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Biological significance of nanograined/ultrafine-grained structures: Interaction with fibroblasts

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ABSTRACT

Given the need to develop high strength/weight ratio bioimplants with enhanced cellular response, we describe here a study focused on the processing–structure–functional property relationship in austenitic stainless steel that was processed using an ingenious phase reversion approach to obtain an nanograined/ultrafine-grained (NG/UFG) structure. The cellular activity between fibroblast and NG/UFG substrate is compared with the coarse-grained (CG) substrate. A comparative investigation of NG/UFG and CG structures illustrated that cell attachment, proliferation, viability, morphology and spread are favorably modulated and significantly different from the conventional CG structure. These observations were further confirmed by expression levels of vinculin and associated actin cytoskeleton. Immunofluorescence studies demonstrated increased vinculin concentrations associated with actin stress fibers in the outer regions of the cells and cellular extensions on NG/UFG substrate. These observations suggest enhanced cell–substrate interaction and activity. The cellular attachment response on NG/UFG substrate is attributed to grain size and hydrophilicity and is related to more open lattice in the positions of high-angle grain boundaries.

1. Introduction

In the replacement or substitution of hard tissue implants, metallic materials including austenitic stainless steels and titanium alloys are widely used biomaterials as devices for bone fixation, partial/total joint replacement and spring clips for the repair of large aneurysmal defects because they are inherently corrosion resistant and possess the necessary mechanical strength and biocompatibility [1–3].

In recent years, surface modification processes such as heat treatment have been explored to induce nanostructuring on the surface and consequently promote osseointegration and modulation of cellular activity. While ultrafine structures may provide benefits of enhanced cellular attachment, stimulate metabolic activity and upregulate protein formation [4–9], it is important that we consider both the surface and bulk properties of the material that is in direct contact with the bone for long-term stability and an acceptable success rate of prosthetic rehabilitation. Thus, processing of bulk nanostructured materials assumes particular significance because it is a viable and potentially transformative approach to favorably modulate the cellular response of biomaterials and constitutes the motivation of the study presented here. Furthermore, nanostructured metals with high strength will enable the use of thinner bioimplants because of high strength/weight ratio. Given the above considerations, the present study aims to combine fundamental aspects of materials science and engineering and cellular and molecular biology to favorably modulate the cell–substrate response on nanograined/ultrafine-grained (NG/UFG) austenitic stainless steels, using a fibroblast cell line to investigate the cellular response. Fibroblast cells are relevant because they are cells of connective tissue that synthesize extracellular matrix (ECM) and collagen.

Regarding the success of a bioimplant in vivo, surface properties are critical for substrate–tissue interaction [10,11]. The chemistry and morphology of the surface can affect the attachment and subsequent growth behavior of cells on bioimplant material and consequently the compatibility between the host tissue and the implant [12,13]. Assuming that the ability of cells to adhere to a foreign surface is related to compatibility, cell adhesion is an important parameter for understanding biocompatibility [14,15]. It is safe to assume that substrate properties determine cell attachment, orientation, migration and metabolism [15–18]. Other surface parameters that modulate cell response include hydrophilicity, roughness and texture [19]. Higher roughness promotes bone-to-implant contact [20,21] and increases removal torque forces [22–24]. The surface properties determine the degree

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of bioimplant integration. Early colonization of the implant surface is likely to promote tissue repair in the peri-implant region, leading to effective integration of the metal implant [25,26]. The adhesion of cells to the substrate through an ECM provides signals that influence their ability to survive, proliferate and express specific developmental phenotypes [27].

The distinct and special properties of materials with sub-micron- to nanometer-sized grains are derived from the high density of grain/interphase boundaries, which are regions of high free energy [28]. Thermomechanical processing (TMP) is one of the primary methods to achieve grain refinement in metals [29–32]. Grain refinement limits imposed by conventional TMP can be overcome by the application of extensive plastic deformation, which leads to the formation of sub-micron or ultrafine structures in metallic materials [33,34]. The laboratory scale methods that have been adopted to obtain NG/UFG materials include equal channel angular pressing [34,35], accumulative roll bonding [36–38], high-pressure torsion (HPT) [39–42], multiple compression [43] and upsetting extrusion [44]. However, the ductility of the UFG materials produced by these methods is low compared to the coarse-grained (CG) materials. In this context, the work presented here is of particular significance because high ductility was obtained in the NG/UFG material.

The objective of the study is to describe processing–structure–functional property relationships in austenitic stainless steel that continues to be used for medical device in view of its long history. The NG/UFG austenitic stainless steels were processed by a novel procedure involving controlled phase reversion of strain-induced martensite in a cold-rolled austenitic stainless steel. Multi-pass cold deformation (~40–65%) of austenite at room temperature led to strain-induced transformation of austenite (face-centered cubic γ) to dislocation-cell-type martensite (bcc α'), which upon annealing in the temperature range of 600–850 °C transforms to NG/UFG austenite through a diffusional reversion mechanism, depending on the temperature–time annealing sequence [45–48].

