Variations in astrocyte and fibroblast response due to biomaterial particulates in vitro

Roche C. de Guzman, Pamela J. VandeVord
Department of Biomedical Engineering, Wayne State University, Detroit, Michigan 48202

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Abstract: The possible involvement of orthopedic biomaterial particles such as cobalt–chrome alloy (Co–Cr), ultrahigh molecular weight polyethylene (UHMWPE), titanium alloy (Ti-6Al-4V), and polymethyl methacrylate (PMMA) in the formation of glial and meningeal scars was investigated using an in vitro system. Cell lines were used as models for astrocytes and meningeal fibroblasts. They were incubated with varying concentrations of particle suspensions, after which proliferative and cytotoxic responses were quantified using MTT assay and Live/Dead microscopy. It was determined that relative particulate toxicity (arranged in decreasing order) to astrocytes is Co–Cr > Ti-6Al-4V > PMMA > UHMWPE, and toxicity to fibroblasts is PMMA > Co–Cr > Ti-6Al-4V > UHMWPE. Cell death caused by PMMA was mainly due to necrosis, while the rest of the particles induced apoptosis. Low quantities of Co–Cr and Ti-6Al-4V stimulate increased astrocyte proliferation rate. However, only the cells treated with titanium alloy caused upregulated transcription of reactive astrocyte markers such as glial fibrillary acidic protein, vimentin, nestin, and type IV collagen, suggesting the potential of titanium alloy alone to trigger glial scarring. None of the biomaterials tested promoted proliferation in fibroblasts implying that biomaterial particles are not directly involved in meningeal scar development.

Key words: reactive astrocyte; fibroblast; proliferation; cytotoxicity; biomaterial particles

INTRODUCTION

Limited studies have been conducted on the response of central nervous system (CNS) elements to biomaterials. In craniofacial and vertebral column surgery, bone fixation devices such as screws, rods, plates, cages, cables, and wires may translocate into the adjacent CNS tissue. Moreover, in chronic implantation of microelectrodes and microdevices for the detection and generation of brain electrical stimuli in seizure treatment and biomechanical neural prosthesis development, metal and polymeric biomaterials come in direct contact with the CNS parenchyma. Long-term implantation of these devices can also lead to the release of biomaterial particles due to shearing forces, cyclic loading, and immunological response. The invasiveness of the above-mentioned clinical and experimental procedures physically injures the CNS and disrupts the blood-brain barrier and surrounding tissues provoking an unwanted host response through glial and meningeal scar formation. Cellular interactions of released biomaterial particles may additionally contribute to these conditions.

In this study, astrocytes and fibroblasts, the main cells responsible for glial and meningeal scars, respectively, were evaluated for their responses to biomaterial particles namely, cobalt–chrome alloy (Co–Cr), ultrahigh molecular weight polyethylene (UHMWPE), titanium alloy (Ti-6Al-4V), and polymethyl methacrylate (PMMA), which are possible components of microscrews, microelectrodes, and bone fixation materials. An in vitro system was used, where C6 cell line was employed as model for astrocytes and Rat2 cells for meningeal fibroblasts.

Glial scar is a structure characterized by the initial involvement of multiple glial cell types (oligodendrocytes, microglia, synantocytes, and astrocytes), participating in a temporal manner, and its most notable effect is the inhibition of neurite regeneration and outgrowth. Its final form is composed mostly of reactive astrocytes that underwent migration, hypertrophy, proliferation, clustering, and formation of dense interweaving processes creating an impenetrable mechanical barrier to contain the damaged site. Reactive astrocytes are identified as cells with upregulated expression of intermediate filament proteins specifically, glial fibrillary acidic protein (Gfap), vimentin...
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(Vim), and nestin (Nes) that make up the majority of the cytoskeletal framework of the network of astrocytic processes and soma of astrocytes. Additionally, extracellular matrix (ECM) proteins like type IV and VIII collagen molecules and the cytokine interleukin 6 (IL-6) have been considered as markers for reactive astrocytosis since production and secretion are enhanced in response to CNS insult. Consequently, we tested if the treatment of biomaterial particles leads to a response similar to reactive astrocytosis by analyzing C6 cell proliferation and transcription of marker genes. Penetration of the meningeal connective tissue layer covering the CNS results in meningeal scar and astrocytic gli limitans development, where meningeal fibroblasts migrate and proliferate to fill-up the injured gap. Hence, we also examined the proliferative response of Rat2 fibroblastic cells to orthopedic biomaterial particles. Finally, we reported the toxicity of both C6 and Rat2 cell lines to the assayed biomaterials in a dose-response fashion. The behavior of these cells may help in the strategy of CNS biomaterial implantation.

