Gene expression profiling of bone cells on smooth and rough titanium surfaces

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Gene Expression Profiling of Bone Cells On Smooth and Rough Titanium Surfaces

J. HARLE[1], V. SALIH[2], I. OLSEN[2], P.M. BRETT[1]

Divisions of [1]Infection & Immunity and [2]Biomaterials & Tissue Engineering, Eastman Dental Institute, University College London, 256 Gray's Inn Road, London, WC1X 8LD, UK.

Abstract

Titanium (Ti) and Ti alloys are widely used as dental and orthopaedic implants, but the effects of the surface characteristics of these materials on the response of cells and target tissues is not well understood. The present study has therefore examined the effects of a rough Ti (RT) and a smooth Ti (ST) surface on human bone cells *in vitro*. Scanning electron microscopy showed attachment and spreading of cells on both surfaces. Expression profiling using ATLAS™ gene arrays showed marked differences in gene responses after 3 hours of culture. A number of osteoblast genes were identified as 'roughness response' genes on the basis of changes in expression on the RT compared with the ST surfaces. The surface roughness of Ti was thus found to have a profound effect on the profile of genes expressed by the bone cells, and suggests that improvements in the biological activity and possibly the clinical efficacy of these materials could be achieved by selective regulation of gene expression mediated by controlled modification of Ti surface.

Keywords: Titanium, surface, bone cells, gene expression

1. Introduction

Titanium (Ti) and Ti alloys have been widely used as components of dental and orthopaedic implants for many years, although the precise effects of these materials on the function of target tissues are not yet known. However, *in vitro* studies of bone-derived cells have shown that the roughness of Ti surfaces has an important influence on osteoblast morphology, proliferation, differentiation and production of soluble mediators [1-3]. In addition, certain intracellular signalling pathways have also been reported to be involved in the response of bone cells *in vitro* to surface roughness [4, 5]. Nevertheless, the range of gene events affected by different surface characteristics of implant materials, including Ti, has thus far not been examined and is fundamental to understanding the cellular effects of environmental stimuli. These changes in gene expression undoubtedly involve a large number of both known and unknown genes whose identification has been hampered, until recently, by the technical difficulties associated with the simultaneous study of multiple gene products.

Gene profiling allows for quantification and comparison of gene expression in different cell/tissue populations with nylon-based cDNA microarrays, which are both sensitive and affordable. In this procedure, specific nucleotide sequences corresponding to known genes are fixed on the nylon membrane, which is then used to hybridise the entire range of radiolabeled cDNA sequences that have been obtained by reverse-transcription of the mRNA extracted from biological samples. A radioactive signal is thus generated on the membrane at the sites at which the same gene was present in the sample. The intensity of this signal thus corresponds to the level of gene expression in the extract and can be detected by x-ray film. The hybridisation of two cDNA samples on identical microarrays can therefore be compared to evaluate the

differential gene expression of over 1000 genes simultaneously in the two samples, at mRNA expression levels as low as 1 in 10,000 total sequences [6]. In the present study we have utilised this gene profiling procedure to examine gene expression by human alveolar bone cells incubated on two Ti surfaces of different roughness.

2. Materials and methods

2.1 Titanium

Ti discs of 15 mm diameter (Straumann Institute, Switzerland), were used in all experiments. The two different surfaces consisted of a smooth Ti surface (ST), formed by mechanical polishing, and a rough titanium surface (RT), formed by sand-blasting and acid-etching. These surfaces had R_a roughness values of 0.6 and 4.0 μm. Discs were cleaned in 10% (v/v) nitric acid, rinsed with distilled water and sterilized with UV light for 1 h prior to use.

