Diabetes mellitus is a metabolic disease that presents a significant public health issue, with its prevalence projected to rise to 439 million people by 2030 worldwide.1 Diabetes causes detrimental complications in multiple organs and is a major risk factor for periodontal disease and tooth loss.2–5 Nearly 20% of US adults over 65 years of age, an age group with the highest incidence of edentulism, are diagnosed with diabetes,6 indicating a need for prosthetic rehabilitation that involves implant therapy. Although it is established that implants have high success rates in medically healthy patients, the implication of implant therapy on patients with systemic diseases is less explored. Numerous studies have reported a higher complication rate for osseointegration in diabetic subjects,7–10 but the study designs are heterogeneous in subject recruitment and parameters measured, with reported success rates ranging from 85.5% to 100%.11 Given that there is a growing population that is diagnosed with diabetes, it is essential to understand the pathologic mechanism of diabetes on osseointegration to determine the ideal method of treatment for these underrepresented patients.

Diabetic Serum Inhibits Osteoblast Adhesion to Titanium Surface Through Advanced Glycation End Products: An In Vitro Study

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Purpose: Diabetes mellitus has been shown to delay osseointegration of titanium dental implants. This study tested the hypothesis that serum derived from diabetes negatively affects osteoblast adhesion to polystyrene and titanium surfaces, partly through the presence of advanced glycation end products (AGEs). Materials and Methods: Twenty-four Sprague-Dawley rats were divided into three groups: normoglycemic control, streptozotocin-induced diabetic group, and diabetic group treated with the AGE inhibitor aminoguanidine. Polystyrene or titanium disks were preincubated in serum derived from each group. Human osteoblasts transfected with green fluorescent protein (GFP) were cultured, and the number of adherent osteoblasts was quantified. High-pressure liquid chromatography (HPLC) was used to fractionate eluates, which were further characterized by western blot with AGE antibody and adhesion assays. In parallel, sera derived from healthy patients, patients with controlled diabetes, and patients with uncontrolled diabetes were utilized for osteoblast adhesion assay and western blot. Results: Diabetic serum significantly reduced the number of adherent osteoblast and osteoblast aggregates on titanium disks, whereas aminoguanidine-treated serum rescued the effect of diabetes on the number of adherent osteoblast aggregates. Fractionated diabetic serum revealed distinct AGE bands at ~100 kDa and 44 kDa, whereas healthy serum did not express any. In human serum samples, both controlled and uncontrolled diabetes led to a significant reduction in the number of adherent osteoblasts on polystyrene and titanium surfaces compared with normoglycemic serum. This correlated with presence of AGEs in western blot in diabetic but not in healthy serum. Conclusion: Osteoblast adhesion on the titanium surface was greatly reduced by the exposure of serum derived from diabetic rats or humans. Recovery of osteoblast aggregates by aminoguanidine treatment suggests that AGEs played a role in this negative effect. The correlating presence of AGEs from the fractionated sera of diabetic rats or humans and impaired osteoblast adhesion on the titanium surface further supports this role.

Keywords: bone healing, dental implant, diabetes mellitus, hyperglycemia, metabolism, osseointegration, osteoblast, streptozotocin, wound healing

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response supports a direct connection with alloplastic materials in a surgically created implant site.\textsuperscript{14} The initial stage of osseointegration is marked by formation of fibrin scaffold, capillary sprouting, and migration of stem cells and preosteoblasts.\textsuperscript{15} The implant surface is immersed in a host serum fibrin scaffold that guides the osteoblast lineage cells between the bone and titanium surface.\textsuperscript{16} The host serum also contains an array of cytokines and chemokines that promote the migration of osteoblast precursor cells to the titanium implant surface.\textsuperscript{14} The number and type of proteins such as fibronectin and osteopontin adsorbed at the titanium interface will determine whether osteoblasts will adhere, proliferate, and differentiate.\textsuperscript{14} Increased osteoblast adherence with the titanium interface directly impacts the extent of matrix mineralization that is critical during osseointegration.\textsuperscript{17,18}

