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14. ABSTRACT
We proposed to determine the changes in osteoblast differentiation *in vitro* when infected with multidrug resistant bacteria and the therapeutic effects of two well characterized antimicrobial peptides. For the first part of the study, an *in vitro* 3 dimensional (3D) model of primary human osteoblasts in a collagen scaffold was developed for infection. The 3D osteoblast cultures were infected with clinical isolates of multidrug resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Custom PCR arrays with genes associated with osteoblast differentiation/maturation were used to study gene expression in primary osteoblasts at several time points 4, 24, 48 and 72 hours post infection. The temporal gene expression patterns of infected osteoblasts in the 3D model were studied by real time PCR. Initial work suggested changes in the expression of osteoblast marker genes, several transcription factors, cell adhesion related genes and proinflammatory cytokines may contribute to an impediment for osteoblast differentiation and maturation when osteoblasts harbor any of these 3 bacteria as intracellular pathogens. Antimicrobial therapy was tested with KSL-W peptide on osteoblasts in 3-D scaffolds and was effective against extracellular *Acinetobacter baumannii* and *Klebsiella pneumoniae* compared to uninfected controls. KSL-W was not effective against *Pseudomonas aeruginosa*. KSL-W was not effective against intracellular bacteria. The outcome of our findings may contribute to accelerate treatment options and management of osteomyelitis due to drug resistant bacteria in the Wounded Warrior.

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INTRODUCTION

The developmental processes for bone tissue is complex and involves several tightly regulated gene expression patterns of bone associated proteins. The expression of these gene and proteins are regulated in a temporal manner both in vivo and in vitro and has previously been studied in both these systems. The process of osteoblast differentiation can be subdivided into 3 stages, namely, proliferation, extracellular matrix synthesis and maturation and lastly mineralization.

Each stage is characterized by the expression of distinctive bone markers. It has been demonstrated by Stein and Lian [1] that during bone formation, the expression of extracellular matrix genes (type I collagen, fibronectin, transforming growth factor-β1), cell cycle or growth-related genes (histone, c-fos, c-myc) increased initially followed by genes associated with mineralization, such as osteocalcin, osteopontin, and bone sialoprotein. In addition, osteoblast-produced of circulating cytokines and growth factors also influence the osteoblast differentiation process.

We proposed to determine the early in vitro gene expression changes in osteoblasts when infected with multidrug resistant Gram negative bacteria. An in vitro 3 dimensional (3D) model of primary osteoblasts in a collagen scaffold was to be utilized for the study. The 3D osteoblast cultures would be infected with clinical isolates of multidrug resistant *Acinetobacter baumannii* (AB), *Klebsiella pneumonia* (KP) and *Pseudomonas aeruginosa* (PA). Post infection, osteoblast differentiation and maturation would be determined in a temporal manner by gene and protein expression. Utilizing these models, the therapeutic effects of two well characterized antimicrobial peptides on osteoblasts infected with these three bacteria would be determined.

The outcome of these findings may contribute to accelerated treatment options and management of osteomyelitis due to drug resistant Gram negative bacteria.

BODY

Milestone 1: *In vitro* infection studies

Two different 3D models for osteoblast infections were proposed. The first model was to establish a 3-Dimensional (3D) model for osteoblast infections utilizing primary osteoblasts in a collagen scaffold (BD biosciences) (n=3).

A second model was proposed which utilized hydroxyapatite discs with specific pore sizes purchased from DynCorp (UK). The company stopped its production of HA discs, and a suitable replacement was not found. Therefore, the collagen scaffold model was selected for further studies.

In this *in vitro* cell culture system, osteoblasts were to be cultured on a collagen scaffold (available from Becton Dickinson). The BD™ 3-dimensional collagen scaffolds are suitable for short- and long-term growth and differentiation of a variety of cell types, including osteoblasts (information from BD Biosciences). The 3D structure mimics the *in vivo* environment to induce tissue formation or to promote tissue repair.

For the proposed work, the collagen scaffolds were to be purchased by Beckton Dickinson, but the product was not available and was uncertain of its availability later. The problem was solved with a collagen scaffold model developed in the laboratory following modifications of Shen et al [2]. The first year's work was dedicated to establish a working model with osteoblast cell line SaOs2. The first year's work described the establishment of the 3D collagen scaffold model with osteoblast cells line SaOS2 for infections. Work was completed successfully to establish the model, and infections were performed with clinical isolates of multidrug resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with SaOs2 cells prior to working with primary osteoblasts. The multiplicities of infection for all three strains were then optimized on the 3-D model with SaOs2 osteoblast cell line.

