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Biomaterials 23 (2002) 4193–4202

Biomaterials

www.elsevier.com/locate/biomaterials

Large-scale gene expression analysis of osteoblasts cultured on three different Ti–6Al–4V surface treatments

Ching-Hsin Ku[a,b], Martin Browne[b], Peter J. Gregson[b], Jacques Corbeil[d], Dominique P. Pioletti[a,c,*]

[a] *Bone Bioengineering Group, Institute for Biomedical Engineering, Swiss Federal Institute of Technology, Lausanne, Switzerland*
[b] *Bioengineering Sciences Research Group, School of Engineering Sciences, University of Southampton, Southampton, UK*
[c] *Orthopaedic Hospital, Lausanne, Switzerland*
[d] *Department of Medicine, University of California, San Diego and San Diego Veterans Affairs, Healthcare System, La Jolla, USA*

Received 24 October 2001

Abstract

To improve implant biocompatibility, we developed a simple cost-effective thermal surface treatment allowing an increase in the oxide layer thickness of a titanium (Ti) alloy used in orthopaedic implants. The goal of this study was to test in vitro the reaction of osteoblasts to the developed surface treatment and to compare it to the osteoblast reaction to two other surface treatments currently used in the practice of implant surgery. Quantification of osteoblast gene expression on a large scale was used in this study. The kinetics of gene expression over 120 h was followed for 58 genes to quantify the effect of the developed surface treatment. Twenty eight genes were further selected to compare the effects of surface treatments on osteoblasts. Based on the genes studied, we could propose a general pathway for the cell reaction according to the surface treatments used: (1) metal ion release changes the time course of gene expression in the FAK pathway; (2) once the accumulation of metal ions released from the Ti surface exceeds a threshold value, cell growth is diminished and apoptosis may be activated; (3) PTK up-regulation is also induced by metal ion release; (4) the expression of Bcl-2 family and Bax may suggest that metal ions induce apoptosis. The developed treatment seems to increase the Ti–6Al–4V biocompatibility as highlighted by the lower impact of this treatment by the different pathways studied, on the lower inflammatory reaction that could be induced, as well as by the lower induced osteoblast apoptosis compared to the two other surface treatments.

Keywords: Osteoblast; Implant; Surface treatment; Gene expression; Apoptosis; cDNA microarray

1. Introduction

When an implant is inserted into bone, it is expected that an apposition of bone to the implant will occur, a process called osteointegration [1]. From histological examinations it has been found that implant loosening is generally associated with the formation of fibrous tissue at the bone–implant interface [2].

It has been hypothesized that the differentiation process of the peri-implant tissue could be driven either by unfavourable mechanical situations [3] or by inflammatory reactions following the phagocytosis of wear particles [4] or by a combination of both [5]. We recently showed that wear particles also influence osteoblast behaviour [6–9]. Release of metal ions by the implant and their effect on osteoblasts has been mostly neglected until recently [10]. Due to the faster process of ion released compared to wear particles generation, interaction of osteoblast–ion would be important to quantify as this phenomenon occurs soon after prosthesis implantation.

To improve implant biocompatibility, we developed a simple, cost-effective thermal surface treatment allowing an increase in the oxide layer thickness of a titanium alloy used for orthopaedic implants [11,12]. This oxide layer acts as a barrier to keep ions from being released into the body. Kinetic analysis showed a decrease in ion release in the developed surface treatment [13].

Information on Ti–6Al–4V alloy treated surfaces affecting osteoblasts is limited. The goal of this study is then to test the osteoblast reaction to the developed

*Corresponding author. Institute for Biomedical Engineering, Bat AAB, EPFL, 1015 Lausanne, Switzerland. Tel.: +41-21-693-8341; fax: +41-21-693-8660.
E-mail address: dominique.pioletti@epfl.ch (D.P. Pioletti).

0142-9612/02/$ - see front matter
PII: S0142-9612(02)00161-8

surface treatment and to compare it to the osteoblast reaction to two other surface treatments currently used in the practice of orthopaedic implant surgery. The strategy of this study is to perform a large-scale analysis of gene expression in order to highlight possible regulation pathways differentiated by the surface treatments.

2. Materials and methods

2.1. Ti alloy surface preparations

Distal sections of forged Ti–6Al–4V alloy femoral stems from the Ti-Mod Freeman hip implant were supplied by Finsbury Instruments (Leatherhead, Surrey, UK). The hip stems were cut into discs of 10 mm diameter and of 1 mm thickness. The samples were polished following a previously described procedure [14]. The samples were then cleaned in 1% Triton solution for 1 h and rinsed in deionized distilled water. This treatment was used as control (C). The practice for surface preparation of surgical implants involves a nitric acid passivation treatment (P) with an immersion of the implants in 30% nitric acid for 1 h [15]. We developed a third treatment that we called aged treatment (A), which consisted of the passivated treatment followed by ageing in boiling deionized distilled water for 10 h [11,12]. The samples were then sterilized and tested for endotoxin contamination [14].

