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Research paper

The role of lysyl oxidase, the extracellular matrix and the pre-metastatic niche in bone metastasis [☆], [☆] [☆]

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ABSTRACT

Most deaths from solid cancers occur as a result of secondary metastasis to distant sites. Bone is the most frequent metastatic site for many cancer types and can account for up to 80% of cancer-related deaths in certain tumours. The progression from a discrete solid primary tumour to devastating and painful bone metastases is a complex process involving multiple cell types and steps. There is increasing evidence that modulation of the extracellular matrix plays an important role in the lethal transition from a primary to disseminated metastatic bone tumour. This review provides an overview of the current understanding on the role of lysyl oxidase, the extracellular matrix and the pre-metastatic niche in bone metastasis © 2016 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Once a primary tumour spreads to distant sites in the body and establishes a secondary tumour, the consequences are devastating, and mostly lethal. Bone is a typical site for cancers to spread to (most commonly breast, prostate, lung, kidney, thyroid and colon cancer) – however why cancer cells preferentially home to bone over other organs in some cancers, but not all, is not fully understood. There is increasing evidence that modulation of the extracellular matrix (ECM) plays an important role in the lethal progression from a primary to metastatic bone tumour. Yet it is not known which occurs first, abnormal extracellular matrix (ECM), which supports secondary tumour formation, or the arrival of cancer cells into the bone that create abnormal ECM. Recently it has been shown that tumour-derived factors circulate the body and exert effects on ECM remodelling within distant organs, creating so-called pre-metastatic niches. This review provides an overview of the current understanding on the role tumour secreted lysyl oxidase (LOX) and modulation of the ECM in bone metastasis.

2. ECM determines cell behaviour

The extracellular matrix (ECM) is made up of over 300 proteins, the majority of which are fibrous proteins (e.g. fibronectin, elastin and collagens) and proteoglycans (e.g. keratin sulphate, heparin sulphate,

chondroitin sulphate). Typically, these proteins are secreted locally and assemble into a complex network of macromolecules, or an “organised mesh”, which has distinctive physical, biochemical and biomechanical properties and which forms the structural framework of most tissues. Previously perceived as being a stable scaffold with merely a supportive role in maintaining tissue morphology, the ECM has now emerged as a dynamic entity and a critical regulator of cell physiology. The versatile nature of the ECM components means that this organised mesh has very unique properties that, through direct or indirect means, regulates almost all cellular behaviour and when tightly controlled is fundamental for embryonic development and organ homeostasis. For example the physical properties of the ECM (rigidity, porosity, topography etc) dictate the tissues architecture and integrity, whilst acting as an anchorage site as well as a migration track or barrier, having both positive and negative influences on cell migration. The biochemical properties of the ECM confer cells with the capability to “sense” and interact with their environment either in a direct (by acting as precursor of biologically active signalling fragments) or indirect (by binding growth factors and limiting their diffusive range) manner that results in signal transduction cascades, gene expression or other changes in cellular behaviour. Finally, the biomechanical properties of the ECM also dictates cellular behaviour due to its huge range in elasticity (from soft and compliant to stiff and rigid) enabling the cell to sense external forces. Thus, the ECM acts as mechanotransducer that translates mechanical tissue loading into cellular signals determining cell behaviour; a process that is particularly critical in the lifelong maintenance of healthy bone.

Given the importance of the ECM in directing almost all cellular behaviour, it is not too surprising that altered ECM deposition, synthesis and post-translational modification leads to a disorganised mesh with differing properties and results in diseases

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such as organ fibrosis and cancer. For more detailed reviews on how increased stiffness translate into regulation of cellular processes such as motility, proliferation and survival please see Cox and Erler 2014 [1] and Pickup et al. 2015 [2]).

