Combined effect of titanium particles and TNF-α on the production of IL-6 by osteoblast-like cells

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Abstract: To clarify the role of tumor necrosis factor (TNF)-α on osteoblast functions in the presence of metal particles, two human osteoblast-like cell lines (MG-63 and SaOS-2) were cultured with TNF-α in the presence or absence of titanium particles *in vitro.* A combination of TNF-α and titanium particles showed additive effects on inhibition of cell proliferation and alkaline phosphatase production. On the other hand, production of interleukin-6, which is well known to induce osteoclastogenesis and to directly stimulate bone resorption, was additively stimulated by the combination of TNF-α and titanium particles. These results suggest that the association of TNF-α and titanium particles may play an important role in the pathogenesis of periprosthetic osteolysis through two different pathways: a reduced periprosthetic bone formation due to inhibition of osteoblast proliferation and alkaline phosphatase production, and osteoblast-mediated activation of osteoclastic bone resorption as suggested by the enhancement of interleukin-6 production. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res, 52, 382–387, 2000.

Key words: TNF-α; Ti particles; osteoblast; IL-6; proliferation

INTRODUCTION

Periprosthetic osteolysis and implant loosening after joint arthroplasty in both cemented and cementless prostheses remain major causes of implant failure. One cause of implant loosening is the proliferation of a fibrous tissue surrounding the implant in which macrophages phagocytosed wear debris.[1,2] Moreover, the interaction of particle wear debris with phagocytic cells results in the activation of cells which in turn produce mediators that provoke a cascade of osteolytic events.[3–9] Among the osteolytic modulators, tumor necrosis factor (TNF)-α stimulates the osteoblasts to produce bone resorptive mediators, interleukin (IL)-6 and prostaglandin E_2 (PGE_2).[7,10]

The fibrous tissue also provides an excess of wear particles in the periprosthetic space.[11] Direct interactions between particles and bone cells are then possible. This fact is important because it has been shown that particles could influence the functions of osteoblasts. After osteoblast-like cells (MC3T3-E1) and rat osteosarcoma cells (17/2.8) phagocytosed particles of hydroxyapatite, reduced cell growth and alkaline phosphatase activity were measured.[12] Biosynthesis of both type-I and type-III collagen was decreased in the human osteoblast-like cell (MG-63) and human osteosarcoma cells, that had been treated with titanium (Ti) particles.[13] Poly(methyl methacrylate) (PMMA) particles were also shown to inhibit osteoblast proliferation and collagen synthesis, whereas osteocalcin and IL-6 production were stimulated.[14]

Despite that the presence of wear particles in conjunction with cytokines seems a common trend in periprosthetic tissue, few data reflecting this situation are available for osteoblasts. We hypothesized that cytokines released by macrophages may alter the functional modulation of osteoblastic cells caused by wear particles. We then investigated the effect of TNF-α, a representative cytokine released by macrophages, on osteoblast-like cell functions in the presence or ab-

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sence of Ti particles *in vitro.* We showed that TNF-α and Ti particles have additive effects on the inhibition of osteoblast proliferation and alkaline phosphatase (ALP) production. This additive stimulation in osteoblast was also measured for the production of IL-6, which is well known to induce osteoclastogenesis and to directly stimulate bone resorption.

MATERIALS AND METHODS

Cells

Human osteoblast-like cell lines MG-63 and SaOS-2 (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified eagle's medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (Irvine Scientific), L-glutamate (4 m*M*), penicillin (100 U/mL), streptomycin (100 μg/mL), and fungizone (0.25 μg/mL) (Biowhittaker, Walkersville, MD), at 37°C and 5% CO_2 in a humidified atmosphere. These cell lines display typical phenotypes of osteoblasts as the polygonal morphology, the formation of calcium phosphate salts, the production of alkaline phosphatase, the formation of calcium phosphate salts, or the production of osteocalcin when these cells are cultured with vitamin D_3.[15–18]

Titanium particles

Commercially pure Ti particles (–325 mesh) purchased from Sigma-Aldrich Chemicals (Milwaukee, WI) were sterilized by autoclave at 135°C for 15 min. A semiautomatic image processing (NIH image software) of particles using optical microscopy allowed us to determine that 80% of the particles had a diameter smaller than 5 μm (diameter ranged from 0.1 to 50 μm). After sterilization of the Ti particles, culture medium was added, followed by ultrasonication for 30 min immediately before use to minimize particulate aggregation. A 1% (wt %) suspension as well as appropriate dilutions of Ti particles in medium described under "Cells" were used in the experiments with cells.