2.2. Cell culture

Primary human osteoblasts (hOB) were isolated from pieces of cortical bone femur obtained during total hip arthroplasty of a female patient (62 yr) following a procedure previously described [16]. The osteoblastic cells were cultured in Dulbecco's modified Eagle's medium (Sigma, Buchs, Switzerland) containing 10% fetal bovine serum (Sigma), 1% PSF (100 ×, 10,000 U/ml Penicillin, 10,000 mg/ml Streptomycin and 25 mg/ml Fungizone) (GibcoBRL, Life Technologies, Basel, Switzerland) under a humidified 5% CO_2/air atmosphere at 37°C. Osteoblasts passages lower than 4 were used for the study. The osteoblasts were seeded at a concentration of 380,000 cells/cm^2 on each sample. In order to have enough material to study gene expression, 6 samples of each treated surface were used (total cells number: 1.62×10^7).

2.3. Gene expression study

Genefilters GF211 (Research Genetics, Huntsville, AL, USA) were used to monitor the expression of 12,626 genes. The collected RNA from the cells (5 mg of total RNA per condition) was processed according to the manufacturer's recommendations. The Pathways™ software, developed by Research Genetics was used to acquire the Genefilters data. Normalization of gene expression and data analysis was performed using Excel (Microsoft Corporation, Redmond, WA, USA) and 2HAPI, a web-based bioinformatics software (http://array.sdsc.edu). The kinetics of gene expression for hOB incubated with the aged Ti–6Al–4V was measured at 4, 24, 48 and 120 h. The gene expression of cells incubated 4 h on the aged sample (A_4) was chosen as the reference. 58 genes were selected and classified in 6 groups according to adhesion, signal pathway and transcription factors, growth factors and cytokines, differentiation, mineralization and apoptosis. This selection represents a panel of major genes expressed by osteoblasts [17,18].

Twenty-eight genes were specifically analysed to compare the difference in gene expression between different surface treatments. The results are presented as the ratio of P/A and C/A for the corresponding gene expression.

For both analyses, the difference in gene expression is based on the experimental finding that upregulation by a ratio higher than 2.5-fold or down-regulation by a ratio lower than 0.4-fold ($=1/2.5$) has to be reached to be considered as significant. Sample control at 24 h was analysed twice with two filters ($C_{24(1)}$ and $C_{24(2)}$) and showed good reproducibility (70% of the gene tested had a gene expression variation lower than 10% between the two analysis) (data not shown).

3. Results

The results presented in Table 1 are intended to evaluate the kinetics of gene modulation of the surface treatment A, while results presented in Table 2 are used to compare gene expression with respect to the different surface treatments (C, P, A). The functional description of the chosen genes is given in Table 3. Sample A_{24} did not have enough RNA for the analysis.

3.1. Kinetics of genes modulation by the surface treatment A (Table 1)

From a quantitative point of view, in comparison with A_4, A_{48} has 213 up-regulated genes and 5 down-regulated genes; A_{120} has 247 up-regulated genes and 5 down-regulated genes. More specifically, 58 genes were selected and described in the following groups.

3.1.1. Adhesion

Collagen alpha-2 type I was up regulated 4.4-fold at 48 h and 3.48-fold at 120 h compared to A_4. Integrin $\alpha_3, \alpha_5, \alpha_6, \beta_1, \beta_5$, and fibronectin were expressed by the isolated hOB, but did not show a significant difference compared to A_4.

Table 1
Kinetic of gene expression for the aged Ti–6Al–4V surface (58 genes)