Diabetes mellitus is associated with impaired bone healing around the titanium-bone interface in the early healing period of bone formation.\textsuperscript{19} This reduced bone formation is directly correlated to a suppression of osteoblast function, which does not exhibit preference with regard to bone type or location.\textsuperscript{20} In the marrow ablation model, reduced osteoblastic activity is associated with the presence of diabetes mellitus and a reduced mRNA expression of osteocalcin and type 1 collagen.\textsuperscript{21} Multiple animal studies have shown poor bone-implant healing with delayed osseointegration directly correlated to inadequate glycemic control.\textsuperscript{21–23} Marchand et al\textsuperscript{24} reported that the hyperglycemia-mediated inhibition of osteoblastic function and subsequent delay in osseointegration is in part due to its impact on angiogenesis. The notion of osteoblast suppression should account for these circumstances of bone loss, as they are directly involved in bone remodeling at the vicinity of the implant.\textsuperscript{20,24}

Diabetes-mediated hyperglycemia can induce changes in serum composition, including advanced glycation end product (AGE) formation. An exposure of the titanium surface to diabetic serum may therefore have a detrimental impact on its interaction with osteoblasts. Thus, in this study, in vitro experiments were carried out to test the hypothesis that diabetic serum alters the titanium surface to suppress osteoblast adhesion through AGES. Using sera derived from diabetic rats and humans, it is shown that osteoblast adhesion and spreading on titanium disks pretreated with diabetic sera were significantly impaired compared with the normoglycemic control group. This defect was associated with increased AGE formation in diabetic serum samples. Taken together, the data of the present study suggest that AGES modify the titanium surface to prevent proper osteoblast adhesion, which subsequently hampers osseointegration in diabetic patients.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with a protocol approved by the Harvard Medical School Committee on Animal Care. Twenty-four male Sprague-Dawley rats (6 weeks old, Charles Laboratories) were assigned to three groups: normoglycemic, diabetic, and aminoguanidine groups, n = 8 each. Low-dose streptozotocin (STZ) was administered to induce type 1 diabetes as previously described.\textsuperscript{19,25} Aminoguanidine (7.35 mmol/kg, Upjohn) was administered intraperitoneally for 28 days. Animals were sacrificed 4 weeks after induction of diabetes.

Serum Collection

Whole blood samples were collected via cardiac puncture in K\textsubscript{3}EDTA blood collection tubes (Vacutainer, Becton Dickinson). The sample was centrifuged at 2,000 rpm for 20 minutes, and serum was collected and stored at –80°C until use.

Cell Culture and Adhesion Assays

An immortalized line of human osteoblast cells (SAOS-2, clone HTB-85, ATCC) transfected with green fluorescent protein (GFP) construct was cultured in Dulbecco’s minimal essential media (DMEM) with 10% fetal calf serum and penicillin/streptomycin in a humidified chamber at 37°C and 4% CO\textsubscript{2}. A confluent layer of cells was trypsinized, and 1 × 10\textsuperscript{5} osteoblasts were seeded on 24- or 96-well plates for adhesion assays. For adhesion assays on titanium (Ti) surfaces, pure Ti disks (15 mm in diameter) with a plasma-sprayed surface on one side (“rough side”) (Institute Straumann) were placed in a 24-well plate and preincubated in 200 µL of serum for 72 hours under static condition, to closely mimic the in vivo condition where fibrin clot remains in close contact with the implant surface for at least 4 days.\textsuperscript{26} Serum was then aspirated out without touching the base. 1 × 10\textsuperscript{5} osteoblasts were seeded for 24 hours, and nonadherent cells were aspirated out.

Microscopic Analysis

Each culture well or Ti disk on a glass slide was placed under a photomicroscope (Nikon Eclipse, E800) equipped with a mercury lamp with blue light (λ = 360 to 400 µm). Five microscopic fields (20×) were taken, and adherent GFP+ osteoblasts as well as homotypic aggregates (> 10 cells) were quantified using ImageTool (UTHSCSA).

Serum Fractionation

Pooled sera from normoglycemic or diabetic animals were fractionated using liquid chromatography (Akta Explorer, Pharmacia), and their eluates were collected.
One hundred μL of fractionated eluates (fraction no. 18 to 34) were used to coat polystyrene culture wells (96-well plate) for 48 hours at 37°C and 4% CO₂. Adhesion assay was carried out for 24 hours. In a separate experiment, 200 μL of fractionated eluates were used to preincubate each Ti disk in a 24-well plate, followed by adhesion assay.

Western Blot
Electrophoresis was performed according to Laemmli protocol on 12% SDS-PAGE gels (BioRad) using fractionated eluates. The gel was blotted on nitrocellulose membrane (Amersham Life Sciences) on an electro-transfer apparatus (BioRad) for 1 hour at 10 V. Immunoblotting was performed using a monoclonal mouse anti-AGE antibody 6D12 (Dojindo Molecular Technologies) and a goat anti-mouse HRP antibody. The membrane was visualized with a chemiluminescence reaction and by exposure to radiographic film (Kodak).