In the second year, work began with primary osteoblasts utilizing the established 3-D collagen scaffold model. To relate to war trauma, primary osteoblasts from healthy male subjects between the ages of 18-45 years were purchased from commercial sources (Lonza and Promocell Inc).

In the third year, work was completed on gene array studies (n=3). Utilizing this model, the peptide KSL-W was tested for bactericidal activities against all 3 pathogens.

Milestone 2: Therapy with antimicrobial peptides

The second stage of the proposed study was to evaluate 2 synthetic antimicrobial peptides, KSL and KSL-W, on infected osteoblasts and study the therapeutic activities of these peptides. We tested the bactericidal activity of KSL-W only against AB and KP in the 3D osteoblast model as it was not effective against PA.

Progress

RNA extractions for gene expression study

Primary osteoblast infections in the 3D model were then performed with the selected multiplicity of infection (MOI's) for each bacterial species for gene expression study. Gene expression studies on primary osteoblasts infected with *Acinetobacter baumannii*, *Klebsiella pneumoniae* or *Pseudomonas aeruginosa* have been completed (n=3). Before completion in December 2012, a second peptide LL-37 will be tested for activity against the 3 pathogens. Also, media from wells of infected scaffolds will be tested for secreted cytokines.

Method

Primary human osteoblasts at 3-4th passage on confluent flasks were harvested and scaffolds loaded as described previously. On day 12-13, infections with AB,KP and pA were performed as described previously. Infected primary osteoblasts on 3Dscaffolds were collected at 4,24,48 and 72 hours. A set of uninfected control osteblasts were included at each time point. 4-5 scaffolds at each time point were pooled together for extraction of total RNA.

Scaffold were digested with 1mg/ml collagenase, and a cell pellet was obtained after centrifugation at 2000RPM. 1ml of RNAzol was added to each pellet and resuspended. The samples were frozen at -80^{0}C until used for the gene expression study.

Total RNA was extracted with the Qiagen miniprep kit (Qiagen Inc). RNA was verified for concentration and purity by Nanodrop and Agilent 2100 Bioanalyzer.

From the literature, a panel of genes involved in osteoblast differentiation and maturation were selected, and a custom real time PCR array was designed (Qiagen, SA Biosciences) to probe the infected/control samples. The list of genes for the custom array is shown in table 1.

GENE SYMBOL	GENE REFSEQ #
ALPL	NM_000478
VDR	NM_000376
BGLAP	NM_199173
CD11	NM_001797
FOXC2	NM_005251
RUNX2	NM_004348
ATF4	NM_001675
STAT1	NM_007315
OSTERIX (SP7)	NM_152860
Dlx3	NM_005220
Dlx5	NM_005221
C-FOS	NM_005252
Msx2	NM_002449
Twist1	NM_000474
HEY1	NM_012258
CDH4	NM_001794
CDH2	NM_001792
IBSP	NM_004967
LEF1	NM_016269
BMP2	NM_001200
BMP7	NM_001719
BETA ACTIN	NM_001101
GAPDH	NM_002046

Table 1. Gene list for RT^2 Profiler^{TM} custom PCR array for osteoblast infection

Total RNA from primary osteoblasts infected with AB, KP and PA were used to probe the arrays at t=4, 24, 48 and 72 hours post infection. An uninfected control was included at each time point. The PCR Arrays consist of sets of optimized real-time PCR primer assays with osteoblast differentiation maturation focused genes

as well as appropriate RNA quality controls on 96-well plate format. The RT PCR array performs gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray.

The RT PCR arrays were completed following the manufacturer's instructions (QIAGEN RT^2 Profiler™). Briefly, total RNA (500ng) was converted to cDNA according to the manufacturer's protocol (Qiagen Inc). cDNA was added to RT^2 qPCR master mix, and was added to the 96 well custom array which included the appropriate primers for each gene. PCR arrays were then run in an ABI 7000 instrument. Data was uploaded according to instructions. The integrated web-based software package for the PCR Array System provided by Qiagen (SA BioSciences) automatically performs all $\Delta\Delta C_t$ based fold-change calculations from the uploaded raw threshold cycle data. The mRNA expression levels obtained for each gene were normalized to the mean expression of GAPDH and beta actin housekeeping genes by using the following equation: relative mRNA expression = $2^{-(\text{Ct of test gene} - \text{Ct of mean housekeeping genes})}$ (where Ct is the threshold cycle). Fold changes for each gene = gene expression of unknown / expression of uninfected control at appropriate time point.