Acc	Gene name	A_{48}/A_4	A_{120}/A_4
	Adhesion		
U70312	Homo sapiens integrin binding protein Del-1 (Del1) mRNA	1.32	0.98
M59911	Human integrin alpha-3 chain mRNA	0.87	1.76
X06256	Human mRNA for integrin alpha 5	0.81	0.69
X53586	Human mRNA for integrin alpha 6	0.93	0.84
X07979	Human mRNA for integrin beta 1	0.55	0.79
M35011	Human integrin beta-5 mRNA	0.80	0.79
X53002	Human mRNA for integrin beta-5	0.68	0.61
X02761	Human mRNA for fibronectin (FN precursor)	1.10	1.26
M10905	Human cellular fibronectin mRNA	1.14	1.42
J03464	Human collagen alpha-2 type I mRNA	**4.40**	**3.48**
	Signal pathway and transcription factors		
L13616	Human focal adhesion kinase (FAK) mRNA	**0.16**	0.73
Z11695	Homo sapiens 40 kDa protein kinase related to rat ERK2 (MAPK1, MAPK2)	0.52	0.72
X80692	Cluster Incl X80692: Homo sapiens ERK3 mRNA (MAPK6)	0.49	0.41
AF002715	Cluster Incl AF002715:Homo sapiens MAP kinase kinase kinase (MTK1)	**0.33**	0.46
D87116	Human mRNA for MAP kinase kinase 3b	**0.22**	**0.23**
J04111	Human c-*jun* proto oncogene (JUN), clone hCJ-1	0.43	0.63
V01512	Human cellular oncogene c-*fos*	2.23	1.52
U02680	Human protein tyrosine kinase mRNA (PTK9)	0.50	0.63
M59371	Human protein tyrosine kinase (PTK) mRNA (EPHA2)	**3.20**	**9.09**
AF015254	Homo sapiens serine/threonine kinase (STK-1) mRNA	**10.10**	—
	Growth factors and cytokines		
M37825	Human fibroblast growth factor-5 (FGF-5) mRNA	**5.85**	**6.37**
M60828	Human keratinocyte growth factor (KFG = FGF-7) mRNA (3853 bp)	1.33	2.80
X03563	Human gene for Insulin-like growth factor I	**4.38**	—
M77349	Human transforming growth factor-beta-induced gene product (BIGH3)	1.31	0.82
X06374	Human mRNA for platelet-derived growth factor PDGF-A	1.41	1.83
M22488	Human bone morphogenetic protein 1 (BMP-1) mRNA	0.79	—
M22489	Human bone morphogenetic protein 2A (BMP-2A) mRNA	—	—
M22490	Human bone morphogenetic protein-2B (BMP-2B) mRNA	0.76	—
U43842	Homo sapiens bone morphogenetic protein-4 (hBMP-4) gene	0.43	0.57
M27968	Human basic fibroblast growth factor (bFGF) mRNA	0.52	0.61
M28983	Homo sapiens interleukin 1 alpha (IL-1) mRNA	—	—
	Differentiation		
J04948	Human alkaline phosphatase (ALP-1) mRNA	1.22	1.34
L40992	Homo sapiens core-binding factor, runt domain, alpha subunit 1 (CBFA1)	—	—
L38517	Homo sapiens indian hedgehog protein (IHH) mRNA, 5′ end	—	—
AF009801	Homo sapiens homeodomain protein (BAPX1) mRNA	—	—
	Mineralization		
J03040	Human SPARC/osteonectin mRNA	1.76	1.49
J04765	Human osteopontin mRNA	0.86	—
J04599	Human hPGI mRNA encoding bone small proteoglycan I (biglycan)	1.42	1.82
J05213	Homo sapiens sialoprotein precursor (IBSP) mRNA	—	—
	Apoptosis		
U37518	Human TNF-related apoptosis inducing ligand TRAIL mRNA	—	—
AF014794	Homo sapiens TNF related TRAIL receptor (TRAIL-R3) mRNA	—	—
M58603	Human NF-κB DNA binding subunit (NF-kappa-B) mRNA (NF-κB1)	0.85	0.92
L19067	Human NF-κB transcription factor p65 subunit mRNA (Rel A)	0.69	0.83
AF018253	Homo sapiens receptor activator of nuclear factor-kappa B (RANK) mRNA	—	—
AF022385	Homo sapiens apoptosis-related protein TFAR15 (TFAR15)	0.52	0.41
Y11588	Homo sapiens mRNA for apoptosis specific protein	—	—
AF053712	Homo sapiens osteoprotegerin ligand mRNA (TRANCE)	—	—
M37435	Human macrophage-specific colony-stimulating factor (M-CSF)	0.90	0.56
M13207	Human granulocyte-macrophage colony-stimulating factor gene (GM-CSF)	—	—
X03656	Human gene for granulocyte colony-stimulating factor (G-CSF = CSF-3)	—	—
M14745	Human bcl-2 mRNA (BCL2 (B-cell CLL/lymphoma 2))	—	—

Table 1 (*continued*)

Acc	Gene name	A_{48}/A_4	A_{120}/A_4
M13994	Human B-cell leukemia/lymphoma 2 (bcl-2) proto-oncogene mRNA encoding bcl-2-alpha protein (BCL2 (B-cell CLL/lymphoma 2))	—	0
M13995	Human B-cell leukemia/lymphoma 2 (bcl-2) proto-oncogene mRNA encoding bcl-2-beta protein	—	—
Z23115	Homo sapiens bcl-xL mRNA	**6.42**	**10.08**
L22473	Human Bax alpha mRNA	1.12	0.68
L22474	Human Bax beta mRNA	1.32	—
L22475	Human Bax gamma mRNA	0.47	—
U19599	Human Bax delta mRNA	0.99	0.67

The column A_{48}/A_4, represents the gene expression of osteoblasts seeded 48 h on aged sample divided by the gene expression of osteoblasts seeded 4 h on aged sample. Similar nomenclature is used for the other columns. "—" means that no signal was measured. The value in bold type means significant expression ($\geqslant$2.5-fold: up-regulation; $\leqslant$0.4-fold: down-regulation). Acc is the Genebank accession number.

3.1.2. Signal pathways and transcription factors

Focal adhesion kinase (FAK) and mitogen-activated protein (MAP) kinases were down-regulated at 48 and 120 h. c-*jun* showed a 0.43-fold decrease and c-*fos* was increased 2.23-fold for A_{48}. Protein tyrosine kinase (PTK9) showed a 0.5-fold decrease at 48 h. PTK receptor was upregulated 3.20-fold at 48 h and increased with time. Stem cell tyrosine kinase 1 (STK-1) was up-regulated 10.10-fold at 48 h but undetected at 120 h.