TNF-α

Recombinant human TNF-α was purchased from Sigma (St. Louis, MO). The range of concentrations used (0–100 ng/mL) was based on previous reports[19–23] and preliminary experiments in which TNF-α concentrated at less than 0.1 ng/mL showed little effect on the proliferation of the osteoblast-like cells.

Proliferation assay

Alliquots (50 μL) of MG-63 and SaOS-2 cell suspensions (5×10^4/mL and 8×10^4/mL, respectively), were seeded onto a 96-well plate, followed by incubation for 24 h. Fifty microliters of variously concentrated TNF-α (for a final concentration of 0.1, 1, 10, 25, or 100 ng/mL) or 25 μL of TNF-α and 25 μL of Ti particle solution (for a final solution of 0.004, 0.016, 0.063, 0.250% for MG-63 and 0.001, 0.004, 0.016, 0.063% for SaOS-2) were added and the culture was incubated for 72 h. For the experiments performed without Ti particles, the control group was the cells cultured without TNF-α, whereas for the experiments performed simultaneously with TNF-α and Ti particles, the control group was the cells cultured without Ti particles. Cell proliferation was determined by a CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).[24] In this assay, a tetrazolium salt is converted into a formazane product, which is directly correlated with an absorbance reading at 520 nm. The cell proliferation (%) was defined by the absorbance of the supernatant of cells treated with Ti and/or TNF-α divided by the absorbance of the supernatant of cells not treated with Ti or TNF-α (×100). All results shown are one of the representative outcomes from at least four experiments with six replicates.

ALP activity

ALP activity was determined using an ALP kit (Diagnostic kit 245; Sigma) Because the levels of ALP in MG-63 cells are extremely low, only SaOS-2 cells were used. Cells were plated at 5×10^4 cells/well in 24-well culture plates with 0.5-mL complete medium [medium described under "Cells" supplemented with 50 μg/mL ascorbic acid (Sigma) and 10 m*M* β-glycerophosphate (Sigma)]. After 48 h, the culture medium was replaced by fresh medium with or without 25 ng/mL TNF-α and/or 0.032% Ti particles. The control group was defined by the cells cultured without TNF-α and Ti particles. The cells were cultured for another 48 h, then the medium was removed and the cells were rinsed three times with phosphate buffered solution followed by the addition of 0.5-mL Triton-X 100 (1%). After freezing and thawing, the cell lysates were sonicated for 1 min on ice. The cell lysates was centrifuged and a volume of 20 μL of each sample was added to 100 μL of *p*-nitrophenyl phosphate solution within a 96-well plate at 25°C and reacted for 3 min. *p*-Nitrophenol is produced in the presence of ALP and the absorbance was measured at 405 nm. The change in rate of absorbance is directly propotional to the ALP activity. Data were normalized for total cell protein measured with a commercial kit (DC protein assay kit; BioRad, Hercules, CA).

IL-6 production

IL-6 was assayed using an ELISA kit (Predicta IL-6 kit; Genzyme, Cambridge, MA). Cells were plated at 5×10^4 cells/well in a 24-well culture dish. After 48 h, the culture medium was replaced by fresh media with or without 25 ng/mL TNF-α and/or 0.125 or 0.032% Ti particles for MG-63 cells and SaOS-2 cells, respectively. The control group was the cells cultured without TNF-α and Ti particles. The cells

were cultured for another 48 h, then the medium was collected, centrifuged, and the levels of IL-6 determined. Data were normalized for total cell protein determined as described above.

Statistical analysis

Data are expressed as the mean ± SEM. For the proliferation assays, a one-way analysis of variance (ANOVA) test was used to analyze the mean variance of cell proliferation. If significant differences were found, a Student's t test was performed to determine which mean was different than mean of control. For the ALP and IL-6 assays, a two-way ANOVA test was used to analyze the effect of TNF-α, Ti particles, and the possible additive effect of both. A 95% confidence level was selected to define significance for all statistical tests.

RESULTS

Particle-free medium test

The 1% Ti particle solution (without cells) was incubated for 72 h, aspirated, and added (without particles) to cells. The cells cultured with this conditioned, particle-free medium had no effect on cell proliferation and the production of ALP IL-6 (data not shown). This excluded the possibility that Ti ions or soluble contaminants on the surface of particles could affect the cell functions.