3. ECM, cancer and metastasis

For cells to become cancerous they must acquire the ability to survive, grow and invade leading to malignant transformation, tumour formation and ultimately overt secondary metastasis. Abnormal ECM can promote these abilities and is a well-documented hallmark of cancer. In fact, it has also been shown that aberrant ECM can precede malignant transformation in many tissues [3]. In addition, many ECM components and their receptors are overexpressed by cancer cells which typically leads to abnormal ECM that may potentiate the oncogenic effect of various growth factors, including ones known to be fundamental in bone remodelling, such as VEGF, IGF-1 and TGF- β . Whether these events occur concomitantly or one proceeds the other is much like asking which came first the chicken (ECM) or the egg (cancer)! It would be interesting to gain further insight into the nature and indeed timing of these changes, as well as understanding similarities and differences in the changes within the ECM and the cancer cells themselves.

Physiological changes in the external environment of the tumour, such as hypoxia can also lead to changes in the ECM due to increased expression of enzymes responsible for the post-translational modification of collagen and other ECM components. This includes the lysyl oxidase (LOX) family of extracellular amine oxidases whose primary function is to post-translationally modify collagens and elastin in the ECM, thereby catalysing the covalent crosslinking of collagen fibres increasing stiffness and tensile strength. The increased stiffness of the ECM is “sensed” by the cancer cells which in turn focus their activities towards invasion, as opposed to proliferation, and drives them to migrate to distant metastatic sites. Consistent with this, the LOX enzyme was shown to be associated with lower distant metastasis-free survival and overall survival in breast cancer patients with ER-negative tumours, and also in head and neck cancer patients [5]. Expression of another LOX family member, LOX Like protein-2 (LOXL-2) was similarly shown to be correlated with metastasis and decreased survival in patients with aggressive breast cancer. Although not required for primary tumour growth, LOXL-2 was required for metastatic colonisation and metastatic growth *in vivo*. Mechanistically, LOXL-2 was shown to regulate the expression and activity of other ECM modifying proteins including tissue inhibitor of metalloproteinase-1 (TIMP1) and matrix metalloproteinase-9 (MMP9), key proteins involved in ECM remodelling [6]. Targeting of LOXL-2 with an inhibitory monoclonal antibody (AB0023) was also shown to be efficacious in both primary and metastatic xenograft models of cancer [7]. Further evidence that the LOX proteins can induce changes in the tumours ECM's physical properties to facilitate metastasis was provided more recently with the observation that LOX-mediated collagen crosslinking creates a growth-permissive fibrotic microenvironment capable of supporting metastatic growth by enhancing tumour cell persistence and survival [8]. Strikingly, not only do LOX enzymes modulate the ECM of the primary tumour, but they can also modulate the ECM of distant organs to form the pre-metastatic “niche” prior to the arrival of tumour cells and development of metastases [9]. The exact mechanisms behind this niche formation are not known; do tumour-derived factors circulate the body and exert differing effects on ECM remodelling within different organs, or do particular tumour-secreted factors initiate specific cascade reactions and signalling events in specific tissues? Pertinent to this review, the

ability of these enzymes to modulate the ECM in the bone to facilitate bone metastasis is only now just being recognised [10].

4. ECM and bone metastasis

When considering the constituents of the ECM, as well as the molecular pathways involved in the dysregulation of the ECM that leads to oncogenic transformation, the similarity and overlap with the constituents of bone and the pathways involved in its homeostasis and remodelling are obvious. Bone is the largest “organised mesh” (by mass) in the human body – providing the ultimate scaffold that dictates the human form and ultimately function. Bone, like any other ECM, consists of fibrous proteins, predominantly type I collagen, and non-collagenous proteins such as proteoglycans, and glycoproteins such as osteopontin and fibronectin. The fact that many cancers have a predilection to metastasise to bone should perhaps not be so surprising then. However, unlike other ECMs bone is physiologically mineralized (thus 100,000–1,000,000 times stiffer than other tissues such as breast tissue). Bone is constantly remodelled throughout life, continually synthesizing osteoid – the unmineralised collagenous matrix which makes up about 50% of the bone's volume. These two properties should in theory preclude and facilitate invasion of cancer cells in the bone respectively, yet this appears not to be so. Stephen Paget first proposed the “seed and soil” hypothesis back in 1889 [11], this seminal paper and the resultant century or more of research has led to concept of the “bone and cancer vicious cycle” whereby once cancer cells arrive in the bone they release factors that stimulate osteoclastic bone resorption (IL-6, IL-8, MMPs TIMPs etc). This bone resorption causes the release of bone stored factors (in particular TGF- β) that favour the growth of the tumour in bone, which inevitably stimulates more osteoclastic bone destruction, and so on. Significant advances in identifying key players in the vicious cycle have been made in the last decade, with many attempts to prevent bone metastasis being focused on targeting this vicious cycle.