3.1.3. Growth factors and cytokines

FGF-5 was up-regulated 5.85-fold and increased to 6.37-fold at 120 h. Keratinocyte growth factor (KGF = FGF-7) was up-regulated 2.8-fold at 120 h. Insulin-like growth factor (IGF-1) was up-regulated 4.38-fold at 48 h and not detected at 120 h. Transforming growth-factor-β-induced gene (BIGH3) and platelet-derived growth factor (PDGF-A) were detected, but did not show significant modulation. Most of the BMPs were not detected (BMP-2A, BMP-3 and BMP-5, data not shown) or down-regulated (BMP-1, BMP-2B and BMP-4). Genes, which have been proved to be involved in the process of osteoporosis, such as basic fibroblast growth factor, (FGF-2 or bFGF) and interleukin 1 alpha (IL-1α) were either absent or down-regulated compared to the reference A_4. The expression of pro-inflammatory mediators including IL-6 and prostaglandin E-2 (PGE-2) were not detected (data not shown).

3.1.4. Differentiation

No significant gene expression for core-binding factor runt domain alpha subunit 1 (cbfa1), Indian hedgehog (IHH) and homeodomain (BAPX1) was observed, except for alkaline phosphatase (ALP), which had a 1.22-fold increase at 48 h and a 1.45-fold increase at 120 h.

3.1.5. Mineralization

SPARC/osteonectin, osteopontin (OPN), and biglycan (bone matrix glycoproteins), which are involved in bone matrix mineralization, were detected. SPARC had a 1.76-fold increase, OPN showed a 0.86-fold decrease and biglycan showed a 1.42-fold increase at 48 h. Sialoprotein precursor (IBSP) was absent.

3.1.6. Apoptosis

NF-κB1 showed a 0.85-fold decrease at 48 h and 0.92-fold decrease at 120 h. NF-κB p65 showed a 0.69-fold decrease at 48 h and a 0.83-fold decrease at 120 h. The apoptosis related protein, TFAR15, showed a 0.52-fold decrease at 48 h and a down-regulation of 0.41-fold at 120 h. Bcl-xL was up-regulated 6.42-fold at 48 h and up-regulated 10.08-fold at 120 h. Bax isoform (alpha, beta, gamma and delta) decreased over time.

3.2. Comparison of gene expression with respect to the surface treatments C, P, A (Table 2)

In order to determine differential hOB gene expression from various treated surfaces, 28 genes were selected from Table 1 and described in the following groups.

3.2.1. Adhesion

Collagen alpha-2 type I expression increased by 1.45- and 1.18-fold for samples P_{48} and C_{48} compared to A_{48}, and decreased by 0.70- and 0.74-fold for samples P_{120} and C_{120} compared to A_{120}, respectively.

3.2.2. Signal pathways and transcription factors

FAK was up-regulated 4.25- and 6.63-fold for samples P_{48} and C_{48} compared to A_{48}. MAPK6 was up-regulated 2.59-fold for sample C_{48} compared to A_{48}. c-*jun* increased by 1.84-fold for samples P_{48} and C_{48}, then decreased over time. c-*fos* expression was down-regulated 0.27-fold for sample C_{48} and increased with time for samples P and C. PTK9 was up-regulated 3.26-fold for sample C_{48} compared to A_{48}. Serine/threonine kinases (STK-1) did not show difference between samples.

Table 2
Gene expressions between the aged treatment and control and passivated treatments (28 genes)