MATERIALS AND METHODS

Cell-line culture

C6 rat astrocytoma cell line (ATCC, Manassas, VA) was maintained in F-12K medium supplemented with 10% horse serum, 2.5% FBS, and antibiotics (100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 250 ng/mL Fungizone). Meanwhile, Rat2 fibroblast cells (ATCC) were cultured in DMEM containing 10% FBS and antibiotics. Both cell types were kept in a humidified incubator (90% RH) with 5% CO₂ at 37°C. Cells growing in tissue culture flasks were trypsinized, counted using a hemocytometer, and resuspended with culture medium in appropriate experimental plates.

Proliferation assay

To determine the number of cells suitable for methylthiazolyldiphenyl tetrazolium bromide (MTT) colorimetric assay, cells were initially plated at a density of 1 × 10^5 and serially diluted in a 96-well plate. After 5 days incubation, 0.5 mg/mL MTT reagent was added to the culture medium. The plates were reincubated for 6 h in the dark at 37°C. Culture media were carefully removed and replaced by dimethyl sulfoxide (DMSO) to solubilize the formazan crystals and cells. Absorbances were read at 570 and 650 nm in a microtiter plate reader. Optical densities of the samples were obtained by subtracting the 650-nm reference wavelength from the 570-nm absorbance reading. Growth curves for C6 and Rat2 cells were generated.

The proliferation responses of C6 and Rat2 cells in the presence of biomaterial particles were determined using the MTT assay. Test biomaterial particulates were generously donated by Dr. Paul Wooley and have been previously characterized. The average diameter (and size range) of these particles was determined to be Co–Cr = 5.7 μm (1–20 μm), UHMWPE = 3.6 μm (2–23 μm), Ti-6Al-4V = 2.3 μm (0.1–68 μm), and PMMA = 4.0 μm (0.1–10 μm). Suspension of cells were seeded into a 96-well plate at 1 × 10^4 cells/well (2.86 × 10^4 cells/cm²). Following 24-h incubation to allow cells to attach on the surface of the plate, test particles, namely, Co–Cr, UHMWPE, Ti-6Al-4V, and PMMA were added serially at 5, 2.5, 1.25, 0.625, 0.3125, and 0 mg/mL. The plates were incubated back at 37°C for 2 days. MTT assay was performed similar to the procedure described earlier. Optical density measurements obtained from wells with particles alone (no cells) were deducted from the particle-treated samples. Concanavalin A (Con A) at 5 μg/mL was used as a positive control for C6 proliferation.

Viability and apoptosis microscopy test

Live/Dead™ (Invitrogen, Carlsbad, CA) viability/cytotoxicity fluorescence microscopy assay was employed to quantify the percent of C6 cells dying due to the presence of orthopedic biomaterial particles. Briefly, cells were cultured and attached on a glass coverslip inside a 35-mm diameter petri dish at 2.75 × 10⁵ cells/dish (2.86 × 10⁴ cells/cm²). Particle suspensions (Co–Cr, UHMWPE, Ti-6Al-4V, and PMMA) at 5 mg/mL were added to the cells. Culture medium was added to the “no particle” untreated control, while 70% MeOH was used for the induced cytotoxic control. After 2 days incubation, cells were washed with PBS thrice. C6 cells were then incubated with Live/Dead stain (2 μM calcein AM and 4 μM EthD-1 in PBS) for 30 min at room temperature (RT). Coverslips were subsequently mounted on glass slides and viewed under an Olympus fluorescence microscope (Olympus America, Center Valley, PA) with bandpass filters for separate calcium (live) and EthD-1 (dead) light emissions analyses.