2.2 Culture of alveolar bone (AB) cells

Fragments of AB were obtained from a male patient aged 26 undergoing routine molar extraction, following a protocol approved by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital (London, UK). They were immediately placed into alpha-minimal essential medium (α-MEM) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10% foetal calf serum (FCS) and 25 μg/ml fungizone, (all Gibco Life Technologies, UK). After cutting into small pieces (1-2 mm^3), they were washed with phosphate-buffered saline (PBS) (Gibco), placed into 6-well culture plates (Becton Dickinson, UK) in complete Dulbecco's minimal essential medium (DMEM), without fungizone, and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells migrated from the

fragments after approximately 7 to 15 days and were grown to confluence before detaching with 0.25% trypsin-1 mM EDTA (Gibco) for 5 min at 37°C. The resulting cell suspensions were centrifuged, washed with fresh medium and seeded into 25 cm^2 culture flasks and maintained in supplemented DMEM. The osteoblastic phenotype was confirmed by flow cytometry analysis of the expression of characteristic bone antigens (osteonectin, osteopontin, bone sialoprotein, alkaline phosphatase) and the formation of mineralised nodules *in vitro* by von Kossa staining, as previously described [7]. The cells were used between passages 2 and 5.

2.3 Scanning electron microscopy (SEM)

The AB cells were cultured for 3 h on each Ti surface, fixed in 3% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3) (both Sigma, UK) at 4ºC overnight then dehydrated in a graded series of alcohols (50%, 70%, 90% and two changes of 100% ethanol), washed with hexamethyldisilazane (TAAB Laboratories, UK) for 5 min and placed in a desiccator overnight. After 24 h, the Ti discs were mounted onto stubs using Araldite (Devcon, UK) and Liquid Dag (Neubauer Chemikalen, Germany) and left to air-dry for 24 h. Specimens were then sputter-coated with gold/palladium using a Polaron E5100 coating device (Polaron CVT, UK) and observed using a Cambridge Stereoscan S90B (Cambridge Instruments, UK). The same treatment was carried out on Ti surfaces that had not been seeded with cells.

The RT and ST discs were seeded with approximately 50,000 cells and incubated in DMEM for 3 h. The cells were detached from the Ti surfaces with trypsin and total RNA isolated using the Quiagen RNeasy® mini kit (Qiagen Sciences, MD, USA), then stored at -70ºC. Samples were treated with 100 U DNase I (Gibco) at 37ºC for 5 min to remove DNA contamination and to reduce non-specific binding to the filters.

2.4 Gene Expression Profiling

Expression profiling was performed using the Atlas™ Human Cancer 1.2 gene array (Clontech, CA, USA) containing 1176 genes, many of which regulate cell growth and differentiation and could thus have an important role in the resultant Ti-cell interaction. 5 µg of total cellular RNA was used for reverse transcribing to cDNA, using [32P]-dCTP (Amersham Biomedical, UK) to radioactively label the sequences. Atlas™ gene array kit protocols and reagents were used according to the manufacturers instructions. The labelled cDNA probes were then hybridised to the Atlas™ gene array filters at 68°C for 18 h, using a rotary incubator to ensure cDNA sample mixing across the microarray surface. The membranes were washed and exposed to autoradiographic film for 72 h, at -70°C to enhance film sensitivity. The films were digitally scanned for image analysis using an Alpha Imager 1200 light cabinet (Alpha Innotech, USA) and intensity values obtained for each gene after subtraction of local background intensity. The AtlasImage™ 2.0 software used a global normalization function to allow comparison between the ST and RT arrays, which was inversely proportional to the sum of background-adjusted values for all genes on each microarray. These background adjusted and normalized values for each gene were thereafter compared between the microarrays. Genes were considered to show marked expression change when there was a greater than 3-fold change in the normalized background-adjusted normalized value, and in addition a 5000 numerical difference in such values.

3. Results

Fig. 1(a) shows SEM images of the ST and RT surfaces. The former was very flat and almost defect-free while the RT surface consisted of many concave pits of varying diameter and depth. Fig. 1(b) shows the morphology of the AB cells after 3 h of incubation on each surface, with the cells adherent to the ST surface beginning to spread and form visible cytoplasmic processes. Very few rounded cells were present. In contrast, a number of rounded cells were visible on the RT surface, although the non-rounded cells appeared to exhibit comparable degrees of spreading and cytoplasmic processes. At 24 h, cells on both the ST and RT surfaces had increased the extent of cell spreading to a comparable level, with few remaining rounded cells (results not shown).

