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ORIGINAL ARTICLE

Lactate modulates gene expression in human mesenchymal stem cells

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Abstract
Purpose Surgical wounds are characterised by elevated tissue lactate concentrations. This accumulated lactate is capable of stimulating collagen synthesis and new vessel growth as well. Recently, it has been shown in vivo that lactate is also able to favour homing of stem cells. The aim of this investigation was to test the hypothesis that lactate has an impact on gene expression of mesenchymal stem cells (MSC).
Materials and methods MSC were isolated from human bone marrow using the density gradient technique and incubated with α-methoxyethoxymethyl containing 10% fetal calf serum at 37°C under 95% air and 5% CO_2. Cultured MSC were characterised by in vitro differentiation assays and fluorescence-activated cell sorting (FACS) analysis. Characterised MSC were treated with 15 mM lactate for different time periods (1, 6 and 24 h and 3 and 7 days). Gene expression analysis was performed using a custom-designed oligonucleotide microarray. A significant alteration of gene expression was defined as a two-fold stimulation or inhibition. The phenotype of MSC was investigated by FACS analysis of specific surface epitope patterns.
Results Gene expression analysis shows 63 up- and 51 down-regulated genes after 1 h of treatment, 45 up- and 47 down-regulated genes after 6 h of treatment, 57 up- and 72 down-regulated genes after 24 h of treatment, 103 up- and 28 down-regulated genes after 3 days of treatment and 50 up- and 101 down-regulated genes after 7 days of treatment with lactate. The majority of the modulated genes are related to the expression of cytokines, transcription factors and cell-cycle- or cellular-matrix-associated proteins. In particular, lactate up-regulates the expression of interleukin-6 (3 days, 4.11-fold), of heat shock protein 70 (3 days, 2.36-fold) and of hypoxia-inducible factor-1α (3 days, 2.09-fold). A down-regulating effect of lactate is observed for superoxide dismutase 2 (1 h, 0.5-fold; 24 h, 0.4-fold; 7 days, 0.32-fold) and BCL2-associated X protein (24 h, 0.42-fold; 7 days, 0.4-fold). Expression of cell surface antigens clusters of differentiation 29, 44, 59, 73, 90, 105, 106 and 146 does not change over the time period of lactate treatment.
Conclusions Lactate modulates expression of genes involved in wound healing. However, lactate does not profoundly change the phenotype of MSC. In addition to providing new insights into the wound healing physiology,

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these data could also be the rationale for new treatment strategies for chronic non-healing wounds.

Keywords Lactate · Mesenchymal stem cells · Wound repair · Gene expression · Microarrays

Introduction

Wounds are characterised by high lactate concentrations [1]. For centuries, the accumulation of lactate was thought to be the result of tissue hypoxia and was therefore considered as an unimportant metabolic by-product [2]. Even though hypoxia can initiate neovascularisation by inducing growth factors [3], sustained periods of severe hypoxia will, conversely, compromise neovascularisation since they inhibit the synthesis of collagen needed for new vessels to withstand the pressure of blood flow [4]. Later it was shown that there is little correlation between lactate and oxygen [5]. Wound lactate levels remain high (5–15 mM) even in conditions of oxygen sufficiency as opposed to the 1–3 mM found in blood and uninjured tissue [6]. Lactate, which is freely diffusible [5, 6], is an instigator of growth factors and cytokines [7, 8] and stimulates both collagen synthesis [1, 9] and new vessel growth [7, 10] in vitro as well as in vivo. Moreover, it has recently been shown that lactate initiates vasculogenesis via stem cell homing [11].

Stem cells display a prolonged self-renewal capacity and an asymmetric replication [12]. Mesenchymal stem cells (MSC) have the potential to differentiate into specific tissue-related cells involved in development of bone and soft tissues [13]. In addition, stem cell accumulation has been detected at the site of repair [12]. However, it remains unclear whether they mainly derive from the tissue itself or enter the wound through the bloodstream since systemically applied MSC have been shown to traffic to the site of injury [14, 15]. Stem cells stimulate healing not only by their ability to differentiate into tissue-related cells but also by producing various chemokines and cytokines themselves [16].