Statistical Analysis

The fold change differences between each unknown gene and control housekeeping genes (value=1) were analyzed using Student's *t*-test. A p value < 0.05 was considered statistically significant.

Gene	4 hr	24 hr	48hr	72hr	Bacteria
ALPL	-3.66	1.75	-3.16	-1.35	AB
	ns	-2.47	ns	ns	KP
	ns	ns	-6.40	-2.18	PA
BGLAP	-1.83	ns	ns	ns	AB
(osteocalcin)	ns	-1.82	ns	ns	KP
	ns	ns	-2.54	ns	PA

Table 2. Temporal gene expression of osteoblast marker gene expression in primary human osteoblasts in 3D scaffold and infected with *Acinetobacter*

baumannii (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls. Values are significant ($p>.05$) fold changes (Students t test) compared to housekeeping gene value=1 (n=3).ns denotes not significant.

Active osteoblasts have a high expression of alkaline phosphatase (ALPL). ALPL is one of the earliest markers for osteoblasts. ALPL is involved in osteoblast maturation, and it is a marker for bone metabolism; *in vitro* studies have shown that expression of ALP increases with the differentiation of osteoblasts [3].

For AB, ALPL levels were downregulated except for 24 hours post infection. For KP, ALP levels were down regulated at 24 hours. When infected with PA*,* ALPL levels were downregulated at 48 and 72 hours post infection (Table 2) suggesting that osteoblast differentiation decreased when infected by the 3 wound pathogens.

Osteocalcin (BGLAP) constitutes the most abundant non-collagenous protein present in bone and an extracellular matrix protein [4]. BGLAP is synthesized in the bone almost exclusively by the osteoblasts has been considered as a bone formation marker. Serum levels of BGLAP correlate closely with bone formation [4, 5] and this marker has been widely used as an indicator of new bone formation in vivo [5, 6]. BGLAP production correlates with the onset of bone mineralization [6, 7]. Our data shows that the expression of BGLAP is not significantly changed compared to controls for any of the 3 pathogens during the course of infection.

Additionally, we tested the supernates for secreted BGLAP from infected osteoblasts at these time points and the levels in infected samples were similar to uninfected controls, suggesting no increase in its secretion by 72 hours (data not shown). The lower expression levels of ALPL and unchanged BGLAP bone marker levels during infection suggest that the intracellular presence of AB, KP or PA in osteoblasts, (though in small numbers) may delay the normal differentiation and maturation processes of osteoblasts.

GENE	4 hr	24 hr	48hr	72hr	Bacteria
STAT1	ns	3.99	2.68	ns	AB
	2.26	2.25	3.58	ns	KP
	ns	3.17	1.86	2.65	PA
SP7 (Osterix)	ns	3.10	ns	ns	AB
	ns	ns	ns	2.28	KP
	ns	ns	ns	ns	PA
Dlx3	-4.41	ns	ns	ns	AB
	-4.53	-1.95	ns	ns	KP
	ns	-2.35	-1.53	ns	PA
Dlx5	-1.99	ns	ns	ns	AB
	ns	ns	ns	ns	KP
	ns	ns	-2.98	-2.62	PA
Twist-1	ns	4.36	ns	ns	AB
	ns	ns	ns	ns	KP
	2.40	ns	2.71	1.90	PA
c-fos	-2.07	3.66	2.12	2.03	AB
	ns	ns	ns	ns	KP
	ns	ns	ns	2.07	PA

Table 3. Temporal gene expression of osteoblast transcription factors in primary human osteoblasts in the 3D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls. Values are significant (p>.05) fold changes (Students t test) compared to housekeeping gene value=1 (n=3).ns denotes not significant.

A number of transcription factors are accountable for the regulation of osteoblast differentiation and function; these include Runt-related transcription factor (Runx2), Dlx3, Dlx5, MSX2 and SP7 (osterix), among others, and were investigated for mRNA expression during infection.