Acc	Gene name	P_{48}/A_{48}	C_{48}/A_{48}	P_{120}/A_{120}	C_{120}/A_{120}
	Adhesion				
J03464	Human collagen alpha-2 type I mRNA	1.45	1.18	0.70	0.74
	Signal pathways and transcription factors				
L13616	Human focal adhesion kinase (FAK) mRNA	**4.25**	**6.63**	1.04	1.22
Z11695	Homo sapiens 40 kDa protein kinase related to rat ERK2 (MAPK1 (mitogen-activated protein kinase 1), MAPK2)	2.32	2.17	0.85	0.76
X80692	Cluster Incl X80692:Homo sapiens ERK3 mRNA (MAPK6 (mitogen-activated protein kinase 6))	2.48	**2.59**	1.60	1.01
AF002715	Cluster Incl AF002715:Homo sapiens MAP kinase kinase kinase (MTK1) mRNA	1.14	1.14	0.93	0.69
D87116	Human mRNA for MAP kinase kinase 3b	1.14	1.46	0.86	1.24
J04111	Human c-*jun* proto oncogene (JUN), complete cds, clone hCJ-1	1.84	1.85	1.09	0.69
V01512	Human cellular oncogene c-*fos*	0.78	**0.27**	1.91	0.42
U02680	Human protein tyrosine kinase (PTK9) mRNA	2.16	**3.26**	1.54	0.81
AF015254	Homo sapiens serine/threonine kinase (STK-1) mRNA	1.30	0.89	0.84	1.48
	Growth factors and Cytokines				
X03565	Human IGF-I mRNA for insulin-like growth factor I	0.58	1.45	2.13	1.47
M37825	Human fibroblast growth factor-5 (FGF-5) mRNA	0.55	0.78	1.80	1.06
S81661	Keratinocyte growth factor (KGF = FGF-7)	**4.90**	**7.07**	0.79	0.62
M60828	Human keratinocyte growth factor (KFG) mRNA	2.41	**2.51**	0.88	0.67
M27968	Human basic fibroblast growth factor (bFGF) mRNA	2.40	**3.19**	1.02	1.13
	Differentiation				
J04948	Human alkaline phosphatase (ALP-1) mRNA	—	0.86	—	—
	Mineralization				
J03040	Human SPARC/osteonectin mRNA	0.95	0.94	0.90	1.34
J04599	Human hPGI encoding bone small proteoglycan I (biglycan)	0.93	0.94	0.84	1.25
	Apoptosis				
M58603	Human nuclear factor kappa-B DNA binding subunit (NF-κB1)	1.51	1.31	0.98	0.88
L19067	Human NF-kappa-B transcription factor p65 subunit (Rel A)	0.97	1.04	0.86	0.87
AF022385	Cluster Incl AF022385:Homo sapiens apoptosis-related protein TFAR15 (TFAR15) mRNA	**2.75**	**2.56**	1.95	1.20
Z23115	Homo sapiens bcl-xL mRNA	—	—	0.90	**0.14**
L22473	Human Bax alpha mRNA	**0.25**	—	2.25	1.99
L22474	Human Bax beta mRNA	0.44	1.23	0.77	1.30
U19599	Human Bax delta mRNA	0.75	0.75	1.72	1.63
U09477	Human clone 53BP1 p53-binding protein mRNA, partial cds (TP53BP1 (tumour protein p53-binding protein, 1))	—	—	—	—
U58334	Human Bcl2, p53 binding protein Bbp/53BP2 (BBP/53BP2) mRNA (TP53BP2 (tumour protein p53-binding protein, 2))	1.31	1.14	0.80	1.01
M35878	Human insulin-like growth factor-binding protein-3 gene (IGFBP3)	1.18	1.08	0.65	0.96

The column P_{48}/A_{48}, represents the gene expression of osteoblasts seeded 48 h on passivated sample divided by the gene expression of osteoblasts seeded 48 h on aged sample. Similar nomenclature is used for the other columns.

3.2.3. Growth factors and cytokines

IGF-I expression decreased by 0.58-fold for sample P_{48} and increased by 1.45-fold for sample C_{48}. FGF-5 decreased at 48 h and increased at 120 h for samples P and C compared to sample A. Keratinocyte growth factor (KGF = FGF7) was up-regulated 4.90-fold and 7.07-fold for samples C_{48} and P_{48}. FGF-2 (bFGF) was up-regulated 3.19-fold for sample C_{48} and was increased 2.40-fold for P_{48}.

3.2.4. Differentiation

ALP was not detected for samples P and C during the test.

3.2.5. Mineralization

SPARC/osteonectin and biglycan had a 0.95-fold decrease for samples P_{48} and C_{48} compared to sample A_{48}.

Table 3
The cellular functions of the chosen genes (based on GeneCards http://bioinfo.weizmann.ac.il/cards/ and [31]

