Collagen Type I Prevents Glyoxal-Induced Apoptosis in Osteoblastic Cells Cultured on Titanium Alloy

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Key Words

Collagen · Osteoblasts · Titanium alloys · Apoptosis · Glyoxal

Abstract

Advanced glycation end products (AGEs) irreversibly cross-link proteins with sugars and accumulate at a higher age and in diabetes, processes which can interfere with the integration of implants into the tissue. Glyoxal is a highly reactive glycating agent involved in the formation of AGEs and is known to induce apoptosis, as revealed by the upregulation of caspase-3 and fractin (caspase-3 being a key enzyme activated during the late stage of apoptosis and fractin being a caspase-cleaved actin fragment). In this study, we investigated the influence of collagen type I coating on the cytotoxic effect

of glyoxal on rat calvarial osteoblastic cells and on human osteosarcoma cells (Saos-2) grown on titanium alloy, Ti6Al4V. Activation of caspase-3 and fractin was measured by counting immunohistochemically stained cells and by flow cytometry with propidium iodide (detection of the apoptosis indicating a sub-G₁ peak). Our results showed an increased number of apoptotic osteoblasts after incubation with glyoxal on Ti6Al4V discs. However, the number of apoptotic cells on collagen-coated titanium was significantly smaller than on uncoated titanium after the same treatment. The present findings demonstrate that osteoblasts treated with glyoxal undergo apoptosis, whereas collagen type I coating of titanium alloys (used for implants) has an anti-apoptotic function.

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Abbreviations used in this paper

AFM	atomic force microscope
AGEs	Advanced glycation end products
BL	uncoated (blank)
CML	ϵ -N-(carboxymethyl)lysine
FC	fibrillar collagen
FITC	fluorescein isothiocyanate
GO	glyoxal
PBS	phosphate-buffered saline
SEM	scanning electron microscope
Ti6Al4V	titanium alloy

Introduction

The ability of an implant to integrate into the bone is a prerequisite for clinical success [Cooper, 1998; Lohmann et al., 1999]. Implant surface coating, roughness and chemistry influence the adhesion, proliferation, and differentiation of osteoblastic cells on implants [Martin et al., 1995; Degasne et al., 1999].

Collagen type I is one of the major extracellular matrix proteins that can be found in fibrous tissue and bone. Pre-coating titanium alloy (Ti6Al4V) with collagen type I promotes adhesion and spreading of rat calvarial osteoblasts and leads to specific cell-implant interactions, which was

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shown by our groups [Geissler et al., 2000; Röhlecke et al., 2001]. We have also shown that collagen contributes to cell spreading and a more rapid formation of focal adhesions [Röhlecke et al., 2001; Becker et al., 2002]. In general, the adhesion of cells to the extracellular matrix and cell-cell interactions have been observed to suppress apoptosis in many cell types, including osteoblasts [Boudreau et al., 1995; Krestow et al., 1999; Hunter et al., 2001].

According to these findings we hypothesized that collagen coating of titanium alloys is able to reduce apoptosis of osteoblasts due to changes in interactions between cells and extracellular matrix. To address this question, we exposed cultivated osteoblast-like cells with glyoxal, a known apoptosis-inducing agent which is involved in the formation of advanced glycation end products (AGEs) [Thornalley and Stern, 1984; Thornalley, 1999].

AGEs are heterogeneous stable end-stage substances formed by the nonenzymatic glycation of proteins, nucleotides and phospholipids. The accumulation of AGEs in vivo has been linked to normal aging and occurs at an accelerated rate in the development of diabetes complications and neurodegenerative diseases [Lyons et al., 1991; McCance et al., 1993]. Diabetes in both humans and rats is accompanied by impaired bone formation [Bouillon et al., 1995; Krakauer et al., 1995; Katayama et al., 1996; Takeshita et al., 1997].