Human Serum Collection and Assays
The collection and use of human serum samples was approved by the Committee on the Use of Human Subjects (CUHS) at Harvard School of Dental Medicine. The research protocol of the human study was presented to each patient in understandable terms in the form of a written consent letter. All patients signed and consented to the use of donated blood for the research. A total of 14 human subjects without a history of previous infectious disease were included; 5 were in the healthy group (HbA1c: 4.3% to 6.1%), 4 were in the controlled insulin-dependent diabetic group (HbA1c: 6.2% to 7.9%), and 5 were in the uncontrolled insulin-dependent diabetic group (HbA1c: ≥ 8%). Other reported medical conditions considered noncontributory for this study were: asthma (ML, NL, FH), Gilbert’s syndrome (ML), heart murmur (WM, NL, WL), cardiovascular disease (GW, FH, FP, WL, NL), blood transfusion (NL, GW, WM), diabetic neuropathy (JW), and thyroiditis (JW). Fasting blood samples were collected and processed as described earlier. Polystyrene and Ti disk preincubation was performed with eluted human sera using liquid chromatography. Each individual serum fraction characterized by distinct peaks on chromatography was used for subsequent osteoblast adhesion assays with a 6-hour incubation time on the polystyrene surface and 24-hour incubation time on the Ti disks.

Statistical Analysis
Data are presented as the mean ± SEM. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test was used to determine statistical difference at $P < .05$. Analysis was performed with the animal as a unit of measurement. The number of animals used was determined by power analysis with $\alpha = .05$, with an expected effect of 40% based on published results on streptozotocin-induced diabetes. All experiments were performed at least three times in triplicate. Data were analyzed on GraphPad software (SAS Institute).

RESULTS
A total of 24 Sprague-Dawley rats were evenly distributed to the normoglycemic, diabetic, and aminoguanidine-treatment groups. After 14 days post-streptozotocin injection, type 1 diabetic rats reached blood glucose levels of 373.2 mg/dL, maintaining high levels until day 42. Aminoguanidine-treated rats also maintained hyperglycemia from days 14 to 42, demonstrating that the drug did not have an impact on regulating glucose levels (Fig 1a). Whereas in the control normoglycemic group, the HbA1c level was 6.5% at the 42nd day, diabetic and aminoguanidine-treated rats had 20.2% and 18.2% HbA1c levels, respectively (Fig 1b). These data confirmed induction of diabetes and the lack of effect by aminoguanidine on glycemic control.

Following confirmation of diabetes induction, osteoblast adhesion assay was carried out on titanium disks that were pretreated with whole rat serum from different groups. Osteoblast count, as quantified by GFP+ expressing cells, demonstrated that titanium disks pretreated with diabetic serum had a significantly lower number of adherent osteoblasts compared with the normoglycemic control groups (Figs 1d and 1e). These data demonstrate that inhibition of AGEs renders diabetic serum more favorable to osteoblast adhesion compared to the control groups (Figs 1d and 1e). These data demonstrate that inhibition of AGEs renders diabetic serum more favorable to osteoblast adhesion, an important early healing process for successful osseointegration.

Next, the molecular component responsible for the reduction in osteoblast adhesion was investigated, and it was hypothesized that AGEs play an important role. Pooled whole serum from control or diabetic rats was fractionated using high-pressure liquid chromatography (HPLC), and elution fractions with distinct peaks were collected. Western blot was performed using anti-AGE antibody. In the normoglycemic control groups, there was little or no detection of glycated proteins in all fractions eluted, whereas the diabetic group showed a distinct AGE product at approximately 100 kDa and

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44 kDa band (Fig 2a). To test if this was associated with osteoblast adhesion, polystyrene culture wells were pretreated with fractionated serum, followed by osteoblast incubation. The normoglycemic group showed a varying number of adherent osteoblasts, with fraction no. 20, 23, 26, and 27 showing the highest number of adherent osteoblasts (Fig 2b). In contrast, culture wells treated with fractionated diabetic serum showed an overall suppression on osteoblast adhesion (Fig 2c). It is interesting to note that fraction no. 19 to 26, which had the highest AGE product signal at 44 kDa overall, demonstrated an even lower osteoblast adhesion count (Figs 2a and 2c). An analysis of pooled data showed that osteoblast adhesion was substantially improved when the polystyrene surface was pretreated with fractionated serum from the normoglycemic group, whereas that of the diabetic group significantly decreased it (Fig 2d).