Gene	Genecard	Cellular function
		Adhesion
Integrin α_3	ITGA3	Acts a receptor for fibronectin, laminin and collagen
Integrin α_5	ITGA5	Integrin alpha-5/beta-1 is a receptor for fibronectin and fibrinogen. It recognizes the sequence R-G-D in its ligands
Integrin α_6	ITGA6	Integrin alpha-6/beta-4 may mediate adhesive and/or migratory functions of epithelial cells. On platelets, integrin alpha-6/beta-1 functions as a laminin receptor
Integrin β_1	ITGB1	Associates with alpha-1 or alpha-6 to form a laminin receptor, with alpha-2 to form a collagen receptor, with alpha-4 to interact with vcam-1, with alpha-5 to form a fibronectin receptor and with alpha-8. Integrins recognize the sequence R-G-D in their ligand
Integrin β_5	ITGB5	Integrins are a large family of cell surface glycoproteins that mediate cell to cell and cell to matrix adhesion
Fibronectin	FN1	Fibronectins bind cell surfaces and various compounds including collagen, fibrin, heparin, DNA, and actin. fibronectins are involved in cell adhesion, cell motility, opsonization, wound healing, and maintenance of cell shape
Collagen α-2 type I	COL1A2	Type I collagen is a member of group I collagen (fibrillar forming collagen)
		Signal pathway and transcriptor factors
FAK	PTK2	Activation of focal adhesion kinases (FAK) may be an early step in intracellular signal transduction pathways. This tyrosine-phosphorylation is triggered by integrin interactions with various extracellular matrix (ecm) adhesive molecules and by neuropeptide growth factors. Potential role in oncogenic transformations resulting in increased kinase activity
MAPK	MAPK1	Phosphorylates microtubule-associated protein-2 (map2). Myelin basic protein (MBP), and elk-1; may promote entry in the cell cycle
c-*jun*	JUN	Transcription factor that binds and recognize the enhancer DNA sequence: TGA(C/G)TCA
c-*fos*	FOS	Nuclear phosphoprotein, which forms a tight but non-covalently linked complex with the c-*jun*/*ap*-1 transcription factor. c-*fos* has a critical function in regulating the development of cells destined to form and maintain the skeleton. It is thought to have an important role in signal transduction, cell proliferation and differentiation
PTK	PTK9	—
PTK-receptor	EPHA2	Receptor for members of the ephrin-a family. Binds to ephrin-a1, -a3, -a4 and -a5
STK-1	STK12	—
		Growth factor and cytokines
FGF-2	FGF2	The heparin-binding growth factors are angiogenic agents in vivo and are potent mitogens for a variety of cell types in vitro. There are differences in the tissue distribution and concentration of these 2 growth factors
FGF-5	FGF5	This oncogene is expressed in neonatal brain. FGF-5 can transform NIH 3T3 cells
FGF-7	FGF7	Growth factor active on keratinocytes. Possible major paracrine effector of normal epithelial cell proliferation
IGF-I	IGF1	The insulin-like growth factors, isolated from plasma, are structurally and functionally related to insulin but have a much higher growth-promoting activity
TGF-β	TGFBI, BIGH3	Binds to type I, II, and IV collagens. This adhesion protein may play an important role in cell–collagen interactions. In cartilage, may be involved in endochondral bone formation
BMPs	BMP4	Induces cartilage and bone formation
PDGF-A	PDGFA	Platelet-derived growth factor is a potent mitogen for cells of mesenchymal origin. Binding of this growth factor to its affinity receptor elicits a variety of cellular responses. It is released by platelets upon wounding and plays an important role in stimulating adjacent cells to grow and thereby heal the wound
IL-1α	IL1A	Produced by activated macrophages, IL-1 stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation & proliferation, & fibroblast growth factor activity. IL-1 proteins are involved in the inflammatory response, being identified as endogenous pyrogens, and are reported to stimulate the release of prostaglandin and collagenase from synovial cells
IL-6	IL6	It plays an essential role in the final differentiation of b-cells into ig-secreting cells, it induces myeloma and plasmacytoma growth, it induces nerve cells differentiation, in hepatocytes it induces acute phase reactants
PGE-2	PTGER1	Receptor for prostaglandin E2 (PGE2). The activity of this receptor is mediated by G-Q proteins which activate a phosphatidylinositol-calcium second messenger system. May play a role as an important modulator of renal function. Implicated the smooth muscle contractile response to PGE2 in various tissues
Cbfa1	RUNX2	Osteoblast-specific transcription factor
IHH	IHH	Intercellular signal essential for a variety of patterning events during development. Binds to the patched (PTC) receptor, which functions in association with smoothened (SMO), to activate the transcription of target genes. Implicated in endochondral ossification: may regulate the balance between growth and ossification of the developing bones. Induces the expression of parathyroid hormone-related protein (PTHRP) (by similarity)
BAPX1	BAPX1	Homeo box-containing gene,Drosophila bagpipe homolog, involved in mesodermal and skeletal development
ALP	ALPPL2	Catalytic activity: an orthophosphoric monoester + h(2)o = an alcohol + orthophosphate (at a high pH optimum)
SPARC	SPARC	Appears to regulate cell growth through interactions with the extracellular matrix and cytokines. Binds calcium and copper, several types of collagen, albumin, thrombospondin, PDGF and cell membranes. There

Table 3 (*continued*)