Early glycation involves the reaction of glucose with N-terminal and lysyl side chain amino groups to form Schiff's base and fructosamine adducts. Glucose (50 mM) degrades slowly at pH 7.4 and 37°C to form glyoxal ($59.5 \pm 3.7 \mu\text{M}$), methylglyoxal ($2.77 \pm 1.22 \mu\text{M}$) and 3-deoxyglucosone ($7.46 \pm 1.48 \mu\text{M}$). The concentrations of these α -oxoaldehydes increased rapidly in an in vitro assay over the initial 2–5 days [Thornalley et al., 1999]. In cells, transmembrane surface receptors for AGE include galectin-3, and the macrophage scavenger receptors p60 and p90 [Thornalley, 1998].

There is evidence that ϵ -N-(carboxymethyl)lysine (CML) modifications of proteins are the predominant AGEs that accumulate in vivo [Berg et al., 1998; Kislinger et al., 1999; Wagner et al., 2001]. CML (which we use in this paper as immunostaining marker for 'aged' proteins) is a chemically defined AGE formed on proteins by glycation and lipid peroxidation as well as by auto-oxidation of lipids, amino acids and carbohydrates [Miyata and Kurokawa, 1999]. In a previous study we could show that CML, bax and active caspase-3 as well as apoptotic events increased after incubation with glyoxal [Reber et al., 2003].

In the present study, we examined the cytotoxic effect of glyoxal on osteoblasts in general, and then tested if collagen coating of titanium alloy reduced the cytotoxic effect of glyoxal.

Apoptotic cells were detected by flow cytometry (propidium iodide showing the apoptosis indicating a sub-G₁ peak) and immunohistochemically using antibodies against caspase-3, a key enzyme during apoptosis, and against fractin, an actin fragment generated by caspase-3 cleavage [Rossiter et al., 2000; Reber et al., 2002] and a valuable marker of apoptosis in various tissues [Pulera et al., 1998; Rossiter et al., 2000; Elibol et al., 2001].

Actin cleavage by caspase-3, in particular, results in the breakdown of the cytoskeleton membrane blebbing, and the release of DNase I, which enters the nucleus and induces DNA fragmentation [Kayalar et al., 1996]. The anti-fractin antibody was raised against the C-terminus 32-kD actin fragment, which is formed by the caspase-3-mediated cleavage at 244 aspartate residue [Yang et al., 1998]. It is specific for the 32-kD fragment, but does not recognize the intact actin.

For clinical relevance we found it important to include both animal primary cultures (rat calvarial osteoblasts) and human cells (osteosarcoma cell line, Saos-2). Cell lines behave sometimes slightly differently compared to fresh primary cultures. On the other hand, it is important to also include human cells which allow a better comparison with the in vivo situation in patients.

Materials and Methods

Preparation of Modified Surfaces

As substrates for the cell experiments, the following surface states were used: Ti6Al4V coated with collagen type I (fibrillar collagen, FC) and Ti6Al4V without coating [uncoated (blank), BL] prepared in collaboration with Dr. S. Bierbaum at the Department of Biomaterials at the TU Dresden.

Experiments were performed on glass coverslips with a diameter of 16 mm (2 cm²) magnetron-sputtered with 50 nm Ti6Al4V alloy. Scanning electron microscope (SEM) and atomic force microscope (AFM) images of these metallic layers showed a cluster structure in the nanometer region. The sputtered Ti6Al4V substrates were cleaned with 1% Triton X-100, acetone and 96% ethanol, rinsed with distilled water, and air dried. The Ti6Al4V discs were coated with FC with an improved adsorption method [Rössler et al., 1999, 2000].

For collagen coatings, collagen fibrils were prepared by mixing in an equal amount of a fibrillogenesis buffer with 1 mg/ml solution of acid-soluble calf skin collagen type I (Fluka, Deisenhofen, Germany), which had been dissolved overnight in 10 mM acetic acid and then centrifuged 10,000 g for 30 min at 4°C. Following overnight incubation at 37°C, the resulting gel was homogenized, fibrils were collected by centrifugation at 5,000 g for 15 min, washed with fibrillogenesis buffer diluted to a working concentration, and centrifuged