Given that NG/UFG steel is likely to have surface properties different from conventional CG structure, the grain size effects on cellular response of CG and NG/UFG austenitic stainless steels were investigated. A comparison of the degree of cell–substrate interaction was assessed by studying the cellular activity of fibroblast cells cultured on NG/UFG and CG austenitic steel substrates. Considering that proteins in the ECM of cells regulate cell–substrate interactions and influence cell adhesion, proliferation, viability and differentiation [26], fibronectin, actin and vinculin, as well as cytoskeletal organization and focal adhesion contacts, were studied. Thus, an important objective of the study was to examine how cells recognize surfaces and interact with them and each other. If the initial cell–substrate interaction is optimal, then greater quantities of ECM would be released than in less stimulated cells. Thus, we hypothesize that a surface to which cells can more readily attach would promote cell adhesion and proliferation, and lead to effective integration of implants. We examined this hypothesis by testing the differences in cellular response to NG/UFG and CG structures and their relationship to surface properties.

2. Experimental

2.1. Cell substrate materials

The experimental material was commercially available 316L stainless steel. Stainless steel strips were obtained from Outokumpu Stainless Oy, Tornio and the nominal chemical composition in wt.% was Fe–0.017C–1.29Mn–17.3Cr–6.5Ni–0.15Mo–0.52Si. To develop NG/UFG structures, stainless steel strips were cold deformed to ~40–65% in a laboratory rolling mill using several passes (generally about 3–15% reduction per pass depending on the available rolling loads, where the contact area determines the rolling load). The reversion annealing was carried out in a Gleeble-1500 simulator in the range of 600–850 °C for periods of 10–100 s to obtain NG/UFG austenitic stainless steel. For experiments described here, the cold reduction was ~62% and annealing was carried out at 800 °C for 1–10 s. The heating rate to the holding temperature was 200 °C s^{-1}, and following annealing, cooled in an airflow at rates of 200–400 °C s^{-1}.

2.2. Characterization of cell substrate materials

The grain structure of CG and NG/UFG austenitic stainless steels was examined using light microscopy and transmission electron microscopy (TEM) operating at 120 kV (Hitachi 7600), respectively. It may be noted that the resolution and magnification of light microscope is inadequate to examine the grain structure of NG/UFG steel, while CG structure can be conveniently examined by light microscope at low magnification, avoiding the need to prepare electron transparent samples for TEM. Thin foils for examining NG/UFG structures by transmission electron microscopy were prepared by punching 3 mm disks from the specimens and twin-jet electropolishing with a solution of 10% perchloric acid in acetic acid as electrolyte. The mechanical properties of the cold-rolled and reversion-annealed austenitic stainless steel specimens were determined by tensile testing samples machined to a profile of 25 mm × 25 mm with a 20 mm gage length. The structure of CG austenitic stainless steel was examined after electropolishing in a solution of 39% nitric at a current density of ~30 mA cm^{-2} and 1.3 V DC for 2–3 min.

The crystallographic texture was studied in the surface layer. Samples (14 mm × 24 mm) were cut, ground close to the investigated layer, and electropolished. Mo Kα radiation was used in the X-ray diffraction, and complete pole figures were measured in back reflection mode. Pole figures (1 1 0), (2 0 0), (2 1 1) and (3 1 0) were used for ferritic (α'-martensite) phase and (1 1 1), (2 0 0), (2 2 0) and (3 1 1) for the austenite. The orientated distribution functions were calculated using a series expansion method ($I_{max} = 22$) from the pole figure data and plotted in contour lines in the Euler space (Bunge's notation) [46,48].

Surface roughness of the CG and NG/UFG austenitic stainless steel substrates was examined by atomic force microscopy (AFM Dimension 3000, Digital Instruments, Santa Barbara, CA) in tapping mode. Surface wettability was measured as contact angle of deionized water using a goniometer system equipped with imaging analysis software (Ramé-Hart, Inc., Germany). An autopipetting system was used to ensure a droplet of water with uniform volume (0.5 μL).

2.3. Fibroblast–metal surface interaction

Polished NG/UFG and CG stainless steel samples were cleaned in an ultrasonic bath with ethanol, followed by washing with deionized water, then wrapped individually in gauze and sterilized in an autoclave. Cell culture studies were performed using mouse fibroblast cell line (L-cell-L929) obtained from the American Type Cell Culture Collection (Manassas, VA, USA). Eagle's minimum essential medium (Invitrogen Corporation, USA) supplemented with 10% horse serum, penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) was used to culture fibroblasts. Cells with 80–85% confluence obtained from T-flask cultures by trypsinization were used to seed onto the disk samples. Briefly, cells were washed with phosphate-buffered saline (PBS), incubated with 0.25% trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) for 5–7 min to detach the cells from the Petri dish, dispersed in trypsin/EDTA, transferred to a centrifuge tube and centrifuged at 322 g for 5 min. The cell pellet obtained after