Gene profiling showed that there were a marked number of gene expression changes in AB cells incubated on the different Ti surfaces. Of the total of 1176 genes detected on the Atlas™ arrays, a total of 10 genes were found to be up-regulated and 16 down-regulated, as determined by the strict expression change criteria described in the Materials and Methods. These results in Table 1. show that these genes comprise a diverse range of functions including cell signalling, DNA synthesis and cytoskeletal/structural filaments. Fig. 2 shows digital images of the ST and RT filters with arrows indicating the position of the neurotrophin 4 gene, encoding a growth factor protein, which showed the greatest degree of up-regulation (nearly 13-fold) as a result of incubation on the RT surface.

4. Discussion and Conclusion

Gene expression changes associated with cell-material interactions define the fundamental molecular mechanisms that mediate cell growth and function following exposure to the implant material. The development of commercially available methods for simultaneously measuring changes in the expression of multiple genes has facilitated the identification of both known and unexpected genes that play a part in many biological functions [6]. However, few studies have used this technology for assessing cell responses to materials, and for identifying genes of key importance in these interactions.

The present study has investigated the effects of two types of Ti, with markedly different surface features, on bone cell morphology and gene expression profile. The surface topography observed by SEM analysis (Fig. 1) confirmed the different degrees of roughness of the RT and ST surfaces, consistent with their respective R_a values as previously reported [8]. This study has also shown that the expression of a number of osteoblast genes was affected by these Ti surface features *in vitro*. Of the total of 1176 genes on the Atlas™ Human Cancer 1.2 gene array, the expression of 10 was found to be up-regulated by more than 3-fold on the RT surface, compared to the ST surface at 3 h. In contrast, 16 genes were found to exhibit down-regulated expression profiles on the RT surface after 3 h. Analysis of the specific DNA sequences which most positively responded to the RT surface compared with the ST surface highlighted the gene neurotrophin 4, which has previously been implicated in the increased expression of the bone-associated proteins alkaline phosphatase and osteopontin during fracture healing and periodontal tissue regeneration [9]. In addition, the results presented here have also highlighted a number of genes that have not previously been associated with the process of wound regeneration/healing or osseointegration. For example, certain genes involved in DNA synthesis/repair and heat shock

proteins were found to be up-regulated in cells incubated on the RT surface, whereas genes corresponding to intermediate filament, histocompatibility and apoptosis-associated proteins were down-regulated by the RT surface.

Expression profiling has thus enabled candidate genes that are differentially expressed on different Ti implant materials of varying surface roughness to be identified, including a number of extracellular matrix proteins, metalloproteinases and adhesion possibly involved in normal wound healing events [10]. The different gene expression profiles of AB cells in response to these Ti surfaces *in vitro* may indicate possible differences in clinical efficacy *in vivo* and thus have important implications for bone repair and regeneration processes.

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Table 1. Gene expression by AB cells on the RT surface compared with the ST surface after 3 h of incubation. The ten most up-regulated genes, i.e. showing greatest expression change, within the exclusion criteria defined in Materials and Methods, are presented, as are the sixteen most down-regulated genes within such criteria. ST expression is defined as 1.00.