To further classify the dead cells as either necrotic or apoptotic, Vybrant® apoptosis assay kit No. 2 (Invitrogen) was utilized. Similar to the above-mentioned procedure, C6 cells were seeded onto a coverslip and treated with 5 mg/mL of test particles (Co–Cr, UHMWPE, Ti-6Al-4V, and PMMA). Camptothecin (Camp) (at 1.25 μM) and 70% MeOH were added to cells for apoptotic and necrotic controls, respectively. Following 2 days of incubation and washing with PBS, particle-treated cells were hybridized with Alexa Fluor® 488 annexin V (1:20 dilution) and propidium iodide (3 μM) in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) for 15 min at RT. Cells were visualized using fluorescent microscopy with filters for green and red fluorescence. Necrotic cells were identified as those generating red signals, while apoptotic cells were distinguished by subtracting the number of red from the green fluorescent-emitting cells.

Quantitative PCR

Total RNA from untreated and particle-treated cell samples were purified using the guanidine isothiocyanate-phe-
nol-chloroform method. Reverse transcription employing the oligo(dT)$_{20}$ primer was subsequently performed to convert mRNA to cDNA template. Quantitative PCR was done to determine the relative amounts of gene transcripts using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Briefly, master mix containing 1X SYBR® Green (Applied Biosystems), and forward and reverse primers (0.4 μM each) were dispensed into wells of a 96-well optical plate. After the addition of cDNA templates, the plate was capped, spun to settle contents, and placed in the retractable plate holder of the Real-Time PCR System. The machine was operated using the profile: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. Serially diluted positive control cDNA samples were also added for the construction of standard curve per gene analyzed. A standard curve was made by plotting log(relative amount) versus threshold cycle $C_T$. The equation of the trend line was used to compute the unknown relative amount from the $C_T$ values obtained experimentally.

The following rat gene primer pairs were employed: (for identification of reactive astrocytes) Gfap, Vim, Nes, procollagen type IV alpha 1 (Col4a1), procollagen type VIII alpha 1 predicted (Col8a1), and IL6; (for prediction of cell proliferation pathway) protein tyrosine kinase 2 (Ptk2), growth factor receptor bound protein 2 (Grb2), Raf1, mitogen activated protein kinase 1 (Map2k1), and Elk1. The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as an internal control. The computed levels of gene transcripts accounting for the expression data according to the standard curve were normalized by dividing with the corresponding amount of Gapdh transcripts.

**Statistical analyses**

Linear regression analysis was performed to construct trend lines for the growth curves of C6 and Rat2 cells. The coefficient of determination ($r^2$) was obtained to determine the strength of a straight-line relationship using Microsoft® Office Excel (Microsoft, Redmond, WA). Dose-response or growth inhibition curve equation, $r^2$, and particle inhibition concentration to 50% of the cells (IC$_{50}$) were generated using BioDataFit 1.02 (Chang Bioscience, Castro Valley, CA). Data points accounting for increased cell proliferation compared to the untreated samples were removed to better estimate the IC$_{50}$ values. The rest of the data points were fitted using one of the following dose-response curve models.

Four parameter (log) \[ y = a + \frac{b - a}{1 + 10^{(x-c/d)}} \]

or

sigmoidal (log) \[ y = a + \frac{b - a}{1 + 10^{(x-c)}} \]

where $y$ is the number of cells, $x$ is the concentration, and $a$, $b$, $c$, and $d$ are the equation-derived constants.

Analysis of variance (ANOVA) at level of significance ($x$) = 0.05 was used for the multiple sample group statistical analyses. Tukey’s multiple comparison test was employed as a post hoc for significantly different ANOVA test groups. Both ANOVA and Tukey tests were performed using SPSS 12.0 (SPSS, Chicago, IL). Linear regression data points and bar graphs were expressed as mean ± 1 standard deviation. MTT assay samples were made in quadruple replicates. Ten frames per group were analyzed in Live/Dead and apoptosis microscopy experiments, while individual samples for real-time PCR were performed in triplicates.