Runx2, a cell-specific member of the Runt family of transcription factors, plays a critical role in cellular differentiation processes in osteoblasts [8] as the crucial osteogenic transcription factor expressed in early bone development. It also persists through different stages of bone formation [8, 9]. Expression of RUNX2 is essential for inducing osteoblast differentiation and also for the regulation and

expression of a variety of osteoblast phenotype genes such osteocalcin, bone sialoprotein, osteopontin, and collagen type I .
When infected with the AB, KP or PA, RUNX2 expression was not significant for AB and KP at all time points suggesting that during infection with either AB or KP normal processes for osteoblasts such as differentiation may not be occurring. Runx2 increased 3.49 fold at 72 hours only when infected with PA [data not shown].

A second transcription factor sp7 (osterix) remained unchanged during infection to PA and increased at 24 hours for AB and 72hours post infection to KP [table 3]. Dlx5 is a bone inducing transcription factor which plays an important role in osteoblast differentiation that is expressed in the later stages [10]. Previous work has shown that over expression of Dlx5 stimulates osteoblast differentiation [11]. We saw that Dlx5 expression when infected with any of the three pathogens resulted in either downregulation or not significantly different from the uninfected osteoblasts.

A role for Dlx3 in the upregulation of bone-related genes to promote osteoblast differentiation has been found and shows that Dlx3 may enhance osteoblastogenesis [12]. They also found that both Dlx3 and Dlx5 expression overlap in part during the matrix maturation stage. During osteoblast differentiation, both Dlx3 and Dlx5 act together to control the expression of osteocalcin gene. Therefore, findings suggest that the Dlx proteins may function in a complex regulatory pathway towards osteoblast differentiation. During infection with AB, KP or PA, we saw that Dlx3 gene was also either downregulated or not significant, again suggesting that intracellular AB, KP or PA may affect the processes of osteoblast differentiation.

The transcription factor MSX2, was significantly induced to AB infection at 24 hours. Both KP and PA infections had no response to MSX2 (data not shown). Studies have demonstrated that Msx2 promotes osteoblast differentiation and/or proliferation [13, 14]. Yet another study by Liu et al. [15] suggested that suggested that Msx2 inhibits differentiation of osteoblast precursors and immature osteoblasts.

An essential transcription factor, whose downregulation is required for osteoblast differentiation, is Twist1. Studies have revealed that the role of Runx2 in

osteoblast differentiation was controlled by Twist1. Twist1 is able to inhibit osteoblast differentiation without affecting RUNX2 expression [16]. Even though infection with KP did not result in changes in TWIST1 expression, its upregulation in AB and PA infections (table3) suggest that differentiation of osteoblasts may be compromised when infected with AB and PA in the first 72 hours of infection.

In bone biology, studies have revealed that Signal transducer and activator of transcription 1 (Stat1) is involved in osteoclastogenesis and osteoblast differentiation in addition to immune regulation [17]. It has been observed that osteoblast differentiation in vitro was significantly enhanced in the absence of Stat1 indicating that Stat1 interferes with osteoblast differentiation. Also, Stat1 acts an attenuator of Runx2 in the cytoplasm [18]. Stat1 has been found to be a negative regulator associated with both bone formation and resorption and it maintains the homeostasis of the skeletal system. Our data indicates that when infected with AB, KP or PA, Stat1 was upregulated at several time points, again suggesting that osteoblast differentiation may be compromised by the intracellular presence of AB, KP and PA.

Among growth factors involved in osteoblast differentiation, bone morphogenetic protein (BMP) family proteins play a crucial role in osteogenesis. BMPs are members of the transforming growth factor-β (TGF-β) superfamily and regulate osteoblast function and development and are vital to this intercellular signaling system and they activate osteoblast differentiation. Studies with recombinant BMP’s 2, 4, 6, and 7 have shown that they are strong inducers of osteoblast differentiation. In addition, BMP2 and BMP7 induce the activation and expression of Runx2 [19-22]. BMP-2 stimulates osteoblast differentiation and bone formation [23-25]. We observed the induction of BMP2 in only AB infected osteoblasts suggesting that it promoted proliferation and differentiation, while BMP7 was induced in both AB and KP at different times. PA infection did not affect the expression of either gene (Table 4).