Gene	Genecard	Cellular function
		are two calcium binding sites; a acidic domain that binds 5–8 Ca^{2+} with a low affinity and a ef-hand loop that binds a Ca^{2+} ion with a high affinity
OPN	SPP1	Binds tightly to hydroxyapatite. Appears to form an integral part of the mineralized matrix. Probably important to cell–matrix interaction
Biglycan	BGN	Found in the extracellular matrices of several connective tissues, specially in articular cartilages. The two glycosaminoglycan chains attached to biglycan can be either chondroitin sulphate or dermatan sulphate
IBSP	IBSP	Binds tightly to hydroxyapatite. Appears to form an integral part of the mineralized matrix. Probably important to cell–matrix interaction. Promotes Arg–Gly Asp-dependent cell attachment
TRAIL	TNFSF10	Induces apoptosis
NF-κB1	NFKB1	P105 is the precursor of the p50 subunit of the nuclear factor NF-kappa-b, which binds to the kappa-b consensus sequence 5′-ggrnnyycc-3′, located in the enhancer region of genes involved in immune response and acute phase reactions. The precursor protein itself does not bind to DNA
NF-κB p65	RELA	p65 is a subunit of the nuclear factor kappa-b, a second messenger, which activates the transcription of a number of genes in multiple tissues. The inhibitory effect of i-kappa-b upon NF-kappa-b in the cytoplasm is exerted primarily through the interaction with p65. p65 shows a weak DNA-binding site which could contribute directly to DNA binding in the NF-kappa-b complex
OPG	TNFSF11	Tumour necrosis factor superfamily, member 11 TNFRSF11A (RANK) and OPG (osteprogerin ligand), localized in T cells bone marrow stromal cells, hypertrophic chondrocyte, stimulated by IL1B and TNFRSF11B,expressed in bone, brain, heart, kidney, skeletal muscle, skin, cooperating with prostaglandin, mediating osteoclastognesis and bone loss through systemic activation of T cells, regulating lymph node organogenesis lymphocyte development and interactions between T cells and dendritic cells, activating the antiapoptotic serine threonine kinase AKT/PKB through a signal complex involving TRAF6 and SRC
RANK	TNFRSF11A	
TRANCE	TNFSF11	
TFAK15	PDCD10	—
CSFs	CSF1	Granulocyte/macrophage colony-stimulating factors are cytokines that act in hematopoiesis by controlling the production, differentiation, and function of two related white cell populations of the blood, the granulocytes and the monocytes-macrophages
Bcl-2	BCL2 (B-cell CLL/ lymphoma 2)	Prolongs the survival of hematopoietic cells in the absence of required growth factors and also in the presence of various stimuli inducing cellular death. Bcl2 blocks apoptosis because it interferes with the activation of caspases by preventing the release of cytochrome *c*. might function in an antioxidant pathway to prevent apoptosis at sites of free radical generation such as mitochondria
Bcl-xL	BCL2L1	Dominant regulator of apoptotic cell death. The long form displays cell death repressor activity, whereas the short isoform (-xS) promotes apoptosis
Bax	BAX	Accelerates programed cell death by binding to, and antagonizing the apoptosis repressor bcl-2 or its adenovirus homolog e1b 19k protein. Induces the release of cytochrome *c*, activation of caspase-3, and thereby apoptosis
TP53BP2	TP53BP2	Impedes cell cycle progression at G2/M
IGF-BP3	IGFBP3	IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture. They alter the interaction of IGFs with their cell surface receptors

3.2.6. *Apoptosis*

NF-κB1 increased 1.51- and 1.31-fold for samples P_{48} and C_{48}, respectively, then decreased at 120 h. NF-κB p65 (Rel A) decreased with time. TFAR15 was up-regulated 2.75- and 2.56-fold for samples P_{48} and C_{48}compared to A_{48}. Bcl-xL was absent for samples P_{48} and C_{48}. It was down-regulated 0.14-fold for sample C_{120} and decreased 0.90-fold for sample P_{120} compared to A_{120}. Bax isoform (alpha, beta, and delta) increased with time and also increased for samples P_{120} and C_{120} compared to A_{120}.

4. Discussion

cDNA microarray technology was used to obtain a high-throughput of information on osteoblasts reaction to different titanium surface treatments. In order to have confidence in the results, we have performed the analysis of one sample (C_{24}) twice with two Genefilters. Although not identical, a comparison of results showed a good agreement of the gene expression values between the two filters.

It has been shown that surface roughness influenced the cell behaviour [19]. We have used XPS and AFM techniques to examine the surface properties of the treated Ti–6Al–4V surfaces [20]. A difference in roughness (R_a) between the passivated and the aged samples could only be observed at a small scale (1 μm^2). The area average R_a was about 0.99 nm (C), 1.29 nm (P) and 0.56 nm (A). Therefore, at the cell level, the roughness could be considered as similar between samples and could not explain the differences in osteoblasts behaviour. These differences are then

certainly due to the kinetics of ions release between surface treatments.

4.1. Kinetics of genes modulation induced by the surface treatment A

FAK and MAPK were down-regulated at 48 h. Expression of these genes is related with integrin expression in a time course of events according to the Pathway 1 [21]

Integrins → FAK → MAPK → c-*jun* and c-*fos* → Proliferation → Differentiation (Pathway 1).

It has been demonstrated that osteoblasts cultured on Ti–6Al–4V produce FAK and MAPK within 24 h [22]. According to our results, down-regulation of FAK and MAPK at 48 h would not contradict the Pathway 1, especially if integrins were not modulated which was the case at 48 h. c-*jun* expression of osteoblasts cultured on Ti–6Al–4V was also shown to be expressed before c-*fos* [22]. In our experiment, we noted a down-regulation of c-*jun* and an up-regulation of c-*fos* at 48 h, which is then in accordance with the time course of events for c-*jun* and c-*fos* expression. It is then reasonable to admit that the osteoblast interaction with the surface treatment A followed the Pathway 1.

The expression of PTK decreased over time. Indeed, PTK has been showed to follow the Pathway 2 in macrophages when metal particles were present [23]

Surface membrane receptors binding particles → PTK and Serine/Threonine kinase → NF-κB → Releasing TNF/IL-6 (Pathway 2).

The osteoblasts also possess an inflammatory signaling response similar to Pathway 2 [24]. Following the decrease of PTK, we did not observe any modulation of NF-κB or IL-6. It is possible that either the concentration of metal ions released from the aged sample is unable to stimulate the PTK pathway in osteoblasts or the induced signal pathway is different between particles and ions.