**RESULTS**

C6 cells proliferate at lower [Co–Cr] and [Ti-6Al-4V], but are inhibited at higher Co–Cr, Ti-6Al-4V, and PMMA levels

Growth curves of both C6 and Rat2 cell lines were obtained by plotting the log(number of cells) versus the net absorbance. Linear trend lines were generated starting at 3125 to $1 \times 10^5$ cells for C6 cells, while at 1563 to $1 \times 10^5$ cells for Rat2 cells (Fig. 1). According to the linear regression lines, a $1 \times 10^4$ starting cell count was sufficient to reflect an increase or decrease of cell number within the 3-day incubation period for both the cell types. Equations of trend lines also allowed the computation of the final number of cells at the end of the experiment.

Reactive astrocytes exhibit binding reaction to the lectin Con A. Since Con A can interact with astrocytes, we tested whether this binding reactivity leads to altered C6 proliferation response. MTT data at 5 μg/mL of Con A revealed a significant increase in cell count (Fig. 2). Hence, Con A can indeed be used as a positive control for C6 cytoproliferation. To find out if orthopedic biomaterial debris particles (Co–Cr, UHMWPE, Ti-6Al-4V, and PMMA) can induce or inhibit C6 and Rat2 cell proliferation, these
cells were incubated with serially diluted particle suspensions. MTT colorimetric assay results showed that both Co–Cr (at 0.3125 and 0.625 mg/mL) and Ti-6Al-4V (at 0.625 and 1.25 mg/mL) particles induced increased proliferation of C6 cells [Figs. 3(A,C)]. C6 cells are more sensitive to Co–Cr compared to Ti-6Al-4V since lower concentrations of cobalt–chrome alloy particles were sufficient to promote increased cell number. In contrast, at greater amounts of both metal particles, the cell counts drop significantly. UHMWPE inhibits C6 cells at 0.625 mg/mL but increasing concentrations lead to cell counts similar to untreated cells [Fig. 3(B)]. This suggests the interplay between toxicity and proliferation signals encountered by C6 cells. PMMA particles were found to be cytotoxic to C6 cells at 0.625 mg/mL and greater, with the increase in toxicity as particle concentration increases [Fig. 3(D)]. To compare the test particles in terms of toxicity to C6 cells relative to their concentrations, dose-response curve models that gave the maximum $r^2$ were fitted into the plot of concentration versus cell number. Table I summarizes the type of model, equation, $r^2$, and IC$_{50}$ of the test particles. The lower the IC$_{50}$ value, the more cytotoxic the material is. According to the predicted results, Co–Cr is the most cytotoxic to C6 cells with IC$_{50}$ = 2.11 mg/mL, followed by Ti-6Al-4V (IC$_{50}$ = 2.24 mg/mL), then PMMA (IC$_{50}$ = 2.48 mg/mL). UHMWPE is the least cytotoxic biomaterial particle since even at 5 mg/mL concentration, it still did not inhibit the proliferation of C6 cells.

**Assayed particles are not proliferative, but instead, cytotoxic to Rat2 fibroblasts**

Our results demonstrated that the test particles are generally toxic to Rat2 cells starting at certain threshold suspension levels (Fig. 4). Rat2 is most sensitive to PMMA followed by Co–Cr, and Ti-6Al-4V/UHMWPE. The MTT result was correlated with the obtained IC$_{50}$ amounts from the dose-response equations (Table I) showing that the following particles (with IC$_{50}$ values) are cytotoxic to Rat2 in decreasing order: PMMA (0.28 mg/mL), Co–Cr (0.93 mg/mL), Ti-6Al-4V (1.31 mg/mL), and UHMWPE (1.39 mg/mL).

![Figure 2.](image1.png) **Figure 2.** C6 cells undergo increased proliferation when Con A is added. The final number of cells is significantly greater in 5 μg/mL Con A-treated (*p = 0.026) compared to untreated C6 cells. No change in cell count was detected at lower Con A concentrations (data not shown).