GENE	4 hr	24 hr	48hr	72hr	Bacteria
BMP2	ns	18.08	5.06	ns	AB
	ns	ns	ns	ns	KP
	ns	ns	ns	ns	PA
BMP7	ns	ns	ns	9.73	AB
	2.20	ns	ns	8.14	KP
	ns	ns	ns	ns	PA

Table 4. Temporal gene expression of bone morphogenic proteins BMP2 and 7 in primary human osteoblasts in the 3D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls Values are significant ($p>.05$) fold changes (Students t test) compared to housekeeping gene values=1 (n=3).ns denotes not significant.

GENE	4 hr	24 hr	48hr	72hr	Bacteria
CDH2	ns	ns	-2.57	ns	AB
	2.36	ns	ns	-3.89	KP
	2.49	ns	ns	-1.78	PA
CDH4	ns	ns	ns	ns	AB
	ns	-2.55	ns	ns	KP
	ns	ns	ns	ns	PA

Table 5. Temporal expression of cell-adhesion related genes in primary human osteoblasts in the 3D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls. Values are significant ($p>.05$) fold changes (Students t test) compared to housekeeping gene value=1 (n=3).ns denotes not significant.

Cell–cell adhesion by cadherins is essential for the function of bone forming cells during osteogenesis. Also, cell adhesion is associated with other biological processes such as immune response, wound healing, and tissue structure maintenance. Osteoblasts express a limited number of cadherins, including the classic N-cadherin, CDH2. The expression profile of N-cadherin in osteoblasts during bone formation in vivo and in vitro suggests a role of this molecule in osteogenesis. Our data shows that both KP and PA induced CDH2 at 4 hours post

infection, but by 72 hours, it was downregulated. For AB, CDH2 was down regulated at 48hours (Table 5). It has been found that interruption of cadherin-mediated adhesion prevents induction of a fully differentiated osteoblastic phenotype [26]. We found that CDH4 was not significantly expressed to any of these pathogens except for the down regulation at 24 hours after infection with KP.

GENE	4 hr	24 hr	48hr	72hr	Bacteria
IL1b	17.46	4.47	ns	ns	AB
	58.59	5.92	ns	ns	KP
	10.73	41.82	25.04	44.99	PA
IL8	29.52	18.35	ns	ns	AB
	27.16	46.47	47.29	ns	KP
	13.79	23.68	24.41	46.58	PA
IL6	5.03	21.17	ns	ns	AB
	19.26	25.25	ns	ns	KP
	21.98	34.47	24.48	ns	PA
TNFa	3.71	1.94	ns	ns	AB
	26.43	2.83	ns	2.40	KP
	29.69	6.85	5.55	7.55	PA

Table 6. Temporal expression of cytokines/chemokine genes in primary human osteoblasts in the 3D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls. Values are significant ($p>.05$) fold changes (Students t test) compared to housekeeping gene value=1 (n=3).ns denotes not significant.

Previous investigations on bacterial arthritis induced by *S. aureus* and *S. agalactiae* have suggested that TNFα, interleukin1β, and IL6, are involved in the pathogenesis of bacterial arthritis [27, 28]. TNFα is known to be involved in osteoclastogenesis and stimulate osteoclast formation and differentiation [29]. Both IL1 and TNFα appear to contribute directly to tissue damage through induction of the release of tissue-damaging enzymes by activation of osteoclasts [30, 31]. Besides stimulating resorption, it is known that IL1b and TNF also inhibit bone formation in vitro [32-36]. IL1b was induced by AB and KP at the early time points of 4 and 24 hours, while it was induced for PA at all time points of infection (Table 6). TNFα expression was induced at the early time points for AB, and it was induced at 72 hours with PA infection. The neutrophil attractant chemokine IL8