To understand if fractionated serum influenced osteoblast adhesion properties on the titanium implant surface, the rough surface of the titanium disk was pretreated with fractionated sera, and osteoblast adhesion assays were performed (Fig 3a). Overall, the titanium disks preincubated in control serum fractions showed a higher number of adherent osteoblasts relative to that of diabetic serum fractions (Figs 3b and 3c). Pooled data showed a statistically significant decrease in osteoblast count in fractionated serum from diabetic animals (Fig 3d).

The above data implicate AGE products in diabetic serum as a factor responsible for impaired osteoblast adhesion. To translate the findings of the present study from the animal model to humans, serum was collected from healthy patients, patients with controlled diabetes, and patients with uncontrolled diabetes. The patient logistical data are listed in Table 1.
**Fig 2** (above and right) Molecular characterization of fractionated sera from normal and diabetic rats. (a) Western blot with anti-AGE antibody in fractionated eluates with distinct peaks. (b, c) Quantification of adherent osteoblast on polystyrene surface preincubated with (b) NG serum fractions and (c) diabetic serum fractions. (d) Quantification of total number of osteoblasts on polystyrene surface after 24-hour incubation. Experiments were performed three times in triplicate using fractionated samples from pooled whole serum; data represent mean ± SEM. *P < .05; one-way ANOVA.

**Fig 3** (below) Diabetic serum exposure predisposes Ti disk to inferior osteoblast adhesion. (a) Ti disk used for 24-well plates, rough side up for cell culture. (b, c) Quantification of adherent osteoblast numbers of (b) NG fractions and (c) diabetic fractions. (d) Quantification of total number of osteoblasts on titanium surface after 24-hour incubation. Experiments were performed three times in triplicate; data represent mean ± SEM. *P < .05; one-way ANOVA.
First, serum samples derived from healthy, controlled diabetes (CD) and uncontrolled diabetes (UD) patients were fractionated. Western blot against AGE products was performed in fractionated eluates. Healthy patients’ fractionated eluates did not express AGEs, whereas CD and UD patients’ sera expressed AGEs corresponding to approximately 50 and 60 kDa and 28, 50, 60, and 70 kDa, respectively (Fig 4a). To investigate a functional role of fractionated sera on osteoblast adhesion, polystyrene culture wells were pretreated with fractionated eluates, followed by osteoblast incubation and adhesion assay. Normoglycemic serum fractions generally promoted an increase in osteoblast adhesion (Fig 4b), whereas serum fractions from CD patients significantly impaired it (Fig 4c). Fractions from UD patients also had a suppressive effect on osteoblast adhesion to the polystyrene surface (Fig 4d) that closely mirrored CD. Further analysis demonstrated that serum fractions from healthy

Fig 4 Molecular characterization of fractionated sera from human subjects and effect on osteoblast adhesion to polystyrene surface. (a) Representative western blot images from serum fraction eluates from healthy (patient MC), controlled diabetes (patient GW), and uncontrolled diabetic patient (patient FP). (b to d) Quantification of adherent osteoblast on polystyrene surface preincubated with (b) healthy serum fraction, (c) controlled diabetic fraction, and (d) uncontrolled diabetic fraction. (e) Quantification of total number of osteoblasts on polystyrene surface after 6-hour incubation. n = 4 to 5; data show mean ± SEM. ***P < .001, one-way ANOVA.
patients promoted osteoblast adhesion compared with uncoated culture wells, whereas serum fractions from CD and UD patients significantly decreased it (Fig 4e).

Next, the experiments were performed on titanium disks that were pretreated with serum fractions from different patient groups. Similar to the findings from the polystyrene surface, osteoblast adhesion was generally improved when titanium disks were preincubated in serum fractions derived from healthy patients, whereas that of diabetic patients, whether in the CD or UD group, generally suppressed it (Figs 5a to 5c). Further analysis demonstrated that healthy serum fractions improved osteoblast adhesion on the titanium surface, whereas this was prevented when the disks were incubated in diabetic (both CD and UD) serum fractions (Fig 5d). Thus, diabetic serum has a detrimental impact on osteoblast adhesion, which is linked to the presence of serum AGEs.