Protein / Gene	Genebank Accession No.	Function	Expression Change RT / ST
Up-regulation on RT compared to ST			
neurotrophin 4	M86528	cell signal protein	12.8
60S ribosomal protein L32	X03342	ribosomal protein	10.6
glutathione-S-transferase-like protein	U90313	DNA synthesis/repair protein	4.9
interferon-regulated resistance GTP-binding protein MXA	M33882	endocytosis protein	4.7
ras-related associated with diabetes protein	L24564	G protein	4.2
axl tyrosine-protein kinase receptor	M76125	protein kinase receptor	4.1
death domain receptor 3	Y09392	death receptor	4.1
cell division protein kinase 6	X66365	cell cycle-regulating kinase	3.9
70-kDa heat shock protein 5	M19645	heat shock protein	3.1
caveolin 1	Z18951	GTP/GDP exchanger	3.0
Down-regulation on RT compared to ST			
rho-GAP hematopoietic protein C1	X78817	GTP/GDP exchanger	0.29
translation initiation factor EIF-2B alpha subunit	X95648	translation factor	0.26
cyclin-dependent kinase 4 inhibitor 2D	U40343	kinase inhibitor	0.21
dishevelled homolog 1-like protein	U46461	intraceelular modulator	0.19
ribosomal protein S6 kinase II alpha 1	L07597	ribosomal protein	0.19
matrix metalloproteinase 16	D50477	matrix metalloproteinase	0.18
type II cytoskeletal 8 keratin	M34225	intermediate filament protein	0.17
fatty acid synthase	S80437	lipid metabolism enzyme	0.17
signaling lymphocytic activation molecule	U33017	receptor	0.14
glutathione S-transferase theta 2	L38503	apoptosis-associated protein	0.13
cyclic nucleotide phosphodiesterase 1B (CAM-PDE1B)	U56976	adenylate cyclase	0.12
DNA recombination & repair protein HNGS1	AF022778	DNA synthesis/repair protein	0.10
HLA-DR antigen	X00497	histocompatibility protein	0.04
CD82 antigen	U20770	oncogene	0.03
cytokeratin 1	M98776	intermediate filament protein	0.02
protein phosphatase EF2 long form (PPEF2)	AF023456	calcium binding protein	0.00

Table 1. Harle et al.

Figure Legends

Fig. 1.

SEM images of the ST and RT surfaces. The surfaces are shown before (a) and after (b) 3 h of incubation with AB cells (Magnification x750 and x200, respectively).

Fig. 2.

Autoradiograph images of the Atlas™ gene array filters showing the relative hybridisation levels of specific genes from AB cells on ST and RT surfaces, after 3 h incubation. ^{32}P-labelled dCTP was used to radiolabel cDNA sequences for hybridisation. The arrows show the relative up-regulation of the signal on RT, compared to ST, for the neurotrophin 4 gene.

