhanced apoptosis.

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