All-trans Retinoic Acid Improves Structure and Function of Diabetic Rat Skin in Organ Culture

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Diabetes increases susceptibility to chronic ulceration. The cause of chronic wound formation in diabetic individuals is multifactorial but may be accelerated by changes in the structure and function of the skin secondary to impaired fibroblast proliferation, decreased collagen synthesis, and increased matrix metalloproteinase (MMP) expression. This study explored cellular and biochemical changes in organ cultures of skin from streptozotocin-diabetic (STZ-D) rats and the effects of all-trans retinoic acid (RA) on these changes. STZ-D rats were killed after 6 weeks. The skin was cut into 2-mm pieces and incubated in organ culture for 3 or 6 days in the absence or presence of 3 μmol/l RA. After organ culture incubation, control and RA-treated tissue was examined histologically after staining with hematoxylin and eosin. In parallel, organ culture–conditioned medium was assayed for MMPs. Additional organ cultures were examined for collagen synthesis using 3H-proline incorporation into trichloroacetic acid–precipitable material and for glycosaminoglycan production based on interaction with the cationic dye 1,9-dimethylmethylene blue and by staining of tissue sections with periodic acid Schiff reagents. Skin from 6-week STZ-D rats demonstrated features of dermal atrophy including thinning and disorganization of connective tissue bundles and increased space between bundles. The addition of RA resulted in cellular reaction and partially reversed the histological features of dermal atrophy. Levels of latent and active MMP-9 and MMP-13 were elevated 4- and 10-fold, respectively, in STZ-D skin and reduced by 50–75% (P < 0.05) by RA. Collagen synthesis was increased by 30% (in STZ-D skin and reduced by 50–75% (P < 0.05) by RA, whereas glycosaminoglycan expression was increased by only 9% (NS). RA also increased proliferation of STZ-D skin fibroblasts (approximately threefold over control; P < 0.05). Together, these data suggest that RA has the capacity to improve structure and function of diabetic skin. Diabetes 51:3510–3516, 2002

It has been appreciated for more than a century that healing of skin wounds, particularly in the lower extremities, is impaired in patients with diabetes (1–5). Wound healing is also impaired in animal models of diabetes (6,7). Although diabetes is a major predisposing factor in the formation of nonhealing wounds, other underlying conditions, including atheromatous vascular disease, sensory loss, systemic malignancy, nutritional disorders, infectious/inflammatory disease, and advanced age, are also associated with impaired healing of skin wounds (reviewed in 8–10). Although the proximal cause of most nonhealing foot ulcers in patients with diabetes is thought to be ischemia/reperfusion injury, impaired vascular function and loss of sensory perception are underlying factors. Dermal atrophy is another feature that may predispose skin to ulcer formation. Fibroblasts from skin that is at risk for the development of nonhealing ulcers demonstrate impaired proliferative capacity in vitro (11–13). Reduced fibroblast growth is associated with reduced collagen synthesis (14–16) and increased matrix metalloproteinase (MMP) production (14,17–19).

The occurrence of nonhealing wounds in the lower extremities of subjects with diabetes results in significant morbidity and is a contributing factor in >70% of the 55,000 annual lower limb amputations (20). A number of experimental approaches have been proposed for the treatment of wounds in patients with diabetes, including the use of vitamin A. It was shown in a 1979 study by Seifter et al. (21) that diabetic rats fed a diet high in vitamin A repaired incision wounds more rapidly than control rats. The authors concluded that proinflammatory retinoid activity contributed to wound repair in the vitamin A–fed rats.

Biologically active retinoids, including all-trans retinoic acid (RA) and all-trans retinol, have been shown to repair skin damaged by chronological aging (22) or photoaging (23,24). Repair of aged skin is associated with induction of fibroblast proliferation and procollagen synthesis and reduction in MMP levels (25). Keratinocyte proliferation also occurs in response to topical retinoid treatment, leading to epidermal thickening (26). It seems reasonable to speculate that similar effects might occur in diabetic skin and that these effects might lead to reduced incidence of wound formation and to improved healing when wounding occurs. To begin examining this possibility, we established organ cultures from the skin of streptozotocin-diabetic (STZ-D) rats (21) and from control rat skin. Organ cultures
of diabetic skin were maintained under control conditions or treated with concentrations of RA that are known to improve histological structure and biochemical function in human skin (24–30). Results of these studies are described in the present report.