Table 1. List of antibodies used for immunohistochemistry

Antibody	Specificity	Species	Dilution	Source
Caspase-3	active caspase-3	rabbit, polyclonal	1:20	PharMingen Heidelberg, Germany
CML	CML	rabbit, polyclonal	1:1,000	Dr. Schleicher University of Tübingen, Germany
Fractin	fractin	rabbit, polyclonal	1:1,000	PharMingen Heidelberg, Germany

again. The pellet was resuspended in the same buffer to a concentration of ~ 1 mg/ml collagen. The titanium-coated discs were incubated in suspension with collagen solution at 25°C for 15 min, washed with distilled water, and air dried. For cell culture experiments, coated (FC) and uncoated (BL) discs were sterilized with 100% ethylene oxide at 42°C for 12 h. Morphological investigations were carried out with a digital SEM (DSM 982 Gemini; Carl Zeiss, Oberkochen, Germany) and an AFM (NanoScope IIIa; Digital Instruments, Santa Barbara, Calif., USA). There was no measurable detachment of collagen after exposure of the coated titanium alloy to the cell culture medium for several weeks [Lowenberg et al., 1988; Rössler et al., 1999; Geissler et al., 2000; Rössler et al., 2000]. Furthermore, members of our groups demonstrated on collagen type I-coated titanium alloys in rat tibiae that no abrasion of collagen type I from the implants occurred during 6–8 weeks of implantation time [Rammelt et al., 2002a, b].

Cell Cultures

The human osteoblast-like cell line, Saos-2 cells, obtained from American Tissue Culture Collection (Manassas, Va., USA), and a p53-negative cell line, were grown at 37°C in 5% CO₂ in McCoy's 5A medium supplemented with 15% fetal calf serum and 10 mg/ml gentamycin.

Primary osteoblasts were obtained from fetal rat calvarial samples by collagenase digestion. The bone samples were dissected to approximately 1-mm³ fragments after removal of the periosteum. They were washed first with Tyrode's solution and then with Ham's F12 medium. They were then digested in 4 ml of collagenase-trypsin solution [137 mg of collagenase type I (Biochrom, Berlin, Germany) and 50 mg of trypsin type III (Sigma, Deisenhofen, Germany) in 10 ml of deionized H₂O containing 8 g of NaCl, 0.2 g of KCl and 0.05 g of NaH₂PO₄·H₂O per 100 ml of deionized H₂O] at room temperature. Cells from the first 45-min digestion were discarded. The second and third 30-min digests (in 4 ml of fresh collagenase-trypsin solution) were centrifuged for 2 min at 600 g. The cell pellets were resuspended in 4 ml Ham's F12 medium containing 20% fetal calf serum. After 10 min of centrifugation at 500 g, the cells were plated in full medium, i.e. 12 ml of Ham's F12 medium containing 12% fetal calf serum, 2.3 mM Mg²⁺, 100 IU/ml penicillin and 100 µg/ml streptomycin sulfate. The medium was changed every 3 days. The osteoblast phenotype was characterized by determining alkaline phosphatase activity, collagen type I synthesis and formation of calcium phosphate deposits. The cells for each experiments were used for 5th and 6th passages. The cultures were maintained at 37°C in humidified air and 5% CO₂. Cell viability was assessed by trypan blue dye exclusion.

Application of Cells to Implants and Exposure to Glyoxal

The titanium-coated discs were placed into 12-well Falcon culture plates (Becton Dickinson, Heidelberg, Germany). Primary osteoblasts and Saos-2 cells were detached using 0.05% trypsin-0.02% EDTA in phosphate-buffered saline (PBS) and plated on the discs to a single-cell density of 10,000 cells/cm². Cells were allowed to settle for 2 h in the incubator at 37°C in PBS, which was then replaced with 2 ml complete medium and nonadherent cells were gently removed. After 24 h in culture, primary osteoblasts were treated for 24 or 72 h with 0 µM (control), 100, 200, 400 and 600 µM glyoxal (40% aqueous solution; Sigma-Aldrich) while Saos-2 cells were treated for the same time with 0 (control), 50, 100, 200 and 300 µM glyoxal. For the sub-G₁ test (see Flow Cytometry below), the Saos-2 cells were treated for 24 or 72 h with 200, 300 and 400 µM glyoxal. Cell viability prior to incubation with glyoxal was assessed by trypan blue dye exclusion.