**DISCUSSION**

The present study investigated the impact of diabetic serum exposure to the titanium surface on osteoblastic adhesion using samples derived from rat and human subjects. The results of the present study demonstrate that diabetic serum derived from rats or humans significantly reduced the number of adherent osteoblasts to titanium surfaces in in vitro models compared with healthy serum. The data showing that the diabetic serum condition did not exhibit less adherent osteoblast counts compared with the uncoated condition suggest that a detrimental impact of diabetes and a beneficial effect of serum pretreatment on cell adhesion may even out. Furthermore, they suggest that pretreatment with diabetic serum had a minimal cytotoxic effect on osteoblast viability. Western blot showed that AGEs were consistently detected in the fractionated diabetic serum eluates, which was closely associated with impaired osteoblast adhesion. HPLC was carried out to identify any specific eluates that exhibit potentiated suppression on osteoblast adhesion, but rather, the results suggest a global effect of diabetes on AGE expression and osteoblast attachment. It is interesting to note that there is a variable osteoblast response to each eluate, which could be investigated in the future for the specific content of protein composition that is either harmful or beneficial for osteoblast function on the Ti surface. Thus, these datasets suggest that the proper osteoblast adhesion to the titanium surface is dependent on the quality of serum that it is exposed to, and that AGEs in diabetic serum renders it unfavorable for normal osteoblast adhesion.

Aminoguanidine to prevent AGE formation effectively rescued the negative impact of diabetic serum on titanium disks for osteoblast adhesion. It is well established that diabetes in vivo suppresses bone formation under the homeostatic condition partly through

![Fig 5](image-url) Detrimental impact of serum exposure from diabetic human subjects on osteoblast adhesion to Ti-surface. (a to c) Quantification of adherent osteoblasts on titanium surface preincubated with (a) healthy serum fractions, (b) controlled diabetic fractions, and (c) uncontrolled diabetic fractions with distinct peaks. (d) Quantification of total number of osteoblasts on Ti-disk surface after 24-hour incubation. n = 4 to 5; data show mean ± SEM. **P < .01, ***P < .001, one-way ANOVA.
upregulation of AGEs.\textsuperscript{30–34} Correction of hyperglycemia and reversal of AGE formation through insulin has a positive effect on osteoblast function on titanium surfaces.\textsuperscript{35} Similarly, the mechanism of diabetic complications in nephropathy, vasculopathy, and retinopathy involves excessive AGE accumulation.\textsuperscript{36–38} AGE inhibition with aminoguanidine, for instance, prevented the negative effects of diabetic retinopathy in a rat STZ model.\textsuperscript{39} While these studies demonstrate a negative intracellular effect through AGE/RAGE signaling, the present study highlights the impact of AGE-rich diabetic serum on an alloplastic material such as titanium, which in turn negatively affects osteoblast adhesion.

The results of the present study demonstrated that the serum samples derived from patients with controlled and uncontrolled diabetes had a similar detrimental effect on osteoblast adhesion to the titanium surface compared with that of healthy patients. One shortcoming of the present study is that the sera derived from patients with controlled diabetes still expressed AGEs, and therefore, they could not be categorized as a rescue group to establish the direct role of AGEs. In some cases, though statistically insignificant, uncontrolled diabetic serum appeared to perform better than that of patients with controlled diabetes (Fig 4e), which could be due to other underlying systemic conditions in the controlled diabetic group that negatively affected the outcome. While the presence of AGEs in both diabetic groups is consistent with the adverse functional outcome, the result suggests that diabetic patients, even when hyperglycemia is controlled, may be at risk for altered implant osseointegration. This may be due to an epigenetic alteration that results from a prolonged exposure to uncontrolled hyperglycemia prior to medical intervention.\textsuperscript{40} The present study did not consider the length of the diabetic period for patients until appropriate hyperglycemic control was introduced, which could be tested in future experiments. Despite the challenges associated with the use of human serum samples, the present rat study demonstrated that AGEs played a direct and critical role in limiting osteoblast adhesion to the titanium surface. Collectively, the present study provides a rationale for potential implant surface modification in conjunction with adjunctive AGE inhibition to overcome this effect.

**CONCLUSIONS**

Titanium disks preincubated in diabetic serum were less effective in promoting osteoblast adhesion in both rat and human models. This negative effect was blocked when diabetic serum from rats treated with aminoguanidine was used. Molecular analysis of diabetic serum revealed multiple fractionated eluates with strong AGE expression. Thus, the authors conclude that diabetic serum predisposes the titanium surface to less favorable osteoblast adhesion through expression of AGEs, which may explain the delayed implant osseointegration in diabetic patients.

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