RESEARCH DESIGN AND METHODS

STZ-induced diabetes. Male Wistar rats with a start weight of 180–200 g were acclimated for 1 week upon arrival in the laboratory. Animals were randomized to control or treatment groups. Diabetes was induced by intravenous injection of STZ as described (31). After 48 h, rats that received an injection were assessed for blood glucose and included in the study if glucose concentrations were >250 mg/dl in heparinized tail vein blood (checked by Glucometer). All treated and control rats were maintained for 6 weeks with ad libitum access to water and suitable rat diet. STZ-treated rats were given daily injections of a small dose of protamine zinc insulin (0.5–3.0 units per day) as required to maintain blood glucose levels between 350 and 450 mg/dl. At the end of the 6-week period, animals were killed by cervical dislocation, after which a section of dorsal flank skin that had been previously shaved was removed and placed directly in organ culture medium. All procedures were approved by the University of Michigan Committee on Use and Care of Animals.

Organ culture procedures and assessment. Tissue for organ culture was cut into pieces ~1.5–2.0 mm on a side and incubated in wells of a 24-well dish. Each well contained 0.5 ml of culture medium. The culture medium consisted of Dulbecco’s modified minimal essential medium (DMEM) containing 200 μg/ml BSA. RA (Sigma Chemical Co., St. Louis, MO) was added at a concentration of 3 μM to the culture medium of half of the wells. Cultures were incubated at 37°C in an atmosphere containing 5% CO2 and 95% air. Fresh culture medium and RA were provided at 3-day intervals. Organ culture–conditioned medium was collected on days 3 and 6 and was used for assessment of MMPs as described below. At the end of the incubation period, tissue pieces were fixed in 10% buffered formalin and stained with hematoxylin and eosin. Histological features were assessed at the light microscopy level and compared with features seen at time 0. Organ culture technology has proved useful in the past for assessing RA effects on aged and photodamaged human skin (32).

Fibroblast isolation. Minced tissue fragments were placed in wells in a 24-well dish, and the dishes were inverted for 1 day to allow the tissue fragments to adhere. After this, the dishes were turned right side up and incubated for another 2 days without additional culture medium. After this, the tissue pieces were treated with DMEM containing 10% fetal bovine serum (FBS). Eventually, cells with a fibroblast-like morphology grew out from the tissue fragments. Although the initial growth of these cells was slow, once colonies were observed, it was possible to remove cells from the surface of the well with trypsin/EDTA and subculture them. In this way, we were able to obtain cultures of fibroblast-like cells (32).

Fibroblast proliferation. Proliferation was assessed by plating 4 × 104 fibroblasts from a 24-well dish in DMEM-FBS. After the cells had a chance to attach and spread, the growth medium was removed and the cells were washed two times in DMEM-BSA. Cells were incubated at 37°C for 2 days in DMEM-BSA with or without 3 μM RA. At the end of the incubation period, cells were harvested and counted.

Substrate-embedded enzymography. SDS-PAGE substrate-embedded enzymography (zymography) was used to assess MMP levels (33). Briefly, SDS-PAGE gels were prepared from 30:1 acrylamide/bis with the added incorporation of gelatin (1 mg/ml) before casting. The gels were routinely 5.75% acrylamide, with the final concentrations of the other components as follows: Tris-HCl at pH 8.8 (325 mmol/l), SDS (0.1%), ammonium persulfate (0.05%), and TEMED (0.05%). Denatured but nonreduced samples and standards were then electrophoresed into the gels at constant voltage of 150 V in an ice bath under nonreducing conditions. After electrophoresis, gels were removed and subjected to the following wash protocol: twice for 15 min in 50 mmol/l Tris buffer containing 1 mmol/l Ca2+ and 0.5 mmol/l Zn2+ with 2.5% Triton X-100 and once for 5 min in Tris buffer alone. The gels were then incubated overnight in Tris buffer with 1% Triton X-100 and stained with Coomassie Brilliant Blue R-250. After destaining, zones of gelatin hydrolysis were detected as regions of negative staining. Volumes of 5–35 μl of undiluted specimen were normally used for these assays. Zones of gelatinolytic activity were proportional to the quantity of culture fluid used.