The aim of this study was to test the hypothesis that lactate is able to modulate gene expression and the phenotype of MSC.

Materials and methods

Bone marrow preparation, MSC isolation, cell culture and characterisation

Our study was approved by the local institutional ethical committee. Bone marrow (BM) was obtained under sterile conditions with informed consent during orthopaedical surgery: 5 ml of the whole BM was collected from a 43-year-old female donor in a sterile heparinised syringe. MSC were isolated using the density gradient technique as described previously [17]. MSC were incubated (37°C, 95% air and 5% CO_2) with a medium containing deoxyribonucleotides, ribonucleotides, ultra glutamine 1 (α-methoxyethoxymethyl, Cambrex Bio Science, Hopkinton, MA, USA), 100 IU/ml penicillin (Cambrex Bio Science, Hopkinton, MA, USA), 100 µg/ml streptomycin (Cambrex Bio Science, Hopkinton, MA, USA) and 10% heat-inactivated fetal calf serum (FCS) (Cambrex Bio Science, Hopkinton, MA, USA). The non-adherent cells were removed and the adherent cells were cultured and characterised by in vitro differentiation assays [13] and fluorescence-activated cell sorting (FACS) analysis. Subsequently, the obtained and characterised MSC were treated with the above-mentioned medium supplemented with 15 mM L-lactate (pH 7.4) for different time periods (1, 6 h and 24 h and 3 and 7 days). MSC incubated in the above-mentioned media without lactate (1, 6 and 24 h and 3 and 7 days) served as controls.

RNA isolation

RNA from stimulated MSC and controls was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel, Dueren, Germany). The RNA quality and quantity were verified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Microarray data generation and statistical analysis

Production and analysis of our oligonucleotide microarrays have been described previously by Zieker et al. [18]. In brief, microarray analysis was performed using oligonucleotide microarrays (65mer) produced at the Max Planck Institute, Tüebingen, Germany. The arrays contained oligonucleotides for about 900 transcripts as well as buffer, control and empty spots, and each feature was printed twice. Using ten oligonucleotide microarrays (including dye-swap), lactate-stimulated MSC were compared with their corresponding control after 1, 6 and 24 h and 3 and 7 days of treatment. Amplification of the sample RNA was performed using Ambion's Amino Allyl MessageAmptrade; II aRNA Amplification Kit (Ambion Inc., Austin, TX, USA). Dye-coupling reaction was performed using Amersham CyDye Post-labelling Reactive Dye Pack (GE Healthcare, Buckinghamshire, UK). After an aRNA fragmentation using Ambion's Fragmentation Reagents (Ambion Inc., Austin, TX, USA), hybridisation was carried

out at 48°C for 14 h. The slides were scanned in a microarray scanner (Genetix Limited, Hampshire, UK). The photomultiplier tube voltage was set to 100% for both red and green channels. The two resulting green and red images were overlaid using ImaGene 5 (BioDiscovery, Inc., El Segundo, CA, USA). Raw data collection was performed using ImaGene v.5.0. Further statistical and bioinformatic analyses were performed using the R language (www.r-project.org) and the 'limma' (Linear Models for Microarray) package from the Bioconductor project (http://www.bioconductor.org/). As a first step in signal extraction for each channel, we used the mean of the pixel distribution for the foreground signal and the median for the background of each spot as estimators of the raw signal values. All spots were used, regardless of their flag status. The data were normalised using loess normalisation on the normexp-background-corrected expression values, followed by a dye-swap normalisation and in-between-array quantile normalisation. Both the loess and quantile normalisation methods were used as provided in the limma package. As a result of the normalisation, for each time point the log2 ratio M of the fold change of the lactate-stimulated MSC cells versus their respective controls was computed. On the basis of the M values, up-regulated genes ($M>1$) and down-regulated genes ($M<-1$) were detected.