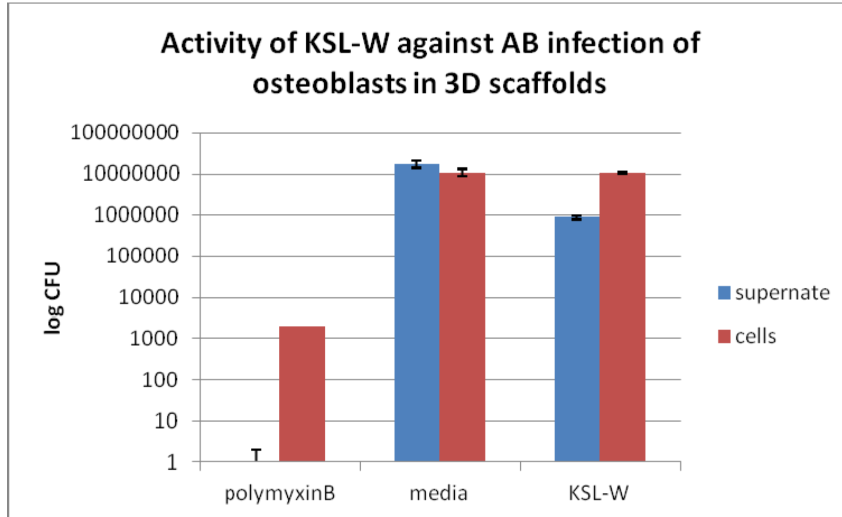
expression increased and then decreased by 72 hours, but remained elevated for all 3 infections (Table 6). A recent study indicated that infection with Chlamydia pneumoniae induced that maximum expression of proinflammatory IL8 (among other cytokines) at 72 hours post infection in SaOs2 osteoblast cell line [37]. The overall expression patterns of these chemokines/cytokines suggest that intracellular AB, KP and PA may elicit inflammatory responses in osteoblasts which in turn may hinder the differentiation and maturation of osteoblasts.

Milestone 2: Therapy with antimicrobial peptides
The second part of the study was to evaluate antimicrobial peptide therapy to infection on osteoblasts in the 3D model.

We determined the bactericidal activity of KSL-W peptide 1 hour post-treatment against extracellular bacteria compared with polymyxin B and untreated controls according to protocols described in the previous reports. For AB, 100ug/ml of KSL-W was effective against extracellular bacteria present in the medium of the 3D osteoblast scaffolds compared to the untreated controls. Polymyxin B (100ug/ml) treatment was very effective in killing extracellular bacteria by 1 hour resulting in 0 CFU. When intracellular AB was determined, both untreated and KSL-W treated cells had similar numbers of intracellular AB, while polymyxin B treated osteoblasts had less intracellular bacteria (Figure 2a). Similarly, for KP, treatment with KSL-W resulted in less extracellular bacteria compared to untreated control.

Again, polymyxin B treatment was very effective against extracellular KP after 1 hour treatment. Intracellular KP was similar to untreated cells, while polymyxin treated cells yielded less bacteria (Figure 2b). Most probably, the reason for higher numbers of intracellular bacteria for AB and KP in both untreated and KSL-W treated cells is continuous re-infection from extracellular bacteria in the medium. PA was not sensitive to the killing of KSL-W (data not shown).

a)



b)

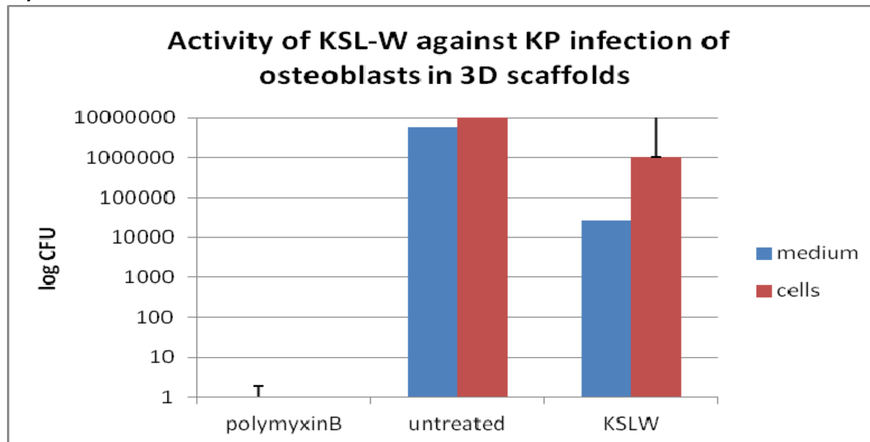


Figure 2. Treatment of infected osteoblasts (in scaffolds) with KSL-W (100ug/ml), polymyxin B for 1 hour; AB infection (a) and KP infection (b). Controls included polymyxin B (100ug/ml) and untreated controls. This experiment represents typical results from a single experiment with triplicate wells/condition. This was repeated independently at least two times.

Challenges in the research project:

Work with primary osteoblasts is challenging as they grow very slow, and have limited ability to divide. To complete the work with primary osteoblasts an extension was requested and it is anticipated that the work will be completed by December 2012.