Western blot analysis. Western blotting was done as described previously (34). Briefly, a mouse monoclonal antibody to rat MMP-13 was obtained from Calbiochem (San Diego, CA). Equivalent amounts of conditioned medium (15 μl) were resolved by SDS-PAGE (12%) and transferred to nitrocellulose membranes by electroblotting using a BioRad mini transfer blotting apparatus.

Membranes were then blocked in Ca2+/Mg2+-free Tris-buffered saline (TBS) containing 5% Blotto. Membranes were then treated with the monoclonal antibody (1:300 dilution) in TBS containing 0.5% Blotto and 0.1% Tween for 1 h. After several rinses in TBS with 0.1% Tween, the membrane-bound antibody was reacted with horseradish peroxidase–conjugated goat anti-mouse antibody at 1:2,000 dilution for 1 h. Protein-antibody complexes were detected by enhanced chemiluminescence (Cell Signaling) and visualized on light-sensitive autoradiographic film (Amerham Biotechnology, London, U.K.).

Glycosaminoglycan production. Skin from diabetic rats was incubated for 3 days in organ cultures in the presence or absence of 3 μmol/l RA. At the end of the incubation period, tissue was homogenized and treated for 24 h with pronase (100 mg per five 2-mm biopsies) at 37°C. At the end of the digestion, the homogenate was pelleted by centrifugation. The supernatant fluid was assayed for sulfated glycosaminoglycans using the Blyscan assay (Biodye Science, Newtownabbey, N. Ireland). The assay measures the binding of the cationic dye 1,9-dimethylmethylene blue to sulfated carbohydrates on proteoglycans and free sulfated glycosaminoglycans. Precipitation of the bound dye is followed by resolubilization and spectrophotometric measurement (35). In parallel, tissue sections from each group were stained by the periodic acid Schiff (PAS) method. In PAS-stained sections, carbohydrate-rich areas are stained a deep crimson.

RESULTS

Plasma glucose values and body weight of control and STZ-D rats. Body weights were similar in rats of all experimental groups at baseline. Six weeks thereafter, body weights were 12% lower in STZ-D rats versus nondiabetic control rats (P = 0.07). Plasma glucose values were significantly elevated in STZ-D rats (377 ± 9 mg/dl) compared with nondiabetic controls (80 ± 5 mg/dl; P < 0.01).

Histological appearance of normal rat skin and diabetic rat skin. Dorsal flank skin was removed from 4 control rats and 17 diabetic rats 6 weeks after STZ treatment. Examination of hematoxylin and eosin–stained sections from normal and diabetic skin revealed that whereas the epidermis was comparable in both normal and diabetic rat skin, dermal features were consistently different. Specifically, diabetic rat skin demonstrated features of atrophy, including reduced dermal thickness, disorganization of connective tissue fiber bundles, and increased space between collagen fiber bundles (Fig. 1C and D). In contrast, thick bundles of connective tissue were observed in the dermis of normal rat skin (Fig. 1A and B). There were also cytological differences between fibroblasts in normal and diabetic skin. In normal skin, cells had a bipolar, spindle-shape appearance in the thick collagen bundles (evident at the higher magnification; Fig. 1B). In contrast, interstitial cells had a rounded, shrunken, crenated appearance in the diabetic skin (Fig. 1D). These differences in histological and cytological appearance of normal and diabetic rat skin are consistent with what has been reported previously (6,7,21).

