The antidiabetic drug metformin acts on the bone microenvironment to promote myeloma cell adhesion to preosteoblasts and increase myeloma tumour burden in vivo

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microenvironment on both tumour growth and osteolytic bone disease was not fully considered [13–20].

The impact of metformin within bone is unclear, with recent research producing conflicting results [21]. Several have published presenting an anabolic effect of metformin on bone [22–25]. In vitro studies have shown an enhancement in osteoblast differentiation after metformin treatment as well as an increase in the expression of important osteoblast markers including collagen-1, osteocalcin, Runx2 or ALP [22,23,26]. In addition, metformin has been proven to inhibit formation of reactive oxygen species (ROS) and apoptosis in osteoblastic cultures exposed to high glucose concentrations [27]. In vivo studies have also demonstrated an increase in bone mineral density and bone micro-architecture, with results showing the ability of metformin to reduce bone loss in a number of disease states [28,29]. Studies of the effect of metformin on osteoclasts are limited, however metformin has been shown to increase circulating osteoclast precursors in postmenopausal women with type II diabetes [30]. Together these studies suggest a potential protective effect of metformin on bone health. However, in contrast, a number of studies found no osteogenic effects of metformin, with no effects on fracture healing [31–34]. These contradictory results likely reflect the complex mechanism of action of metformin combined with different in vitro and in vivo disease models yet highlight the potential for metformin to directly impact the bone microenvironment.

Multiple myeloma is exquisitely dependent upon cellular interactions within the bone microenvironment to support both tumour growth and survival and the development of the osteolytic bone disease [35]. The discovery that myeloma cells home to osteoblastic haematopoietic stem cell niches places osteoblasts as major regulators of the tumour bone niche [36,37]. While at later stages of disease osteoblasts are suppressed, contributing to myeloma bone disease, at early stages osteoblasts are known to support myeloma cell homing, dormancy and disease progression. As such, the reported osteogenic effects of metformin may have the potential to impact the overall response of myeloma to treatment with metformin. The aim of this study was to determine whether metformin has direct effects on the bone microenvironment that subsequently impact myeloma development and progression. Using in vitro and in vivo pre-treatment myeloma-bone model systems allowing us to separate direct and indirect effects of metformin, we demonstrate the unforeseen effects of metformin to promote myeloma cell adhesion and tumour burden via altering the bone microenvironment.

Methods

Metformin treatment in vivo

All procedures were conducted in accordance with the Animals Scientific Procedures Act of 1986 (UK) and approved by the University of Oxford Animal Welfare and Ethical Review Body (Home Office Project License 30/2996 and PCCCC8952). For in vivo studies, weight-matched, 5,6 week old female C57BL/KaLwRij were used (Harlan Netherlands B.V.). For specific experiments males were also used as indicated in figures legends. Within each study, mice were age, sex and weight matched between treatments. Mice were housed under standard conditions in a temperature and humidity controlled environment. Mice were sacrificed at day 25 post-inoculation. Bone marrow and spleen cell suspensions were filtered (70 µm filter) and analysed for percentage GFP positive cells, using Image J. When appropriate, an Incucyte® analysis software following 3 days proliferation kit were used for proliferation assays (V22885 and C34557, Thermo Fisher). GFP-myeloma cells were stained following manufacturer’s protocol on day 0 before seeding them as single culture or cocultured with 2T3. Some cells were used for flow cytometry to determine the highest level of stain at day 0. After 4 days of culture, cells were collected and studied again by FACS in order to determine the level of staining [37].

Cell viability was assessed using Alamar Blue (0.1 mg/ml). Quantification was performed on a BMG labtech FLUOstar plate reader.

Proliferation and viability assays

Vybrant Dl cell labelling solution and CellTrace™ Violet cell proliferation kit were used for proliferation assays (V22885 and C34557, Thermo Fisher). GFP-myeloma cells were stained following manufacturer’s protocol on day 0 before seeding them as single culture or cocultured with 2T3. Some cells were used for flow cytometry to determine the highest level of stain at day 0. After 4 days of culture, cells were collected and studied again by FACS in order to determine the level of staining [37].

Osteoblast mineralisation

2T3 cells were seeded and osteogenic media was used for 20–21 days. Osteogenic media was prepared using alpha MEM and including 50µg/ml ascorbic acid and 20 µM β-glycerophosphate. Media was made fresh every week and changed every 2–3 days. For mineralisation assay and quantification, a 2% alizarin red staining solution was prepared using water. Cells were fixed for 10 min with 10% formalin, washed with milliQ water and then alizarin red was added for 30 min. Nikon Eclipse TE300 inverted microscope was used to image 3–5 fields per well. Image J was used for quantification.
B. Gámez et al.