Cell proliferation

Preliminary results showed that MG-63 and SaOS-2 cell proliferations were directly correlated with the absorbance values of the formazane product at 520 nm (R^2 = 0.984 and 0.987 respectively, n = 6). The absorbance values at 520 nm also correlated with the number of living cells, which were counted directly on a hemocytometer with trypan blue after co-culture with titanium particles (up to 1.0%) and/or TNF-α (up to 100 ng/mL) for 72 h (data not shown).

Effect of TNF-α on cell proliferation

Cell proliferation was statistically decreased by TNF-α concentrated at more than 0.1 ng/mL for MG-63 (Fig. 1(a); $p < 0.05$) and more than 10 ng/mL for SaOS-2 (Fig. 1(b); $p < 0.05$) for 72 h. All values were normalized by the control, TNF-α-free, group.

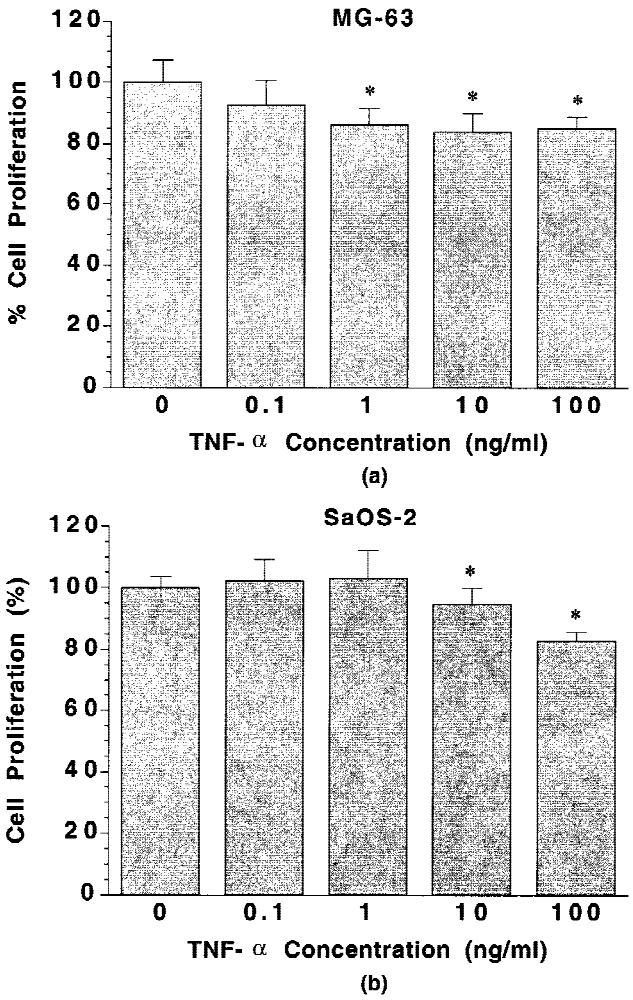

Figure 1. The effect of TNF-α on osteoblast like cells after 72 h of incubation. (a) MG-63 cells, (b) SaOS-2 cells, treated with various concentrations of TNF-α (0, 0.1, 1, 10, and 100 ng/mL). The results of proliferation versus concentration of TNF-α were plotted (mean ± SEM, n = 6). The data were normalized with respect to the control group (0 ng/mL TNF-α). *$p < 0.05$ compared with control.

Effect of TNF-α on cell proliferation in the presence of Ti particles

Ti particles concentrated at 0.063% or more for MG-63 [Fig. 2(A)] and at 0.016% or more for SaOS-2 [Fig. 2(b)] statistically decreased the cell proliferation ($p < 0.05$, for Fig. 2(a,b). The sensitivity of Ti particles was seen more in the proliferation of SaOS-2 than MG-63 cells. The addition of TNF-α concentrated at 25 ng/mL markedly increased this adverse effect induced by particles. Furthermore, the ratio of the inhibitory effect caused by TNF-α, when compared with the TNF-α-negative group, increased as the concentration of the titanium particles increased; e.g., although 22% of the proliferation was decreased by TNF-α at 0 wt % of Ti particles, 36% of the proliferation was decreased by TNF-α in the presence of 0.250 wt % of Ti particles in