Figure 2 demonstrates histological features of diabetic rat skin after 6 days in organ culture in the absence or presence of 3 μmol/l RA. Normal skin maintained for the
same period of time is presented for control. Normal skin maintained in organ culture for 6 days had the same general appearance as freshly biopsied skin (Fig. 2A). As expected, diabetic skin maintained in the absence of RA expressed the same atrophic features as seen in freshly biopsied skin from the same animals. The epidermis was actually thinner after 6 days in organ culture than it was at time 0, and a number of pyknotic cells were visible. The dermis showed the same “wispy” thin connective tissue fiber bundles as seen in freshly biopsied skin (Fig. 2B). In contrast, incubation for 6 days in the presence of RA resulted in evidence of cellular reactivation in both epidermis and dermis. Increased epidermal thickening was evident (45 ± 15 μm in RA-treated diabetic skin vs. 22 ± 8 μm in untreated diabetic skin; n = 3; P < 0.05). Epidermal thickening may reflect, in part, increased glycosaminoglycan synthesis because when we assessed glycosaminoglycan amounts, levels were slightly higher in the RA-treated diabetic skin than in control diabetic skin (4.63 ± 0.91 vs. 4.24 ± 1.01 μg per biopsy, respectively; n = 3). However, increased keratinocyte proliferation must be the primary underlying factor for the increase in epidermal thickness. In the untreated diabetic skin, there was a single layer of thin, pyknotic keratinocytes. After RA treatment, two or more layers of healthy epidermal keratinocytes were evident. Normal stages of differentiation including a well-defined granular layer could be seen. Cells in both the epidermis and the dermis demonstrated cytological features associated with regeneration, i.e., lightly stained cytoplasm and large, oblong nuclei (Fig. 2C). The appearance of RA-treated diabetic skin was similar to that of normal skin after organ culture (compare Fig. 2A and C).

**MMP expression in normal and diabetic rat skin.** Normal and diabetic rat skin was maintained in organ culture for 3 days in the absence or presence of 3 μmol/l RA. At the end of the incubation period, organ culture-conditioned medium was collected from all four groups and analyzed for MMP activity by gelatin zymography. Figure 3 shows results from a typical experiment. When conditioned medium from diabetic rat skin was assessed, the enzyme profile was complex. The 72-kDa gelatinolytic enzyme (MMP-2, Gelatinase A) was represented by both active and latent forms. In addition, gelatinolytic bands at
92, 86, and 83 kDa were seen, consistent with the presence of latent and active MMP-9 (Gelatinase B) forms. Although there was no significant difference in expression of MMP-2 between control and RA-treated groups, the expression of MMP-9 forms was significantly reduced in the presence of RA (Fig. 3, lane 2). Finally, lower molecular weight gelatinolytic forms (at ~60 kDa and 40 kDa), consistent with the presence of MMP-13 (see below), were also observed in organ culture fluid from untreated diabetic skin. These were also sharply reduced in the presence of RA.

Conditioned medium from normal rat skin demonstrated a high level of gelatinolytic activity at 72 and 68 kDa (consistent with latent and active MMP-2). Organ cultures maintained in the absence or presence of RA (Fig. 3, lanes 3 and 4, respectively) exhibited comparable amounts of activity. In conditioned medium from normal skin samples, there was little activity in the 92-kDa region of the gel and essentially no activity in the lower molecular weight range.

In additional studies, organ culture fluid from normal and diabetic rat skin was incubated with 10 mmol/l EDTA in the overnight incubation buffer. In the presence of the divalent cation chelator, virtually 100% of all gelatinolytic activity was lost (not shown). The presence of EDTA-sensitive gelatinolytic activities at 72 and 92 kDa is diagnostic for MMP-2 and MMP-9, respectively. The identification of the lower molecular weight gelatinolytic forms is less certain. The major MMP with collagenase activity in rodents is MMP-13. This enzyme, which also has gelatinolytic activity, has a latent form at 60 kDa and lower molecular weight (40 kDa) active form (37,38). Western blotting was used to confirm the presence of MMP-13 in organ-cultured fluid. Western blotting demonstrated that immunoreactive MMP-13 was detectable in organ culture fluid from diabetic rat skin (60 kDa) but was barely detected (>95% reduction) in normal skin culture fluid (not shown).

**Effects of RA on incorporation of ³H-proline into TCA-precipitable material.** Organ cultures were established with skin from eight diabetic and normal rats and incubated for 3 days in the absence or presence of 3 μmol/l RA. At the end of the incubation period, conditioned medium was collected and analyzed by gelatin zymography. Lanes 1 and 3, DMEM-BSA; lanes 2 and 4, DMEM-BSA with 3 μmol/l RA. Findings shown here are representative of results from four diabetic and five normal rats.