Immunocytochemistry and Quantitative Microscopy

For immunocytochemistry, the medium was removed and cells were washed 3 times with PBS. Cells were then fixed with 4% buffered paraformaldehyde for 5 min at room temperature. After washing with PBS, cells were permeabilized with 0.5% Triton X-100 in PBS for 6 min and then incubated with 1% bovine serum albumin for 10 min. Cells were incubated overnight at 4°C with polyclonal, antisera against caspase-3 (active caspase-3, rabbit, polyclonal; 1:20; PharMingen, Heidelberg, Germany), fractin (rabbit, polyclonal, 1:1,000; Dr. Schleicher, University of Tübingen) or CML (rabbit, polyclonal, 1:1,000; PharMingen) (table 1). After washing, the cells were exposed to a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:100; Dianova, Hamburg, Germany) for 1 h at room temperature. After rinsing 3 times with PBS, bisbenzimid (Hoechst; 1:1,000) was added for 15 min. Cellular fluorescence was evaluated with a conventional fluorescence microscope (Leica, Bensheim, Germany). The FITC wavelengths for excitation average at 490 nm and emission at 525 nm, bisbenzimid wavelengths for excitation at 365 nm and emission at 480 nm. Pictures were captured with a Nikon Coolpix 995.

After staining with bisbenzimid, apoptotic cells exhibited in blue condensed nuclei and fragmented chromatin. Quantification of apoptotic cells in each culture condition was performed by counting the number of cells with fragmented chromatin within six random fields of sight (0.5985 mm²). Experiments were independently repeated 9 times. Data are presented as mean $\pm$ SD. Differences between Ti6Al4V collagen type I (FC) and Ti6Al4V uncoated (BL) were evaluated with the paired Student t test. The Student t test is a parametric test for the comparison of the average values of two inde-

Fig. 1. Percentage of apoptotic cells (with fragmented chromatin, staining with bisbenzimid) of calvarial (**A, B**) and Saos-2 (**C, D**) cells after treatment for 24 h (**A, C**) and 72 h (**B, D**) with different glyoxal (GO) concentrations. Cells were cultured on BL and FC-coated titanium alloy. The histogram shows the influence of FC on the antiapoptotic behavior of calvarial cells and Saos-2 cells after incubation of cells with various GO concentrations. Representative data from nine experiments are shown. * $p < 0.05$; ** $p < 0.01$ FC vs. BL.

pendent samples out of the normally distributed universe test statistic t . Calculations were performed using the statistical package SPSS 11.0 (SPSS, Professional Statistics, Chicago, Ill., USA) and significance was accepted at * $p < 0.05$; ** $p < 0.01$ FC vs. BL.

Flow Cytometry

Apoptosis of cells plated on Ti6Al4V-coated discs with (FC) or without (BL) collagen type I was assessed by flow cytometry (sub- G_1) as described [Nicoletti et al., 1991] after treatment with 300 μM of glyoxal. Control cells were grown on the same plates, but were not exposed to glyoxal. Briefly, cells were fixed by adding 70% ethanol. Fragmented DNA was extracted with a buffer containing 0.192 M Na_2HPO_4 and 4 mM citric acid, pH 7.8. Staining was performed with 20 $\mu g/ml$ propidium iodide and 200 $\mu g/ml$ RNase A (Becton Dickinson) in PBS, pH 7.4 for 30 min at room temperature. Cells were analyzed using a FACSCalibur (Becton Dickinson) [Darzynkiewicz et al., 1992]. Apoptotic cells were revealed by a hypodiploid DNA peak with a weaker fluorescence than the normal G_1 peak [Gong et al., 1994].

Results

Glyoxal-Induced Apoptosis in Rat Calvarial Cells and Saos-2 Osteoblastic Cells

Figure 1 shows the percentage of calvarial (fig. 1A, B) and Saos-2 (fig. 1C, D) apoptotic cells after treatment with different glyoxal concentrations for 24 h (fig. 1A, C) and 72 h (fig. 1B, D) by counting the bisbenzimid-stained fragmented and condensed nuclei.