A

| 2T3 | CTR | 0.5  | 2   | 5   | 10  |
|-----|-----|------|-----|-----|-----|
|     |     |      |     |     |     |
| MM1S|     |      |     |     |     |

GFP intensity

| ST2 | CTR | 0.5  | 2   | 5   | 10  |
|-----|-----|------|-----|-----|-----|
|     |     |      |     |     |     |
| MM1S|     |      |     |     |     |

B

C

2T3 CONTROL

2T3 METF 5mM 48h

D

5TGM1-GFP

Fluorescence intensity

| CTR | Metf |
|-----|------|
| 1   | *    |

E

MM1S-GFP

% High Dil positive cells

| CTR | Metf | w/2T3 | w/2T3 pretreated |
|-----|------|-------|------------------|
| 50  | 70   | 30    | 10               |

F

5TGM1-GFP

% High Dil positive cells

| CTR | Metf | w/2T3 | w/2T3 pretreated |
|-----|------|-------|------------------|
| 2   | *    | **    | ***              |

G

MM1S w/2T3

| SN | ATT |
|----|-----|
| CTR|     |
| Metformin|

p21 Hs

β-actin

(caption on next page)
Fig. 1. Metformin-pretreated 2T3 preosteoblasts increase myeloma cell adhesion and reduce proliferation in coculture. (A) 2T3 preosteoblasts and (B) ST2 stromal cells were treated with increasing doses of metformin (0.5–10 mM) for 48 h. Cells were then washed and MM.1S myeloma cells seeded on top. 72 h later supernatant was removed and cells imaged (4x magnification). GFP quantification is shown in each figure. (C) STGM1 or MM.1S myeloma cells were cocultured with metformin-pretreated 2T3 preosteoblasts (5 mM) as described previously. Images were visualised either by brightfield or fluorescence microscopy (4x magnification). (D) Quantification of fluorescence intensity of STGM1 cells attached to 2T3 after 3 days of coculture (n = 5). *p < 0.05. Metf – metformin (B) MM.1S myeloma cells were cultured in the presence and absence of 2T3 preosteoblasts that had been pretreated or not with metformin. Data are expressed as the proportion of Dil high positive cells at day 4. ***p < 0.001 as compared to control. ###p < 0.001 as compared to myeloma cells cultured with 2T3. Representative graph of 6 independent experiments. (F) STGM1-GFP cells were cultured in the presence of 2T3 preosteoblasts that had been pretreated or not with metformin. Data are expressed as the proportion of high violet staining, *p < 0.05, **p < 0.01 and ***p < 0.001 as compared to control. ##p < 0.01 as compared to myeloma cells cultured with 2T3. Representative graph of 3 independent experiments. (G) 2T3 cells were treated with metformin for 48 h, washed with media and then MM.1S-GFP myeloma cells were seeded on top and cocultured for 72 h. Supernatant (SN) and attached (ATT) myeloma cells were removed following treatment with metformin, a significant increase in DiL/violet-positive, non-dividing myeloma cells was detected after 4 days of coculture (Fig. S1). To better understand the nature of the 3D systems). Secondary rabbit antibody against 

###siRNA experiments

Osteopontin siRNA, control siRNA and control siRNA-fluorescein conjugate were used for transient transfection (sc-36,130, sc-37,007, sc-36,869, Santa Cruz Biotechnology). 4 μl of RNAiMAX lipopectamine (13,778,075, Thermo Fisher) and 30 pmol siRNA were used per 6-well. In order to increase levels of OPN repression in some longer experiments, cells were seeded, next day transfected and retransfected again 48 h later when also metformin was added. Then media was removed and myeloma cells were seeded on top (direct coculture). Nikon Eclipse TE300 inverted microscope was used to image 3–5 GFP fields per well. Image J was used for quantification.

###RT-qPCR

RNA isolation was carried out using RNAeasy mini kit (74,104, QIAGEN) following manufacturer’s protocol. RNA was then reverse transcribed using Precision DNase kit (DNASE-50, Primerdesign) and Precision nanoScript2 Reverse Transcription Kit (RT-NanoScript2, Primerdesign). Sybr Green qPCR was performed using Fast SYBR®Green Master mix (Thermo Fisher). The following primers were used: Mm OPN (F:AGCCACAACAGTTTACACGAGG, R:CTGAGAAATGAGCAGTTAG TATTCCTG), Mm GAPDH (F:TCAACAGCAACTCCCACTCCTCA, R:A CCCTGTTGCTGTAGCCGGTATTCA), Mm β-actin (F:GCAAGCAGGAG- TAGCATGA, R: CCATGCACATTTGTCTCTT).