![Figure 3.](image2.png) **Figure 3.** Proliferation response of C6 cells in the presence of orthopedic biomaterial particles. A: Co–Cr: $p = *0.027$, **0.008, *0.000. B: UHMWPE: $p = *0.035$. C: Ti-6Al-4V: $p = *0.017$, **0.013, *0.022, **0.000. D: PMMA: $p = *0.000$, *0.025. The symbol (*) corresponds to groups with significant increase in cell number; while (+) indicates decrease in cells as compared to untreated C6 cells.
The cell count slightly increased in samples with 5 mg/mL titanium alloy compared with those with 2.5 mg/mL [Fig. 4(C)].

PMMA-treated C6 cells die mostly via necrosis, while Co–Cr, Ti-6Al-4V, and UHMWPE particles cause apoptosis.

MTT assay indirectly measures the number of cells at the end of the experiment. However, to determine the fraction of living versus dead cells at a particular instance, Live/Dead microscopy test was done. After 2 days incubation, C6 cells with 5 mg/mL PMMA exhibited 25.6% ± 17.9% cell death, which was significantly higher (p = 0.021) compared to cell death in samples with no particles (2.0 ± 1.0%) (Fig. 5). Other particle-treated C6 samples did not provide changes in cell death percentage compared to the untreated. Yet, MTT data at 5 mg/mL of Co–Cr [Fig. 3(A)] and Ti-6Al-4V [Fig. 3(C)] displayed reduction of cell number, thus cells were killed during the process. This implies that cell death occurred at an earlier time point and majority of the dead cells got cleared away from the substrate. In PMMA-treated cells, significant fraction of cell death was still occurring after 2 days in culture, suggesting a different mode of death compared to metal particle-treated cells. To prove this, we performed the apoptosis microscopy assay. Results showed that the main mechanism of PMMA-induced cell death is through

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<th>Table I</th>
<th>Computed IC&lt;sub&gt;50&lt;/sub&gt; Values</th>
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<tr>
<td><strong>Cell Line</strong></td>
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<td>C6</td>
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<td>UHMWPE*</td>
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NA, not applicable.

IC<sub>50</sub>, particle concentration to inhibit 50% of the cells.

Toxicity to C6: Co–Cr > Ti-6Al-4V > PMMA > UHMWPE; to Rat2: PMMA > Co–Cr > Ti-6Al-4V > UHMWPE.

*IC<sub>50</sub> for UHMWPE with C6 was not determined because the concentration range used did not inhibit proliferation.

Figure 4. Proliferation response of Rat2 cells in the presence of orthopedic biomaterial particles. A: Co–Cr: \( p = ^{*}0.000 \). B: UHMWPE: \( p = ^{*}0.019, ^{++}0.000 \). C: Ti-6Al-4V: \( p = ^{*}0.029 \). D: PMMA: \( p = ^{*}0.003, ^{*}0.004, ^{++}0.000 \). The symbol (‘) corresponds to groups with significant decrease in cell number compared to untreated Rat2 cells.
necrosis while the rest of the tested particles are via apoptosis (Fig. 6). Also, images demonstrated that necrotic cells in PMMA-treated C6 cells were prevalent in the center of the aggregate, while the fewer apoptotic cells were found in the periphery. The aggregate also contained PMMA particles. Hence it can be hypothesized that cells undergo necrosis because of physical obstruction and deprivation of nutrients within the aggregate.