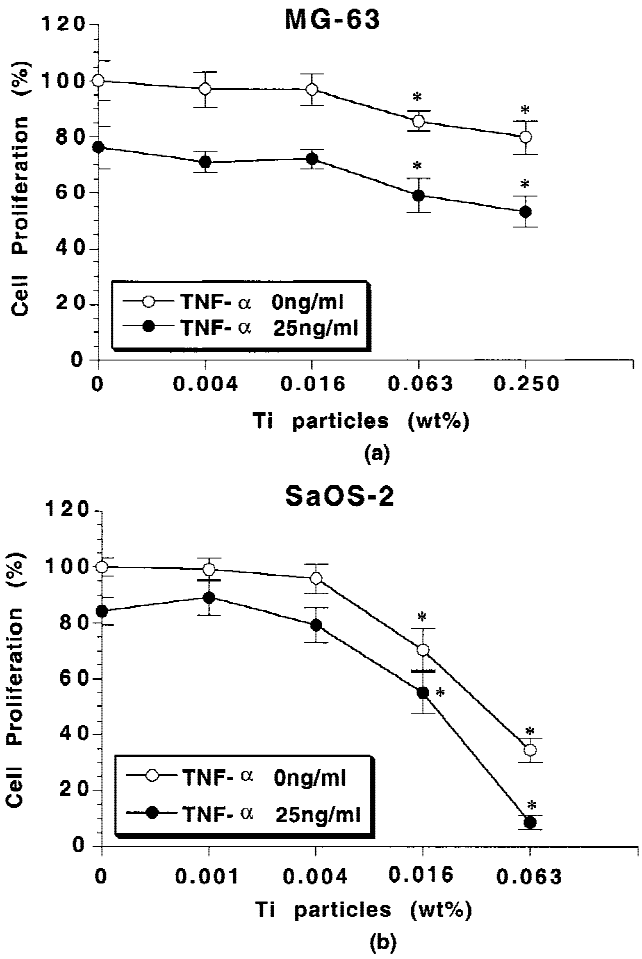

Figure 2. The effect of Ti particles and TNF-α on osteoblast-like cell proliferation after 72 h of incubation (mean ± SEM, $n = 6$). The data were normalized with respect to the proliferation obtained without Ti particle and TNF-α. (a) MG-63 cells, (b) SaOS-2 cells. The concentrations of Ti particle (wt %) used in the system are indicated on the horizontal axis. *p < 0.05 compared with control (without particles) in each group.

the MG-63 cell system [Fig. 2(a)]. In the SaOS-2 cell system [Fig. 2(b)], 77% of the proliferation was decreased by TNF-α in the presence of 0.063 wt % of Ti particles, whereas 16% of the proliferation was decreased by TNF-α at 0 wt % of Ti particles.

Effect of TNF-α and Ti particles on ALP activity

ALP activity of SaOS-2 cells decreased when incubated with TNF-α concentrated at 25 ng/mL. The combination of TNF-α and Ti particles (0.032%) markedly decreased the ALP activity of SaOS-2 cells (Fig. 3). This additive effect of TNF-α and Ti particles was significantly relevant.

Effect of TNF-α and Ti particles on IL-6 production

IL-6 production by MG-63 cells cultured with TNF-α markedly increased when compared with the

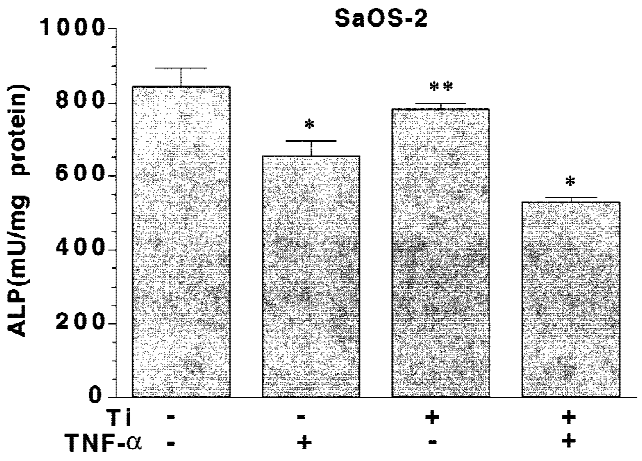

Figure 3. The effect of Ti particles (0.032%) and TNF-α (25 ng/mL) to ALP activity on SaOS-2 cells after 48 h of incubation (mean ± SEM). "+" indicates the presence of corresponding stimulus, whereas "–" indicates its absence. Using a two-way ANOVA analysis, an additive effect of TNF-α and Ti particles on the ALP activity was demonstrated (*$p <$ 0.001; **$p < 0.042$; $n = 3$).

control (Table I). A statistically synergistic effect on IL-6 production by the MG-63 cells was measured when these cells were incubated with a combination of Ti particles and TNF-α. IL-6 production from SaOS-2 cells was only detected when incubated with TNF-α. However, Ti particles with TNF-α markedly increased the production of IL-6 by SaOS-2 cells.