In the overnight incubation buffer. In the presence of the divalent cation chelator, virtually 100% of all gelatinolytic activity was lost (not shown). The presence of EDTA-sensitive gelatinolytic activities at 72 and 92 kDa is diagnostic for MMP-2 and MMP-9, respectively. The identification of the lower molecular weight gelatinolytic forms is less certain. The major MMP with collagenase activity in rodents is MMP-13. This enzyme, which also has gelatinolytic activity, has a latent form at 60 kDa and lower molecular weight (40 kDa) active form (37,38). Western blotting was used to confirm the presence of MMP-13 in organ-cultured fluid. Western blotting demonstrated that immunoreactive MMP-13 was detectable in organ culture fluid from diabetic rat skin (60 kDa) but was barely detected (>95% reduction) in normal skin culture fluid (not shown).

**Discussion**

Topical retinoid treatment improves clinical features of human skin that has been damaged by the passage of time (chronological aging) (22) or by excessive exposure to solar ultraviolet irradiation (photoaging) (23,24). Improvement in clinical features is associated with cellular and molecular effects in both the epidermis and the dermis. Epidermal effects include stimulation of keratinocyte proliferation, leading to epidermal thickening. Induction of glycosaminoglycan synthesis also occurs (39), and this may contribute to thickening of the epidermis. In the dermis, there is activation of quiescent fibroblasts with a resultant increase in types I and III procollagen elaboration. A reduction in both age-associated and ultraviolet irradiation-induced MMP expression is seen in epidermis and dermis (25–30). These cellular and molecular effects are thought to underlie the improvement in clinical fea-
FIG. 5. Effects of RA on proliferation, MMP elaboration, and collagen synthesis by fibroblasts from diabetic rat skin. Fibroblasts were incubated for 3 days in DMEM-BSA in the absence or presence of 3 μmol/l RA. At the end of the incubation period, conditioned medium was harvested and assayed for MMP expression by gelatin zymography. Cells were harvested and counted. Additional cells were incubated with 1 μCi of 3H-proline 1 day before harvest. Incorporation of 3H-proline into TCA-precipitable material was assessed. A: Proliferation assay. Values shown are means and SD based on n = 6 samples. Statistical significance, assessed with the Student’s t test, indicated significance at the P < 0.05 level. B: MMP profile. Virtually all of the detected activity represents latent MMP-2. Similar levels were observed (on a per-cell basis) in the control and RA-treated cells. C: Incorporation of 3H-proline into TCA-precipitable material. Values shown are means and SD based on fibroblast cultures from nine diabetic rats.

Tissues that follow retinoid treatment. Retinoid-induced repair of skin damaged as a result of aging/photoaging follows from retinoid binding to specific nuclear receptors in target cells (40,41) and interference with signaling through mitogen-activated protein kinase pathways. Specifically, in the presence of RA, accumulation of c-jun protein is reduced (42). Because c-jun participates in formation of the AP-1 transcription factor complex, leading to induction of MMP (27,28) and inhibition of procollagen gene transcription (43), the net result is an increase in collagen synthesis and a decrease in collagen breakdown.

Diabetic skin manifests some of the same defects seen in aged skin. These include reduced growth capacity of the dermal fibroblast population (11–13) associated with abnormalities in collagen metabolism (6,7,21). On the basis of similarities with aged skin, we hypothesized that retinoid treatment may have therapeutic benefit in diabetic skin in a manner analogous to its effects in aging/photoaging. This study, therefore, explored cellular and biochemical changes in organ-cultured skin from 6-week STZ-D rats and the effects of RA on these changes. Organ culture technology has proved useful for assessing retinoid-induced changes in human skin (32,44), and the same approaches used in these past studies were used here. To summarize, STZ-D rat skin demonstrated significant defects in structure/function as compared with skin from nondiabetic control animals. These defects were (at least partially) amenable to treatment with RA.

The epidermis of the skin from diabetic rats was thinned relative to the control, and in the presence of RA, this was completely reversed. Increased glycosaminoglycan production may be responsible, in part, for increased epidermal thickness, but increased keratinocyte proliferation must play a major role. Whereas a single layer of mostly pyknotic keratinocytes characterized the epidermis of the control diabetic skin, there were two or more layers of healthy epidermal cells after RA treatment. Normal features of epidermal differentiation, including a prominent granular layer, were also seen. Thus, we attribute the thickened epidermis primarily to an RA-induced proliferative response in the keratinocyte population.