###Western blotting

Cell lysates were resolved and transferred to PVDF membranes. Primary antibody against osteopontin (AF808, R&d systems) was used at 5 μg/mL, human p21 at 1:1000 (2947, Cell signalling) and β-actin (AS53, Sigma) at 1:5000. cPARP antibody was used at 1:1000 (9544, Cell signalling). Binding was detected with horse peroxidase-conjugated antibodies (7074 & 7076, Cell signalling, 1:5000) and images obtained with UVITEC Fineallance.

###Micorcomputed tomography

Tibias were fixed in formalin and stored at 4 °C until needed. Bones were then mounted vertically in PBS and placed in the micro-CT scanner (SkyScan 1172, SkyScan) and scanned at an isotropic pixel size of 9 μm, 37 kV voltage, 228 mA current and using a 0.5 mm aluminium filter. Images were reconstituted and analysed using the SkyScan CT analyser software (Bruker) to quantify cortical bone lesions.

###Immunohistochemistry

OPN was detected in long bones using mouse OPN primary antibody at 15 μg/mL (AF808, R&d systems). Secondary rabbit antibody against goat was used at 1:200 dilution (P0160, Dako). Peroxidase reaction was performed using RTU Vectastain kit (PK7100, Vector) and impact DAB peroxidase substrate kit (SK4105, Vector). Then slides were counterstained with hematoxylin and mounted with DPX.
Myeloma cell adhesion to metformin-pretreated preosteoblasts is mediated through osteopontin

We have shown that metformin has a direct effect on preosteoblasts that increases the subsequent adhesion of myeloma cells. Adhesion of myeloma cells to osteoblasts within the endosteal niche is known to be a pivotal mechanism to support myeloma localisation. Osteopontin (OPN) is reported to positively regulate solid tumour cell proliferation and metastasis and has been recently implicated in tumour dormancy [40]. Since OPN is a chemotaxant expressed by osteoblasts and well known to play a role in bone cell homing, including multiple myeloma [40,41], we sought to investigate whether the effect of metformin to support myeloma cell adhesion and promote myeloma development was mediated via OPN. Treatment of 2T3 and MC3T3 preosteoblasts with metformin revealed a significant increase in OPN gene and protein expression (Figs. 2A&B, S7). In contrast, treatment of ST2 bone marrow stromal cells with metformin had no effect on osteopontin gene or protein expression (Fig. 2A&B). To further study the role of osteoblast-derived OPN in myeloma cell adhesion, loss-of-function experiments were performed using small interference RNA strategies. OPN specific siRNAs were used to successfully reduce OPN gene expression in 2T3 preosteoblasts (Figs. 2C, S8) and to reduce OPN protein in response to treatment with metformin when compared to scrambled control (Fig. 2D). Genetic knockdown of osteopontin expression in preosteoblasts was found to reduce the increase in myeloma cell adhesion in response to metformin pretreatment of 2T3 preosteoblasts (Fig. 2E&F). Taken together, these results indicate that osteoblast-derived OPN is at least partially responsible for the indirect effects of metformin to promote myeloma cell attachment to preosteoblasts.

Metformin pretreatment increases myeloma tumour burden and bone disease in vivo

Our in vitro studies suggest that metformin has an indirect effect on myeloma cells to increase myeloma cell adhesion and quiescence, mediated at least in part through preosteoblast-derived OPN. To determine whether this indirect effect can be observed in vivo, we combined the well-characterised STG1 murine model of myeloma with a metformin pre-treatment strategy, allowing us to exclude direct effects of metformin on myeloma cells. C57BL/KaLvRij mice were treated with metformin for 4 weeks at which point treatment was halted, prior to inoculation of STG1-GFP myeloma cells (Fig. 3A). C57BL/KaLvRij mice were treated with 2.5 mg/ml metformin in drinking water for 4 weeks, a dose previously shown to be clinically relevant. Metformin was well tolerated, with no detectable adverse effects, changes in body weight or water consumption and no changes in glucose levels (Fig. S9). Metformin pretreatment had no effect on either circulating levels of RANKL and OPG or local concentrations in the bone marrow plasma at 4 weeks (Fig. S10).