In calvarial cells, glyoxal for 24 h induced apoptosis in a dose-dependent manner: 100, 200, 400 and 600 μM

glyoxal induced apoptosis in 55, 67, 70 and 85% of the cells, respectively (fig. 1A). After 72 h incubation with glyoxal at the indicated concentrations, the apoptotic cell rate was further increased to 70, 85, 90 and 92% (fig. 1B).

A similar dose- and time-response to glyoxal was observed in Saos-2 cells (fig. 1C, D). Treatment over a period of 24 h with 50, 100, 200 and 300 μM glyoxal induced 5, 12, 32 and 50% apoptosis of Saos-2 cells, respectively (fig. 1C). After 72 h glyoxal treatment, the corresponding apoptosis rates increased to 9, 15, 64 and 85% (fig. 1D). Higher concentrations or longer incubation with glyoxal induced 100% cell death. In general, Saos-2 cells responded to lower glyoxal concentrations than calvarial cells.

Furthermore, fewer calvarial cells and even fewer Saos-2 cells underwent apoptosis on collagen-coated titanium discs (FC) than on uncoated titanium discs (BL) (compare fig. 1A and B with fig. 1C and D). Thus, the collagen coating had a protective effect, with a decreased apoptotic rate in Saos-2 cells (10–30% less) compared to uncoated surfaces. In addition, these results revealed that even a small concentration of glyoxal is sufficient to induce apoptosis of human cells.

The sub- G_1 tests by flow cytometry corroborated our microscopical observations. Higher concentrations and longer incubation with glyoxal resulted in increased apoptotic rates (fig. 2). Moreover, the number of apoptotic cells on collagen-coated titanium was much smaller than on uncoated titanium. For instance, after treatment with

300 μ M glyoxal for 24 h, propidium iodide fluorescence was much higher in Saos-2 cells grown on uncoated than on collagen-coated discs. Data are shown in figure 2.

Glyoxal-Induced Apoptosis: Upregulation of Caspase-3, Fractin and CML

Double immunofluorescence (bisbenzimid and caspase-3 or fractin) demonstrated that treatment with glyoxal caused the activation of caspase-3 (fig. 3) and fractin (fig. 4) in calvarial and Saos-2 cells, as expected in apoptotic cells.

In the absence of glyoxal, cell cultures grown on collagen-coated or uncoated titanium discs included fewer apoptotic cells, as revealed by staining for activated caspase-3 and fractin (data not shown). Double immunofluorescence showed that fractin-positive cells were identical to those undergoing nuclear fragmentation (fig. 4).

Fig. 2. Apoptotic Saos-2 cells after treatment for 24 h with 300 μ M glyoxal (GO) detected by sub- G_1 test. --- = Untreated control. BL (—) and FC (.....) represent treated cells either 300 μ M glyoxal. The sub- G_1 test shows the influence of FC on the antiapoptotic behavior of Saos-2 cells after incubation of cells with 300 μ M GO.

The experiment revealed the lowest apoptotic rate in controls (the controls grew only on culture plates without glyoxal), followed by those on FC-coated titanium alloy. The highest apoptotic cell rate we determined was on BL titanium alloy.

Fig. 3. Apoptotic calvarial cells after 24 h treatment with 200 μ M glyoxal (GO) cultured on BL (**A, B**) and FC (**C, D**) titanium alloy detected by reactions with bisbenzimid (**A, C**) combined with anti-caspase-3 immunocytochemistry (**B, D**). Identical cells are shown in **A + B** and **C + D**. The arrows marked identical cells. The immunocytochemistry demonstrates that only apoptotic cells (**A, C**) produced anti-caspase-3 (**B, D**). Double immunofluorescence showed that more apoptotic cells were present on BL (**A, B**) than on FC (**C, D**). Anti-caspase-3 expression was quantitatively decreased on FC correlating with a lower number of apoptotic cells. Scale bar for **A–D** is 10 μ m.

Fig. 4. Apoptotic calvarial cells after 24 h treatment with 200 μ M glyoxal (GO) cultured on BL (**A, B**) and FC (**C, D**) titanium alloy detected by reactions with bisbenzimid (**A, C**) combined with anti-fractin immunocytochemistry (**B, D**). Identical cells are shown in **A + B** and **C + D**. The arrows marked identical cells. The immunocytochemistry demonstrates that only apoptotic cells (**A, C**) produced anti-fractin (**B, D**). Double immunofluorescence showed that more apoptotic cells were present on BL (**A, B**) than on FC (**C, D**), where anti-fractin expression was quantitatively decreased on FC correlating with a lower number of apoptotic cells. Scale bar for **A-D** is 10 μ m.