But the big question on most bone oncologists lips, and which remains largely unanswered, is why do certain circulating tumour cells particularly home to bone in the first place? Whilst there are many theories from hijacking stem cell niches to osteomimicry, there is one in particular that is at the same time an intriguing and horrifying concept – that is the ability of the primary tumour to modify the bone's ECM to facilitate the initiation of the vicious cycle of bone metastasis prior to the arrival of tumour cells.

In an effort to determine whether the primary tumour might be responsible for “fertilising” the bone “soil” ready for the cancer “seed”, we recently found that a hypoxic gene signature, and more specifically the secreted enzyme lysyl oxidase (LOX) was closely associated with bone metastasis, specifically in estrogen receptor negative (ER-) breast cancer patients. We further investigated the role of LOX in bone metastasis *in vivo* and found that tumour-bearing mice showed increased bone loss and the formation of focal osteolytic lesions over time. Whilst this would not be too surprising an observation in a mouse that has overt bone metastases – we found these changes occurred from as little as 2-weeks post tumour implantation (when no metastases are present) and that they could be recapitulated by injection of cell-free conditioned medium (secreted factors) from cancer cells into mice. These changes were LOX-dependent, as cancer cells expressing shLOX injected into mice showed significantly less osteolytic lesions. Our data clearly showed that early osteolytic lesions are formed in the absence of tumour cells by hypoxia-induced tumour-secreted factors. Mechanistically these macroscopic changes, which would directly alter the physical, biochemical and biomechanical properties of bone ECM, were as a result of LOX

modulating bone homeostasis leading to unbalanced coupling in favour of bone degradation and the formation of pre-metastatic osteolytic lesions. The physiological consequence of these ECM changes were increased metastatic burden in the bone, with micro-CT analysis revealing a positive correlation between lesion number and tumour burden. This demonstrated that LOX-mediated pre-metastatic changes to bone ECM led to the generation of pre-metastatic niches within the bone microenvironment that support colonisation of circulating tumour cells and the formation of overt bone metastases [13]. Furthermore, LOX may well be a useful marker for predicting the likelihood of metastases to the bone in ER- breast cancer patients and identifying these patients for early adjuvant bisphosphonate treatment. Similarly, in the future anti-LOX therapy, may prevent secondary bone cancer from occurring. Interestingly, a recent meta-analysis from the Early Breast Cancer Trialists' Collaborative Group recently confirmed that adjuvant bisphosphonates reduce the rate of breast cancer recurrence in the bone and improve breast cancer survival, albeit with definite benefit only in women who were postmenopausal when treatment began [14]. For more information on this see our recently published Cancer Research Review [15].

Increasing evidence for the role of the LOX family of proteins in other cancers that metastasise to bone, specifically prostate cancer, has recently been provided. Caley and colleagues demonstrated that the tumour-associated collagen receptor Endo180 and the crosslinking of collagen by stromal-derived LOX controls tumour cell movement and as such is a potential target for limiting metastatic progression in prostate cancer [16]. In another study by Alsulaiman and colleagues whose initial aim was to provide evidence to support previous work that suggests LOX pro-peptide (LOX-PP), an 18kDa pro-peptide that is formed as Pro-LOX is processed by procollagen C-proteinases to yield the mature LOX enzyme, acts as a tumour suppressor. However, unexpectedly they found that LOX-PP was able to stimulate osteoblast differentiation as well as osteoclast formation and differentiation either in the presence or absence of exogenously added RANKL and M-SCF. Intramedullary injections of PC3 cells expressing LOX-PP showed increased osteoclast resorption and bone destruction *in vivo*, whilst the effect to enhance the development of osteoblastic lesions in the DU145 cells expressing LOX-PP was absent in their model [17]. This apparent paradox, that the effects of LOX-PP in all soft tissue tumour studies so far have demonstrated a beneficial outcome, whilst in mineralized tissue, LOX-PP shifts the balance towards a destructive, pro-cancerous effect could be due to the very unique properties of bone ECM. Delineating the differences between the effects of the same protein on different ECM will have beneficial therapeutic value for both types of cancer.