Pretreatment with metformin resulted in a significant increase in tumour burden, reflected by a doubling of the proportion of myeloma cells within the bone marrow and spleen, and elevated serum paraprotein (Figs. 3B, C, S11, S12). Myeloma-bearing mice developed a characteristic osteolytic bone disease, with microCT analysis demonstrating significantly more osteolytic lesions in mice pretreated with metformin (Fig. 3E&F). While we found no increase in OPN in the bone marrow plasma of mice treated with metformin for 4 weeks (Fig. 4A), metformin pretreatment elevated OPN levels in the bone marrow plasma, but not serum, of myeloma-bearing mice (Fig. 4B&C). Immunohistochemistry provided further evidence for an increase in osteopontin in the bone marrow of mice pre-treated with metformin (Fig. 4D).

Discussion

In recent years there has been widespread interest in investigating metformin as an anti-tumour and anti-aging therapeutic. While preclinical studies support a direct anti-tumour effect of myeloma, to date the contribution of the bone microenvironment has been largely ignored. Given the interdependence of myeloma cells with the bone microenvironment, particularly with bone-lining osteoblasts, and the conflicting reports of metformin in bone biology, we aimed to investigate whether metformin could have an indirect effect in myeloma via bone cells. In the current work we show how metformin acts upon preosteoblasts to increase the subsequent adhesion of myeloma cells, with this indirect effect on myeloma cells dependent, at least in part, upon elevated OPN levels in metformin-treated preosteoblasts. In vivo, we implemented a pretreatment model where mice were treated with metformin prior to tumour inoculation, allowing us to eliminate direct anti-tumour effects. Unexpectedly, metformin pretreatment was found to increase myeloma tumour burden and myeloma bone disease, with metformin treatment associated with an increase in osteopontin within the bone microenvironment. Altogether, our results show that metformin treatment increases OPN expression and osteogenic potential in preosteoblasts, increasing their capacity to harbour myeloma cells and potentially expanding the endosteal niche. In vivo, this may translate to myeloma cells adhering more efficiently to these niches and thus, worsening the disease outcome.

Osteoblasts play a critical role in the homing and maintenance of cancer cells within the bone marrow. It is known that osteoblasts contribute to several cancers by creating a favourable environment for the cells to home to the bone and metastasize [42]. In the same way, they contribute to myeloma by supporting myeloma cell growth and survival. This happens in part by the ability of osteoblasts to secrete IL-6 when they are in coculture with myeloma cells [43]. Osteoblasts are also involved in cell-to-cell interactions which facilitate homing of myeloma circulating cells [44]. Thus, the bone niche has been acknowledged as the site for myeloma cells to home and initially reside within the bone marrow and it has opened a large area of research to further study and understand the disease [36]. The bone marrow niche plays a vital role in
Fig. 3. Pretreatment with metformin increases myeloma tumour burden and osteolytic bone lesions. (A) C57BL/KaLwRij mice were treated with 2.5 mg/ml metformin or vehicle control for four weeks prior to cessation of treatment and inoculation of \(1 \times 10^6\) 5TGM1 myeloma cells. (Control \(n=8\), myeloma, \(n=17\), metformin \(n=17\)). The proportion of GFP-positive 5TGM1 myeloma cells in bone marrow (B) and spleen (C) was quantitated by flow cytometry (\(\ast\) \(p<0.01\), \(\ast\ast\ast\) \(p<0.001\) as compared to myeloma control). (D) Serum paraprotein was quantitated by IgG2b ELISA (\(\ast\ast\ast\) \(p<0.001\) as compared to non-tumour bearing mice). (E) Representative images of osteolytic bone lesions. (F) MicroCT analysis and quantification of osteolytic bone lesions (\(\ast\) \(p<0.05\), \(\ast\ast\ast\) \(p<0.001\) as compared to non-tumour bearing mice; \# \(p<0.05\) as compared to myeloma-bearing control. Data are presented as mean ± SEM.
homing, differentiation and proliferation of malignant plasma cells, with several studies highlighting the importance of osteoblasts in myeloma cell quiescence and dormancy [36,37]. We provide evidence to suggest that metformin impacts the bone marrow niche with a significant increase in myeloma cell adhesion to preosteoblasts in response to prior treatment of osteoblasts with metformin. Interestingly, the bone marrow stromal cell line ST2 did not support changes in myeloma cell adhesion highlighting the specific effect of metformin on osteoblasts. Our studies elucidate changes in myeloma cell proliferation in response to metformin effects on preosteoblasts. The use of proliferation markers allowed us to explore the level of division of myeloma cells in coculture with pretreated osteoblasts. These results showed that myeloma cells presented a reduction in their proliferation rate together with an increase in p21 expression. Thus, the direct effects of metformin on preosteoblasts result in indirect effects on myeloma cells, including an increase in attachment.