Fig. 5. Apoptotic calvarial cells after 24 h treatment with 200 μ M glyoxal (GO) cultured on BL (**A, B**) and FC (**C, D**) titanium alloy detected by reactions with bisbenzimid (**A, C**) combined with anti-CML immunocytochemistry (**B, D**). Identical cells are shown in **A + B** and **C + D**. The arrows marked identical cells. The immunocytochemistry demonstrates that only apoptotic cells (**A, C**) produced anti-CML (**B, D**) expressions. Double immunofluorescence showed that more apoptotic cells were present on BL (**A, B**) than on FC (**C, D**), where anti-CML was quantitatively decreased on FC correlating with a lower number of apoptotic cells. Scale bar for **A-D** is 10 μ m.

The effect of glyoxal on the accumulation of the AGE product CML in apoptotic cells was also assessed by double immunocytochemistry (fig. 5). These double immunofluorescence assays showed that CML-positive cells were identical to those undergoing nuclear fragmentation. Again, the number of these cells (Saos-2) was higher on uncoated titanium discs than on collagen type I-coated discs.

Discussion

An implant material attains and maintains contacts with interfacial tissue through its surface. Since reactions *in vivo* are governed by molecular mediators, it is conceivable that these mediators play a role in the process leading to implant integration or rejection. When a substrate or an implant is inserted into the living tissue, it is exposed to cells and a large variety of ionic and molecular species that ultimately affect the course of the events at the interface between the tissue and the implant.

A very early event in the implantation process is the adsorption of proteins onto the substrate. Collagen type I is the predominant matrix protein in the bone tissue and plays an important role in bone cell-matrix interactions. The initial adhesion and differentiation of osteoblasts are improved by coating titanium alloy with collagen type I [Geissler et al., 2000; Röhlecke et al., 2001; Becker et al., 2002]. The adhesion of cells to the extracellular matrix and intercellular contact have been observed to suppress apoptosis in many cell types. For example, glyoxal treatment in epithelial lung L132 cells induced apoptosis as a consequence of the loss of cell contacts and simultaneous downregulation of cell adhesion molecules, such as β_1 integrin, CD44 and ICAM-1 [Röhlecke et al., 2000]. The collagen framework is also important for the survival of cells shown in human cartilage [Kim et al., 2001]. Correspondingly, collagenase treatment leads to an increased apoptosis.

Both p53 and pRb, the latter being a product of the retinoblastoma gene, are implicated in certain pathways of apoptosis. Saos-2 cells are defective in the expression of both p53 and pRb. Nevertheless, our findings indicate that glyoxal can induce apoptosis of these p53/pRb-negative cells. In addition, we found that caspase activation is involved in glyoxal-mediated apoptosis, as caspase-3 activity and fractin expression were increased in glyoxal-treated, bisbenzimid-positive cells.

In diabetic rats and humans, marked abnormalities in bone metabolism have been described, characterized by a decreased number of osteoblasts, decreased bone formation and slow turnover, all of which contribute to osteoporosis [Vaeghe et al., 1990; Bouillon et al., 1995]. Treatment with glyoxal promotes the formation of CML residue on proteins, which can mimic diabetes-induced damage [Berg et al., 1998; Rico et al., 1998]. Our present results prove that glyoxal, indeed, induces the formation of the AGE product CML and CML accumulation is concomitant with a markedly increased apoptosis.

The present findings show (as important findings for implant modification) that the coating of titanium alloy with collagen type I exerts a protecting, antiapoptotic effect on osteoblasts. The mechanisms behind this effect are still unclear, but one could propose that collagen exerts a scavenging action on radicals generated by AGEs [Loske et al., 1998] or that it directly cross-links with AGEs, as previously suggested [Mentink et al., 2002]. Thus, implants coated with collagen could have beneficial effects especially in patients with longer implant duration, advanced age and diabetes.

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