The proposed work was to test antimicrobial peptides to treat infected osteoblasts. The findings from this work indicated that all three species of bacteria can survive intracellularly in osteoblasts. The tested peptide KSL-W does not penetrate eukaryotic cell membranes, and future studies are needed to modify the peptide or encapsulate the peptide to reach the intracellular bacteria in the osteoblast.

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of a 3D collagen scaffold with primary osteoblasts to study osteoblast differentiation by gene expression.
- Developed a working 3D model of osteoblasts on a collagen scaffold to study the interaction of osteoblasts and bacteria.
- Significant finding- All three Gram negative bacteria that are associated with osteomyelitis in war wounds, namely, *Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Klebsiella pneumoniae* invade osteoblasts and can survive intracellularly.
- Preliminary findings suggest that several genes associated with osteoblast differentiation and maturation is impacted when infected with these Gram negative MDR bacteria and may negatively affect osteoblast maturation.
- Treatment with KSL-W was able to reduce extracellular bacteria compared to untreated control; KSL-W was not able to kill intracellular AB or KP; KSL-W was not effective against PA.

REPORTABLE OUTCOMES

- Manuscripts in preparation

CONCLUSION

This study resulted in an important finding in that Gram negative bacteria associated with war related osteomyelitis can survive intracellularly in osteoblasts.

In addition, our investigations suggest that intracellular *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* have the ability to reinvade healthy osteoblasts.

For all pathogens, osteoblast marker genes ALPL and BGLAP showed reduced and unchanged expression. In addition, post infection the master transcription factor Runx2 which is the activator of osteoblast differentiation was not significantly different from the uninfected controls for AB and KP and increasing for PA at 72 hours. The expression of several other genes also may depend on Runx2, and could contribute to the lack of expression observed for several other genes. Another important gene related to osteoblast differentiation Dlx5 was either downregulated or had no significant changes when infected with AB, KP or PA. The transcription factor Twist1 needs downregulation for osteoblast differentiation, yet we saw upregulation when infected with AB and PA, and not significantly changed with KP.

Interestingly, the growth factor gene BMP2 was upregulated only for AB, and BMP7 was upregulated for AB and KP. Infection with PA did not result in significant changes to either BMP2 or 7.

The cell adhesion genes were also impacted by infection as N-cadherin (CDH2) was down regulated/not significant at the later time points except for the initial upregulation at 4 hours for KP and PA.

AB infection elicited an early response for IL1b, IL8, IL6 and TNFα. KP infection was similar except IL8 and TNFα were induced at 48 and 72 hours post infection respectively. When infected with PA, inflammatory responses were more vigorous with induction of all these genes at all time points except IL6 at 72 hours. Thus, proinflammatory responses in osteoblasts suggest that intracellular infection with these bacteria does result in changes to the bone cells which may affect their differentiation and maturation processes.

Even though the osteoblasts harbor reduced numbers of intracellular bacteria the infections may cause delays in osteoblast maturation at 3 days post infection. The long term effects of such infections on the bone cells are unknown.

The effect of KSL-W was tested and it was effective in decreasing the extracellular bacterial load in the media for both AB and KP. Intracellular bacteria were not reduced by KSL-W treatment. As appropriate, therapies should then be focused towards the eradication of intracellular bacteria. For example, cell penetrating peptides should be tested against these bacteria. An antimicrobial peptide formulation in nanoparticle encapsulation may be more appropriate for treatment of intracellular AB, KP and PA infections of the osteoblasts.

"So what" section:

Osteoblasts and other cell types in vivo have a typically 3D structure from which they are able to migrate, attach and proliferate. In order to study osteoblasts in vitro, a natural matrix such as collagen to act as support for seeded osteoblasts is vital. In order to study the interaction of bacteria with osteoblasts, a 3D model of infection is essential as it has been reported that osteoblasts in a 3D environment is more physiologically relevant. An osteoblast 3D model for infections has not been reported in the literature yet.

The important finding from the present work is that *Acinetobacter baumannii, Pseudomonas aeruginosa and Klebsiella pneumoniae* have the ability to reside as intracellular pathogens in osteoblasts. The intracellular bacteria may also affect osteoblast maturation and differentiation, and could potentially delay bone wound healing. Most antibiotics used to treat osteomyelitis do not penetrate osteoblasts. Antimicrobials such as peptides that can penetrate the host cells should be developed to eradicate these intracellular pathogens. These findings could potentially change treatment options for osteomyelitis in soldiers infected with these Gram negative pathogens.

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