DISCUSSION

Prostheses made of titanium alloys are extensively used for the management of joint disorders. Although *in vitro* studies have revealed that Ti particles are less toxic than Co–Cr particles,[25,26] titanium alloys have a certain premature failure rate[27,28] suggesting that debris from titanium prostheses have harmful effects. One of the reasons for this may be the higher concentration of metal particles in tissue around loose titanium-alloy implants compared with those with loose implants made of other alloys.[29–32] In our laboratory, we also found that Ti particles have harmful effects on the viability of neonatal rat calvarial osteoblasts in a dose-dependent manner.[33]

In the present study, we investigated the *in vitro* effect of TNF-α on osteoblast functions in combination with Ti particles at concentrations representative of what can be found during biopsy studies.[30] We have demonstrated that TNF-α and Ti particles have additive effects on inhibition of cell proliferation and decrease the production of ALP on human osteogenic cell-lines MG-63 and SaOS-2. Moreover, the production of IL-6, which is well known to induce osteoclastogenesis and to directly stimulate bone resorp-

TABLE I
Levels (pg/mg Protein) of IL-6 Released by Osteoblastic Cells Stimulated by Titanium Particles with or without TNF-α for 48 h

	Treatment			
	Ti Particles[a]	−	−	+	+
Cells	TNF-α[b]	−	+	−	+
MG-63		103 ± 13	8128 ±4 10*	165 ± 18**	9542 ± 512*
Saos-2		ND	416 ± 82	ND	895 ± 214

[a]Ti particles; 0.125% for MG-63 cells and 0.032% for Saos-2 cells.
[b]TNF-α: 25 ng/mL.
ND, not detected.
Data are mean ± SEM; $n = 6$.
*$p < 0.007$.
**$p < 0.013$.

tion,[20,34,35] was additively stimulated. *In vitro* studies aiming to quantify the reaction of bone cells to particles should then also be concerned with the possible modulation by cytokines that would be present in the corresponding *in vivo* situation.

The data obtained with TNF-α are consistent with several other reports in which TNF-α inhibits ALP production but increases IL-6 synthesis in osteoblastic cells.[21–23] It has been suggested that TNF-α stimulates IL-6 production of osteoblastic cells by activation of transcription factor NF-κB: gene regulatory factor.[23,36] It has also been reported that TNF-α regulates IL-6 production of osteoblastic cells through sphingomyelin hydrolysis to produce sphingosine 1-phosphate as a second messenger and protein kinase C activation as an autoregulator.[21]

In a previous study, Chiba et al.[19] showed that an approximately 5-mm^3 interface membrane retrieved from patients with loose cementless hip prostheses cultured with 2 mL of medium produced up to more than 9 ng TNF-α per g of tissue in the culture medium. This concentration of TNF-α in the medium could be concentrated more *in loco*. Furthermore, the concentration of TNF-α up to 100 ng/mL used in the present study is similar to what has been used in previous reports in which the effect of TNF-α on the functions of the osteoblast-like cells was investigated.[20–23]

Using calcium phosphate cement particles and TNF-α at concentrations similar to the present study, no additive effect on viability was noted with osteoblasts obtained from neonatal Sprague-Dawley calvarial rat.[37] Despite comparisons between results obtained with different cell lines, it could be assumed that viability of SaOS-2 and MG-63 cells would not be affected by Ti particles and TNF-α at the concentrations used in this study.

In conclusion, we showed that by combining TNF-α and Ti particles, the effects on osteoblastic cells in terms of decreased proliferation and ALP production, and increased IL-6 production, are additively enhanced. It could be hypothesized from these results that the exposure of osteoblasts to TNF-α in combination with Ti particles could contribute to the pathogenesis of peri-implant osteolysis. Further *in vivo* investigations to clarify this hypothesis should be performed.

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