**Figure 5.** Live/Dead assay showing the cytotoxicity of 5 mg/mL PMMA to C6 cells (*p = 0.021* compared to untreated). Cells emitting green fluorescence in the calcein quadrant were alive, while dead cells fluoresce red signals in the ethidium homodimer-1 (EthD-1) zone. Differential interference contrast (DIC) image was overlapped to clearly identify which cells were dead or alive. Shown here are cells with (A) no particle and (B) PMMA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

**Ti-6Al-4V-treated C6 cells generally behave like reactive astrocytes**

Sudden increase in proliferation of astrocytes is one of the hallmarks of reactive astrogliosis,\(^5,^{14}\) which is a major component of glial scarring. Thus, we tested whether the proliferative behavior of C6 cells in the presence of metal alloy particles is similar to induction of reactive astrocytes in traumatized CNS. MTT assay data showed that the peak cell number increase occurred when C6 cells were treated with 0.625 mg/mL of Co–Cr and 1.25 mg/mL of Ti-6Al-4V. To determine the changes in gene transcription, total RNA molecules of these particle-treated cells, as well as untreated control C6 cells, were extracted, reverse transcribed, and tested for real-time PCR. Results revealed significant elevation of Gfap, nestin, and Col4a1 in Ti-6Al-4V-treated cells but not in cells with Co–Cr particles (Fig. 7). Vimentin was also detected at slightly higher level in cells with titanium alloy compared to the untreated control but was not statistically significant. The upregulation of these genes suggests that the C6 cells respond to titanium particles similar to activated astrocytes following injury.\(^5,^{11}\)
C6 cells attach and appear to phagocytose metal particles

One of the physiological functions of astrocytes is phagocytic activity for the removal of dead cells, debris, and foreign particles. Inspection of metal particle-treated C6 cells under an inverted microscope demonstrated that the cells surround and physically interact with Co–Cr and Ti-6Al-4V particles (Fig. 8). The altered and more circular morphology of cells with particles suggests the engulfment of these dense spherical particulates. The

Figure 6. Apoptotic assay demonstrating that the main mode of cell death in PMMA-treated cells is via necrosis (\( p = 0.01 \) compared to Camp-apoptosis control) while others involve apoptosis. Total dead cells were quantified by counting the number of green cells in the annexin V image. Necrotic cells were those producing both red and green signals; simplified by just counting the red cells in the propidium iodide (PI) image. Thus, apoptotic cells (cells emitting green only) were identified by subtracting the necrotic from the total dead cell count. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 7. Transcription of genes implicated in reactive astrocystosis in C6 cells with or without metal alloy particles. Addition of Ti-6Al-4V to C6 cells induced upregulation of Gfap (*\( p = 0.043 \)), Nes (**\( p = 0.003 \)), and Col4a1 (**\( p = 0.006 \)). Co–Cr particles did not alter the transcription of any of the assayed genes. Levels of Col8a1 and IL-6 transcripts were found to be very low in all samples.
The diameter of resting C6 cell body was found to be 12.6 ± 1 μm (mean ± 1 standard deviation) via measurement of phase-contrast images. However, C6 cells appear to engulf the bigger Co–Cr and titanium alloy particles with sizes 13.4 ± 4 and 18 ± 6 μm, respectively. Furthermore, multiple cell-particle aggregations were observed in all of the four assayed biomaterials. Initially, 5 mg/mL particle suspensions were evenly distributed on the surface of the substrate. After 2 days incubation, C6 but not Rat2 cells cause particle clustering. This implies C6 adhesion to particles during their random crawl, and C6-particle interaction signals cell-to-cell clustering while dragging the particles with them.

**DISCUSSION**

Positive regulation of cell proliferation was observed in both Co–Cr and Ti-6Al-4V particle treatment of C6 cells [Figs. 3(A,C)]. The dose-response relationship displayed a hormesis-like effect, where there was activation or increased rate of cell proliferation at low amounts of metal alloy particles; further increase in dosage returned the response to the “no observed adverse effect level” followed by the cytotoxic descending curve. Astrocytes are capable of detecting various stimuli due to their numerous receptors. Consequently in an effort to restore homeostasis, they respond by the expression of multiple genes and undergo structural, biochemical, functional, and behavioral alterations. Although astrocytes can transform heterogeneously into possibly hundreds of still uncharacterized forms depending on the type of insult, Liberto et al. grouped the response as either “reactive” (anisomorphic) or “activated” (isomorphic) astrogliosis. The former and more aggressive reaction deemed destructive to tissues and associated with glial scar, while the latter is linked to production of substances for recovery.