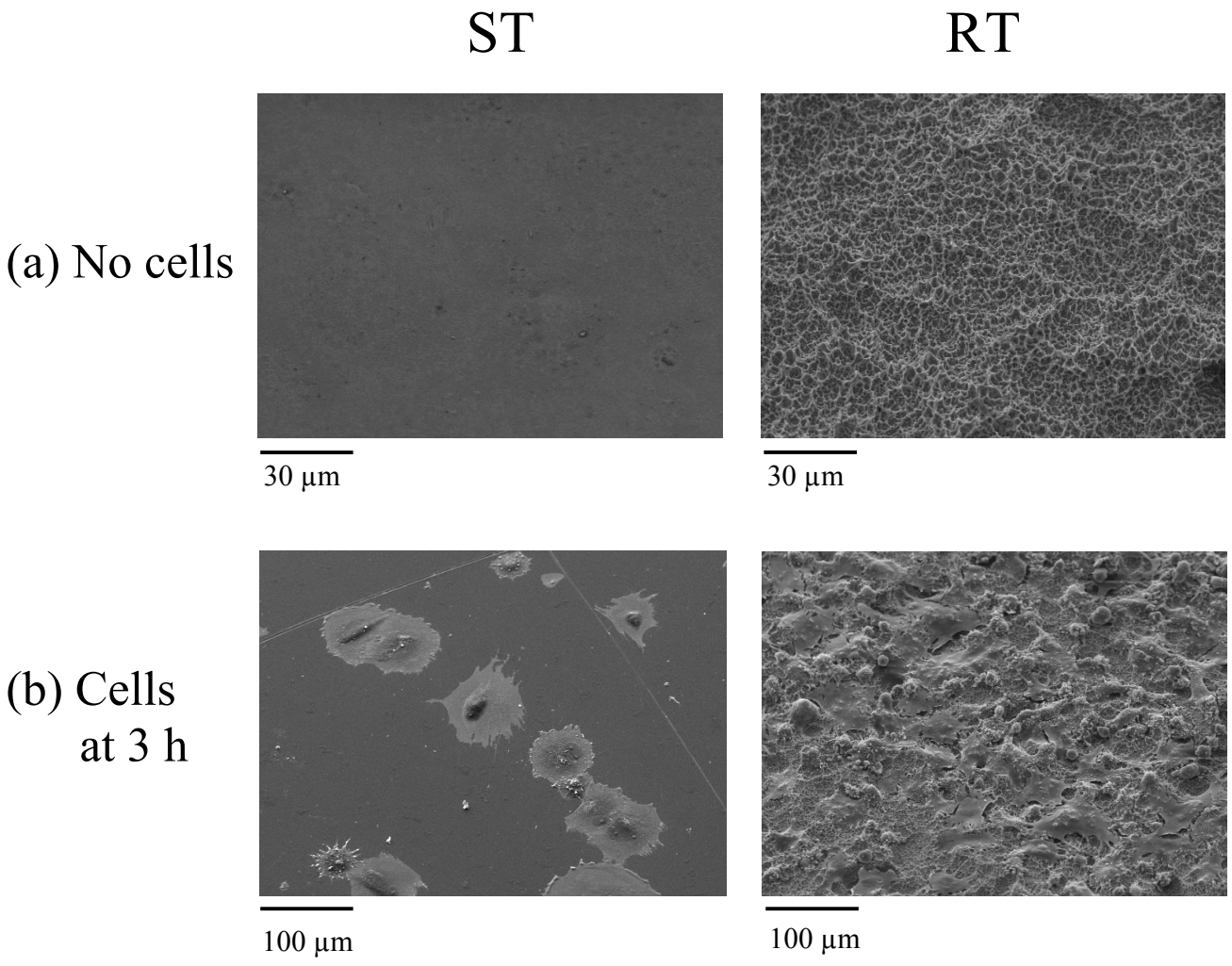

Fig. 1. Harle et al.

ST RT

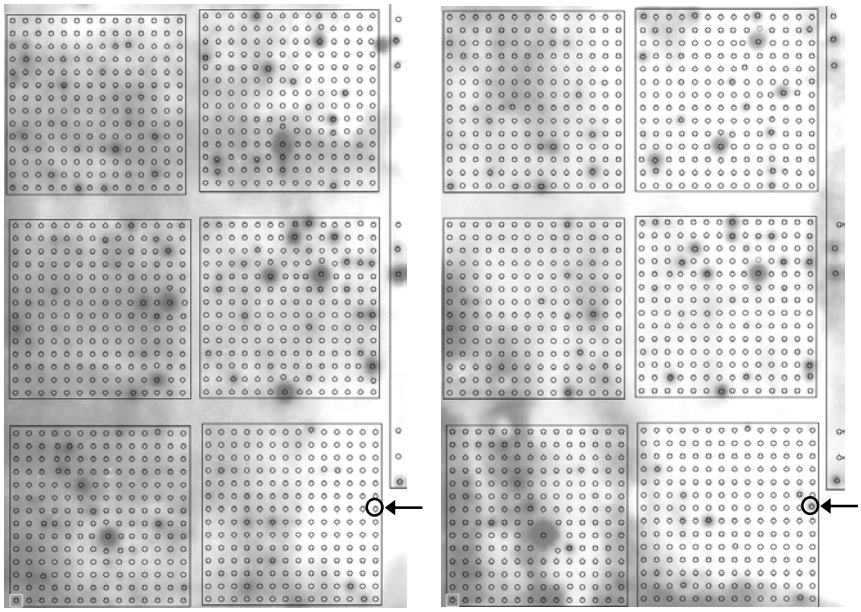

Fig. 2. Harle et al.