Given our findings, and the reported osteogenic effects of metformin, it could be postulated that metformin may have a role in expanding the endosteal niche where dormant and/or quiescent myeloma cells reside. It is known that after tumour inoculation into C57Bl/KaLwRij mice, only a few colonizing myeloma cells will adhere to endosteal bone whereas the majority of myeloma cells will continue circulating through the bone marrow [37]. Thus, we speculated whether metformin, by the changes observed on osteoblasts in vitro, was able to create a more favourable bone marrow environment for the initial retention and subsequent expansion of tumour cells. To determine whether an indirect effect of
metformin could impact the development of myeloma, we used the 5TGM1 mice model. C57/KaLwRij mice were treated with metformin for 4 weeks, with subsequent inoculation of myeloma cells following cessation of metformin treatment, to eliminate direct antitumour effects of metformin. The dose of metformin was previously shown to be clinically relevant [45,46] and was well tolerated. No effect of metformin on blood glucose levels was observed, which may reflect that the animals were not diabetic, or on a high-fat diet, and were not fasted prior to blood glucose analysis. Interestingly, pretreatment with metformin resulted in an increase in tumour burden and osteolytic bone lesions, suggesting that the effects of metformin on the host microenvironment indirectly promote myeloma. It is intriguing to consider and warrants further investigation as to whether the increased tumour burden in vivo may be indicative of an in vivo influence to activate quiescent myeloma cells that is not present in our in vitro culture systems, where metformin pretreatment increases adhesion and reduces proliferation, or whether it is a result of increased homing and/or adhesion of myeloma cells to bone. Notably, we observe an increase in tumour burden in both bone marrow and spleen, reflecting the haematopoietic nature of both sites in mice, but also suggesting the contribution of a systemic effect of metformin to promote myeloma cell homing. Current clinical evidence to support metformin use in MGUS and myeloma comes from diabetic patients, and diabetes has been associated with poor clinical outcomes in myeloma [10]. We acknowledge that our findings are limited to a non-diabetic myeloma model, and as such, do not interrogate the relationship between diabetes and myeloma development. While there are limited reports of such tumour-enhancing effects of metformin, the antioxidant antidiabetic agents saxagliptin, sitagliptin and α-lipoic acid have also been shown to accelerate metastasis in murine experimental metastasis models [47].

OPN is known to positively regulate solid tumour cell proliferation and metastasis and is a chemoattractant well known to play a role in bone cell homing, including multiple myeloma [40,41,48,49]. Metformin was found to increase osteopontin expression in a panel of osteoblasts and to induce cell attachment of different myeloma cell lines. Moreover, the genetic knockdown of osteopontin expression in osteoblasts resulted in a reduction of cell adhesion. In vivo, pretreatment with metformin significantly elevated OPN within the bone marrow microenvironment of myeloma-bearing mice, as measured by ELISA. Immunohistochemistry provided further support, with a clear increase in OPN expression in the bone marrow of non-tumour-bearing mice treated with metformin. In addition to cells of the bone microenvironment, myeloma cells are also known to express OPN [50,51], and it cannot be excluded that the increase in OPN reflects the elevated tumour burden observed following metformin pre-treatment. Notably, no such increase was observed in circulating OPN, suggesting that metformin has specific effects to elevate OPN in vivo within the myeloma-bone microenvironment. While a limitation of our study is the use of murine systems, the high homology between murine and human osteopontin supports the clinical relevance. While our data support an effect of metformin on myeloma progression, the high homology between murine and human osteopontin supports the clinical relevance. While our data support an effect of metformin on myeloma progression, the high homology between murine and human osteopontin supports the clinical relevance. While our data support an effect of metformin on our study demonstrates that pretreatment of myeloma-bearing mice with metformin has significant effects to worsen myeloma development, with increased tumour burden and bone disease. The interdependence between myeloma cells and cells of the bone microenvironment has long been known to support both tumour growth and bone disease. The importance of this cellular crosstalk is exemplified by our current study, where an unexpected in vivo drug response can be explained, at least in part, by indirect effects mediated by osteoblasts and highlighting the importance of studying myeloma pathogenesis within a physiologically-relevant setting.

CRediT authorship contribution statement

Beatriz Gámez: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. Emma V. Morris: Methodology. Sam W.Z. Olechnowicz: Methodology. Siobhan Webb: Conceptualization, Methodology. James R. Edwards: Methodology. Aneka Sowman: Methodology. Christina J. Turner: Methodology. Claire M. Edwards: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101301.

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