Gene transcription data of upregulated Gfap, vimentin, nestin, and type IV collagen revealed that Ti-6Al-4V, but not Co–Cr, exposure possibly incited a reactive astrocytic phenotype. This implies that at low particle dosage, titanium alloy can evoke more harm than Co–Cr. In vivo, titanium and titanium dioxide implantation in the CNS have been shown to produce glial scars and it causes more adverse acute inflammatory reaction compared to cobalt–chrome–molybdenum alloy. The difference in astrocyte response to Co–Cr and Ti-6Al-4V particles can be attributed to initial activation of distinct molecular signaling mechanism leading to cell proliferation. Since we used an axenic clonal cell-culture system, induction of increased cell proliferation and reactive astrogliosis may be caused by autocrine signaling and autoactivation. An example of this phenomenon is through endothelin autocrine signaling in astrocytes. The mitogen endothelin, secreted by astrocytes, activates the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and cytoskeleton-dependent pathways leading to cell proliferation and differentiation. In our unpublished preliminary study, we analyzed the transcription of genes involved in ERK/MAPK and cytoskeleton-dependent pathways in untreated and metal alloy-treated C6 cells at different time points. Cells with Co–Cr particles were found to have elevated Ptk2, Grb2, Raf1, and Map2k1 mRNA compared to untreated control. For Ti-6Al-4V-treated C6 cells, Ptk2 level remained similar to cells with no biomaterial particles, but increase in gene transcripts Grb2, Raf1, Map2k1, and Elk1 were detected. Although increased amount of these transcripts does not necessarily mean more protein activation to drive the transduction of the signal cascade, this indicates greater availability for possible time-dependent activation. Moreover, upregulation of Ptk2, Grb2, Raf1, Map2k1, and Elk1 were indirectly implicated in various cell behavioral changes including survival and cell proliferation in subset of cells. We proposed that one of the mechanisms of Co–Cr-mediated cell proliferation involves upregulation and activation of the Ptk2 focal adhesion kinase (Fig. 9), which subsequently activates the ERK cascade: Raf1, Map2k1 (Mek1), and Mapk1 (Erk2) or Mapk3 (Erk1), via association with the adaptor protein Grb2. Positive regulation of cell proliferation and dif-
Differentiation into reactive astrocyte upon Ti-6Al-4V exposure is proposed to take a different induction route, independent of Ptk2, possibly due to mitogens and growth factors acting in autocrine fashion to "turn on" the ERK cascade. The transcription factor Elk1 may be activated leading to the production of proteins involved in cell proliferation and differentiation.

Cytotoxicity with biomaterial particles was observed in both C6 (Fig. 3) and Rat2 (Fig. 4) cells. Oxidative stress or oxidation-reduction reaction initiated by toxic substances may be the reason for this response. Continued accumulation of toxicants drives the net outcome toward more oxidative environment leading to metabolic disruption and cell damage. Unlike fibroblasts, astrocytes have the capacity to counteract oxidative stress by activating their antioxidant protective systems. Hence, IC$_{50}$ values (Table I) for C6 cells were higher compared to Rat2 fibroblasts, implying that C6 cells can tolerate higher quantities of toxicants. The relatively large IC$_{50}$ units, in milligrams per milliliter, indicate that the bulk of the material does not influence oxidative stress toxicity, but the released soluble and minute particles do.