Several genes involved in the apoptosis process [25–27] such as TRAIL, TRAIL-R3, RANK, TRANCE, CSFs were not detected while NF-κB1, NF-κB p65, TFAR15 had their expression decreased. In addition, the up-regulation of Bcl-xL, which is an inhibitor of apoptosis [28], increased with time. Conversely, the expression of Bax, which can accelerate apoptosis [28], decreased with time. These results could indicate that the surface treatment A did not stimulate the activation of apoptosis at 120 h. Therefore the survival of osteoblasts could be enhanced on the aged surface treatment. However, these results need to be confirmed by direct apoptosis measurement such as TUNEL assay.

Without the expression of NF-κB, or CSFs, osteoblasts are unable to secrete cytokines such as IL-1 or IL-6 involved in the differentiation and apoptosis of osteoclasts [25, 26, 29]. This confirms the observation of IL-1 or IL-6 expressions not being detected in our experiment. Based on the genes studied in this experiment, no stimulating effect of osteoblasts on osteoclasts differentiation or activation through cytokine expression seems to be involved in the 120 h of this experiment.

4.2. Comparison of gene expression with respect to the surface treatments C, P, A

Considering the genes involved in signal transduction, FAK was up-regulated 4.25-fold for sample P_{48} and 6.63-fold for sample C_{48} while MAPK6 was up-regulated 2.59-fold for sample C_{48} compared to A_{48}. For samples P_{48} and C_{48} c-*jun* increased and c-*fos* decreased compared to A_{48}. In addition, c-*jun* decreased with time while c-*fos* increased in all samples. All these modulations seem to indicate that samples P and C delay the time course of gene expression in the Pathway 1 compared to the sample A. By extrapolating the time course of gene expression in the Pathway 1, there should be a decrease of cell proliferation for the samples C and P compared to A. This is what we found in a previous study [14], however using a different cell line rendering direct comparison delicate. This delay in the gene expression time course could be due to the decrease of aluminium ions being released by the surface treatment A [12] as it has been suggested that aluminium may alter the timing/regulation of the proliferation/differentiation transition point [30].

The expression of PTK9 was differentially modulated according to the surface treatments with an up-regulation for C_{48} and P_{48} compared to A_{48}. In our previous work [11], we showed that the surface treatment C releases the highest amount of metal ions followed by the surface treatment P then the surface treatment A. Lending evidence that the up-regulation of PTK followed exactly the same classification. The up-regulation of PTK was then directly related to the amount of ions released. According to the Pathway 2, the surface treatment A would then be more favourable by inducing less inflammatory reaction than the surface treatment P or C.

Recently, it has been demonstrated that Ti particles induce osteoblast apoptosis [6]. Despite osteoblast reaction to particles and ions may be different, we investigated the possibility of apoptosis induced also by ions. In the present study, no modulation of Bcl-xL was detected for samples P_{48} and C_{48} compared to A_{48}, while it was down-regulated for P_{120} and C_{120} compared to A_{120}. On the other hand, the gene expression of Bax isoform (alpha, beta, and delta) increased with time. Osteoblast apoptosis could then be initiated from the

high ratio of Bax to Bcl-xL. Based on these observations, osteoblasts may undergo more apoptosis on the surface treatment P and the C at 120 h than on the surface treatment A as a result of increased apoptotic factors expression.

Based on the genes selected in this study, we propose a general pathway of cell reaction according to the surface treatments used:

(1) Metal ion release changes the time course of gene expression in the FAK pathway (Pathway 1).
(2) Once the accumulation of metal ions released from the Ti surface exceeds a threshold value, cell growth is diminished and the apoptosis process may be activated.
(3) Metal ion release upregulates PTK (Pathway 2).
(4) The expression of Bcl-2 family and Bax may suggest that metal ions induce the apoptosis process.

5. Conclusions

The significant gene expression of collagen, FAK, MAPK, FGF-5, IGF-I and Bcl-xL demonstrated that primary human osteoblastic cells were active on the aged Ti–6Al–4V implants.

Although several apoptotic genes were expressed during the test, none of them continuously increased with time, except Bax, which suggests that Bax plays a significant role in the effects of metal ions on apoptosis.

Considering the experimental design, it has been found that most of the significant modulation took place before 48 h. Therefore, to determine the critical point of gene expression in the different pathways future genomic biocompatibility testing should be performed before 48 h following cell seeding.

Finally, the developed surface treatment A seems to increase Ti–6Al–4V biocompatibility. This is highlighted by the lower impact of this treatment on the different pathways studied, by the lower inflammatory reaction, as well as by the lower induced osteoblast apoptosis compared to the surface treatment C and P.

Acknowledgements

We would like to thank Davey Smith, MD for critical review of this manuscript. This study was financially supported by the Bioengineering Research Group of the School of Engineering Sciences at University of Southampton, the Orthopaedic Hospital of Lausanne and a grant from Leenaards Foundation (No. 309). This work was also supported by the Center for AIDS Research Genomics Core laboratories (AI36214) and the San Diego Veterans Affairs Healthcare System (JC).

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