Retinoid treatment of diabetic rat skin also resulted in MMP suppression. In comparison with organ cultures of normal rat skin, which expressed high levels of a 72-kDa gelatinolytic activity (i.e., MMP-2) but little detectable activity at other molecular weights, diabetic skin expressed equivalent amounts of 72-kDa gelatinase but also high levels of gelatinolytic activity in the 92-kDa region (MMP-9) as well as a gelatinolytic activity at ~60 kDa. Both the 92-kDa and 60-kDa forms (but not the 72-kDa form) were suppressed by RA treatment. We hypothesize that elevated MMP expression in diabetic skin contributes to collagen breakdown (visible histologically) as well as to destruction of other components of the extracellular matrix.

Two issues related to these findings need to be addressed. The first is the role of individual enzymes present in the organ culture fluid. At least three different MMPs (MMP-2, MMP-9, and MMP-13) were detected in culture fluid from diabetic skin organ cultures. On the basis of the known specificities of these enzymes (45,46) as well as our recent findings from aged/photoaged skin (47,48), we propose that the collagenolytic enzyme (in this case, MMP-13) is primarily responsible for initial fragmentation of the intact collagen and that the gelatinolytic enzymes (especially MMP-9) further degrade the fragments generated by the collagenase. It is of interest in this regard that our recent studies have shown that type I procollagen synthesis is reduced in the presence of high molecular weight collagen fragments. When the collagen fragments are further degraded by MMPs with gelatinolytic activity, procollagen production remains high (48). Thus, the gelatinolytic enzymes may, in fact, play a beneficial role by removing fragments of collagen that inhibit new collagen formation. At the same time, however, MMP-2 and MMP-9 (as well as MMP-13) may promote tissue damage by degrading noncollagenous components of the extracellular matrix, including fibronectin, laminin, and elastin (45,46). Additional studies will be needed to elucidate fully how each individual enzyme contributes to the overall changes seen in control and retinoid-treated diabetic skin. Unfortunately, the lack of MMP inhibitors with sufficient specificity to distinguish among various MMPs makes this challenging.

Another issue is the cellular source of the enzymes elaborated in organ cultures of control and RA-treated diabetic rat skin. Past studies have demonstrated that both
epidermal keratinocytes and dermal fibroblasts are capable of synthesizing MMP-2, whereas keratinocytes are responsible for the majority of MMP-9 (49–51). Our findings with dermal fibroblasts in monolayer culture, i.e., high level of MMP-2 but no detectable MMP-9, are consistent with this. Alternatively, however, MMP-2 is constitutively expressed in most tissue (including skin), whereas MMP-9 is subject to regulation at the gene level. Perhaps the lack of detectable MMP-9 in isolated dermal fibroblasts reflects a lack of induction. The lack of detectable MMP-13 in monolayer cultures of dermal fibroblasts may also reflect a lack of the proper stimulus for induction. In human skin, factors elaborated in the epidermis, including heparin-binding epidermal growth factor and interleukin-1, serve as important inducers of MMPs in the surrounding stroma (52). Additional studies will need to be done to determine which cell types are responsible for which enzymes and which factors are responsible for expression and activation.

Diabetes is one of the major predisposing factors for the development of nonhealing skin wounds, particularly in the lower extremities (10). Although repeated episodes of ischemia/reperfusion injury are thought to be the proximal cause of most wounds in the feet of patients with diabetes, decreased vascular function and loss of peripheral sensory perception constitute underlying risk factors. The role that atrophic changes in the connective tissue play in sensitizing a tissue to chronic wound formation is not clear. It is known, however, that in other situations in which there is dermal atrophy—e.g., in aged/photoaged skin (25), after long-term treatment with corticosteroids (55)—sensitivity to wound formation increases. Thus, it would not be unreasonable to suggest that dermal atrophy, like chronic vascular insufficiency, increases the risk of chronic ulcer formation in response to repeated bouts of ischemia/reperfusion.

In a like manner, it would not be unreasonable to suggest that to the extent that dermal atrophy predisposes to chronic wound formation, prevention of atrophic features with topical retinoid treatment would be beneficial. This has not been established clinically. Given the data presented here, we believe that studies to determine whether topical treatment with RA leads to reduced wound formation or improved healing in this rat model of diabetes are warranted. If successful, one would predict similar efficacy in other situations in which dermal atrophy is present. Such studies are currently underway.

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