The biomaterial particles used in this study were as follows (arranged in increasing average diameter): Ti-6Al-4V (2.3 μm) < UHMWPE (3.6 μm) < PMMA (4.0 μm) < Co–Cr (5.7 μm). At a constant volume, smaller particles provide greater surface area compared to the bigger ones, thus they release more toxicants that interact with cells. But due to variability in particle chemical composition, density, and other physical factors, the relative toxicity of the assayed particles cannot be determined solely by comparing their average diameters. In C6 cells, at constant mass per volume, Co–Cr and Ti-6Al-4V particles were determined to be the most and second most toxic among the tested materials, respectively. Both metal alloys exhibit toxic and necrotic effects when implanted in CNS parenchyma due to oxidation-reduction cycling reactions from released metal ions and compounds via corrosion and oxidation. UHMWPE was the least toxic in C6 cells. An earlier study has demonstrated that polyethylene in the cerebral cortex do not induce any reactive response. The inhibition of cell proliferation observed at 0.625 mg/mL of UHMWPE may be due to toxicity other than oxidative stress such as receptor-ligand interaction, while recovery at higher concentration may be caused by proliferation signaling response of astrocytic cells. Metal ions and particles are known to cause oxidative stress that kills cells via apoptosis; consistent with our finding that apoptosis is the main mode of cell death (Fig. 6). Additionally, necrosis was observed significantly in PMMA-treated C6 cells (Fig. 6), and it occurred slowly when compared with apoptosis (Fig. 5). Microscopic examination provided evidence that dense aggregates of PMMA and C6 cells (Fig. 6) led to necrosis most likely via physical obstruction of particles causing nutrient deprivation. PMMA bone cement implanted in the skull induced a slight reactive astrocyte response. Thus, it is suggested that the formation of gliosis is not caused by direct activation of astrocyte by PMMA but by some other mechanisms.

The Rat2 proliferative response to biomaterial particles followed the threshold model, in which the "no observed adverse effect level" zone was initially observed, but beyond a threshold concentration, cytotoxicity was exhibited. Rat2 cytotoxicity occurred at a much lower particle concentration compared to C6 cells. According to IC$_{50}$ data (Table I), PMMA caused the greatest toxicity to Rat2 cells, possibly because of PMMA’s ability to induce cell-particle aggregations that kills cells via necrosis. Metal alloy particles are less cytotoxic to Rat2 cells compared to PMMA but nevertheless induce cell death significantly at higher quantities. Data on 5 mg/mL
Ti-6Al-4V [Fig. 4(C)] suggest the activation of Rat2 cell proliferation. A possible explanation of this behavior is that Rat2 cells may utilize titanium alloy particles as substrate for growth and colonization. However, titanium alloy at high dose is also inducing its cytotoxic effect, thus the net result was cell count similar to untreated cells.

Astrocytes are phagocytic cells. They are known to engulf dead cells for removal, as well as foreign biomaterials such as polyactic-co-glycolic acid (PLGA) microspheres, dextran beads, and colloidal carbon. C6 cells in this study likewise appeared to phagocytose Co–Cr and Ti-6Al-4V particles (Fig. 8). Apoptotic astrocytes can be phagocytosed by healthy astrocytes. At 2 days incubation with 5 mg/mL Co–Cr and Ti-6Al-4V, minimal percentage of dead cells was observed (Fig. 5). But MTT data indicated the decrease in cell number during the 2-day metal alloy particle exposure. Hence, it is suggested that the apoptotic cells were cleared by phagocytic astrocytes at an earlier time point. Chang et al. demonstrated astrocyte phagocytic activity as early as 3 h postincubation with apoptotic cells. Fast clearance of apoptotic cells in vivo via phagocytosis was suggested to prevent the exposure of adjacent healthy tissues to inflammatory molecules to contain the damage and to maintain tissue homeostasis.

In summary, C6 cells selectively respond to orthopaedic biomaterial particles by proliferation, differentiation into reactive astrocyte form, and phagocytosis. Both Co–Cr and Ti-6Al-4V may activate the cell division pathway, but only titanium alloy particles provoke a reactive astrocytic morphology alteration. Biomaterials induce cytotoxicity via oxidative stress or receptor-mediated signaling. Additionally, bulk particles cause physical obstruction leading to decreased cellular respiration and necrotic death. This is the mode of cell death prevalent in PMMA-treated C6 cells. Rat2 fibroblasts exhibit cytotoxicity in a threshold dose-response manner to all of the tested biomaterials. Biomaterial particles did not enhance the proliferation rate of Rat2 cells, implying that the meningeal scar formation cannot be activated by just the presence of biomaterials alone. This study suggests that UHMWPE particles can be used in the CNS without causing extensive cell death or glial and meningeal scar formation.

References