FACS analysis

FACS analysis was performed with FACScan (BD Biosciences, San Jose, CA, USA) using BD CellQuest Pro software. At subconfluency (1×10^6 cells), the cells were detached with Accutase™ (PAA Laboratories, Cölbe, Germany) and washed (phosphate-buffered saline (PBS)+AccuMax™ (PAA Laboratories, Cölbe, Germany)). Each probe contained a cell suspension with 5×10^5 cells in FACS buffer (PBS+1% bovine serum albumin (Sigma, Taufkirchen, Germany)+0.1% FCS (Cambrex Bio Science, Hopkinton, MA, USA)). The phycoerythrin-conjugated antibody (anti-human clusters of differentiation (CD) 29, 44, 59, 73, 90, 105, 106 and 146) was added. After an incubation time of 20 min and two washing steps, the probe was ready for analysis. All antibodies were from BD Biosciences.

Results

Microarray analysis

We detected a number of genes that were up- or down-regulated comparing MSC stimulated with lactate among their corresponding MSC controls without lactate (see Fig. 1). After 1 h, 114 genes are differentially expressed. Sixty-three genes are up- and 51 genes are down-regulated in lactate-stimulated MSC. After 6 h, 45 genes are up- and 47 genes are down-regulated. After 24 h, 57 genes are up- and 72 genes are down-regulated. After 3 days, 103 genes are up- and 28 genes are down-regulated. Finally after 7 days, 50 genes are up- and 101 genes are down-regulated in lactate-stimulated MSC. Interestingly, the sets of differentially regulated genes between the time points differ substantially. The majority of the clustered genes are members of the cell cycle, cell adhesion and communication, transcription factor, cytokine, apoptosis and oxidative metabolism families. In particular, the following genes are up-regulated by lactate after 3 days: 4.11-fold up-regulation of interleukin-6 (IL-6), 2.36-fold up-regulation of heat shock protein 70 (HSP70) and 2.09-fold up-regulation of hypoxia-inducible factor-1α (HIF-1α). In addition, a down-regulating effect of lactate on superoxide dismutase (SOD)-2 is observed after 1 h (0.5-fold), 24 h (0.4-fold) and 7 days (0.32-fold) and on BCL2-associated X protein (BAX) after 24 h (0.42-fold) and 7 days (0.4-fold).

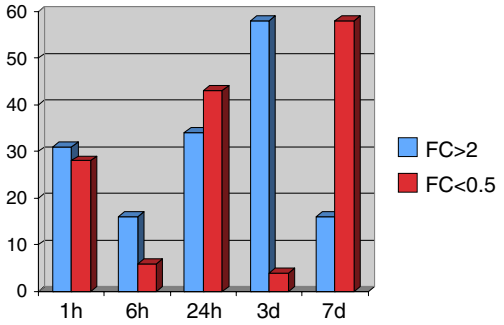

Fig. 1 Up- and down-regulated genes in lactate stimulated MSC. For each time point, the number of up-regulated and down-regulated genes, respectively, with a fold change larger than 2 (log ratio $M>1$) and smaller than 1/2 (log ratio $M<-1$), respectively, and log2 expression level larger than 8 were computed

FACS analysis

Lactate does not induce significant alterations in expression of cell surface epitope patterns (CD 29, 44, 59, 73, 90, 105, 106 and 146) (Table 1).

Discussion

This study investigates the effect of lactate on gene expression and phenotype of MSC. We are able to show that lactate alters gene expression but does not change the phenotype of MSC with respect to the investigated cell

Table 1 FACS analysis was performed in lactate-stimulated MSC. Cell surface epitope patterns from anti-human CD 29, 44, 59, 73, 90, 105, 106 and 146 were investigated. Data are expressed as mean percentage of positive counts±SD

		Control	Lactate (15 mM)
1 h	CD 29	99±0	99±0
	CD 44	97±0	98±0
	CD 59	100±0	100±0
	CD 73	98.5±0.71	99±0
	CD 90	99.5±0.71	100±0
	CD 105	96±0	97±0
	CD 106	13.5±0.71	33.5±24.75
	CD 146	32.5±0.71	26.5±16.26
6 h	CD 29	98.5±0.71	98.5±0.71
	CD 44	97.5±0.71	98±0
	CD 59	99±0	99±0
	CD 73	98.5±0.71	98.5±0.71
	CD 90	99±0	99±0
	CD 105	86±12.73	97±0
	CD 106	13.5±6.36	7±1.41
	CD 146	40±0	32±4.24
24 h	CD 29	93.5±3.54	98±0
	CD 44	97±1.41	98±0
	CD 59	96±4.24	99.5±0.71
	CD 73	90.5±4.95	96.5±0.71
	CD 90	99±0	99±0
	CD 105	88±11.31	97±0
	CD 106	31±7.07	16.5±13.44
	CD 146	30.5±7.78	34±1.41
3 days	CD 29	99±0	99±0
	CD 44	99±0	99±0
	CD 59	100±0	100±0
	CD 73	98.5±0.71	99.5±0.71
	CD 90	99.5±0.71	100±0
	CD 105	98±0	98±0
	CD 106	49.5±9.19	48±12.73
	CD 146	37±2.83	41.5±0.71
7 days	CD 29	99±0	99.5±0.71
	CD 44	99±0	98.5±0.71
	CD 59	100±0	100±0
	CD 73	99±0	99±0
	CD 90	100±0	100±0
	CD 105	97.5±0.71	96 ± 0
	CD 106	57.5±2.12	70±.83
	CD 146	24.5±0.71	36.5±0.71

surface epitope patterns. Genes favouring wound healing are found up-regulated while genes involved in apoptosis are found inhibited.

Wound healing is a complex biological phenomenon. Many cellular and biochemical reactions take place in order to restore tissue integrity following injury [4]. Bone-marrow-derived stem cells have been found in all tissues in adult animals and it is now accepted that they play a significant role in tissue regeneration and homeostasis [12]. By their expression of specific cell surface markers, these particular cells can be divided in two subgroups, namely hematopoietic and mesenchymal stem cells [13]. Bone-marrow-derived stem cells enter the injured tissue by a process called homing [19]. MSC contribute to the healing process by two possible mechanisms: a paracrine way and the in situ differentiation into specific tissue-related cells to replace the damaged tissue [20]. The paracrine mode of action has been popularised by studies in which MSC-conditioned media injected into myocardial infarct sites resulted in decreased infarct areas and less apoptotic cells [21, 22]. Similarly, MSC were capable of improving renal function due to changing the production of pro- and anti-inflammatory cytokines of kidney cells in a rat model [23].

Lactate has been known for many years to contribute to wound healing both by stimulating collagen synthesis and by angiogenesis [4, 9]. Moreover, since lactate has recently been shown to play a role in stem cell homing [11], we sought to investigate whether or not lactate also has an impact on gene expression and phenotyping of MSC. We found that lactate temporarily increases the expression of genes of pro-inflammatory cytokines (IL-6 and HSP70) and genes involved in angiogenesis (HIF-1α) in adequately oxygenated MSC. These findings confirm the hypothesis that MSC are capable of acting due to a paracrine mechanism since a sustained expression of HIF-1α and an in vivo delivery of HSP70 were able to stimulate healing [24, 25]. Similarly, expression of genes associated with apoptosis (BAX) is inhibited. BAX is an apoptotic signalling molecule involved in the regulation of anoxia-induced cell death [26]. Thus, lactate seems to be able to express impending hypoxia while not necessarily incurring the hazards of hypoxia itself [4]. Furthermore, lactate attenuates the expression of the SOD gene which might result in an increased availability of oxidants. This is in accordance with other studies where oxidants have been shown to stabilise HIF-1α [27] and stimulate healing [28]. However, lactate does not change the phenotype of MSC with respect to the investigated cell surface epitope patterns.

In accordance with a previous report [11], this study demonstrates the impact of lactate on stem-cell-mediated tissue repair. The next step will be to analyse the

mechanism of lactate-induced stem cell trafficking and the effect of lactate on transcriptome-wide expression of mesenchymal stem cells.

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