Other proteins reported to play a role in bone marrow alteration and bone metastasis include (but not limited to) CXCR4, IL-11, IL-6, IL-8, TGF- β s, BMPs, CTGF, various MMPs and PTHrP. However, most of these have been shown to be important once the tumour cells arrive in the metastatic site and not before. Determining whether these molecules also play a role in pre-metastatic niche formation warrants further investigation.

5. Future perspectives

Identification of LOX as a modulator of the ECM to form pre-metastatic niches and subsequently bone metastasis arose as we were investigating the hypoxia tumour secretome using existing patient cohort data and cutting edge proteomics. Although condoned by some researchers as “fishing” experiments, the “hypothesis-free” comparative or subtractive analysis approach has proved to catch some important targets. At the start of the 21st century use of gene microarrays enabled the interrogation of

between 15,000–25,000 genes at a time and led to the identification of regulators of breast cancer to bone metastasis such as interleukin-11 and CTGF [18] as well as ECM genes such as *POEM* [19]. In just over a decade, technology has improved our capacity to comprehensively profile genes as well as secreted proteins associated with bone metastasis. This has led to the identification of novel biomarkers and candidate therapeutic targets such as LOX family members [13,15,20,21], cystatins (CST1, CST2, and CST4), plasminogen activators (PLAT and PLAU) and collagen functionality proteins (PLOD2 and COL6A1) [20].

The ethos of publically sharing data sets [21], the increasing availability (and reduction in cost) of deep-read RNA sequencing and whole genome sequencing methodology, plus the progress in systems biology/bioinformatics in the last 5 years or so will mean that these sophisticated fishing expeditions are bound to net a considerable haul of potential therapeutic targets.

In summary, in an effort to contain tumours to their primary site, and thereby limit the high morbidity and mortality associated with metastasis, the targeting of tumour secreted molecules and prevention of pre-metastatic niche formation offers a powerful therapeutic option. (Fig. 1).

At the site of a primary tumour, cells become cancerous and acquire the ability to survive, grow and invade leading to malignant transformation, tumour formation and ultimately overt secondary metastasis. (1) Abnormal ECM can promote normal cells to become cancerous (blue arrows). In addition as cells undergo oncogenic transformation, many ECM components and their receptors are overexpressed by cancer cells which typically leads to abnormal ECM (red arrows). Whether these events occur concomitantly or one proceeds the other is much like asking which came first the chicken (ECM) or the egg (cancer)! Once a primary tumour has established circulating tumour cells will eventually arrive in distant sites such as bone (osteotropism). (2) Once a tumour cell is present in the bone a “vicious cycle” occurs whereby cancer cells release factors which enhances the osteoblastic stimulation of osteoclast resorption. This resorption of bone changes the bone ECM, releasing stored growth factors which promote cancer cell growth, and so on. (3) More recently it has been shown that primary tumour cells release proteins and ECM components which modulate the bone ECM prior to the arrival of cancer cells and initiation of the vicious cycle. (3) Once the changes in the distant metastatic site have taken place cancer cell recruitment takes place and the result is overt metastasis (with or without involvement of the vicious cycle), which in the case of secondary bone cancer has lethal consequences.

The main outstanding questions

- Why do certain cancer cell types preferentially home to bone?
- Specifically what is it about mineralized tissue that makes it such a favourable place to grow?
- Which occurs first - abnormal extracellular matrix (ECM) which drives oncogenic transformation, or cancerous cells which create abnormal ECM?
- Does bone remodelling need to, at least in part, occur before cancer cells can successfully colonise? Or, do cancer cells start the remodelling only when they arrive in the bone marrow. Or both?
- Do tumour-derived factors circulate the body and exert differing effects on ECM remodelling within different organs, or do particular tumour-secreted factors initiate specific cascade reactions and or other signalling in specific tissues?
- Are there any built-in mechanisms within the bone which may restrict, or accelerate aberrant ECM remodelling?

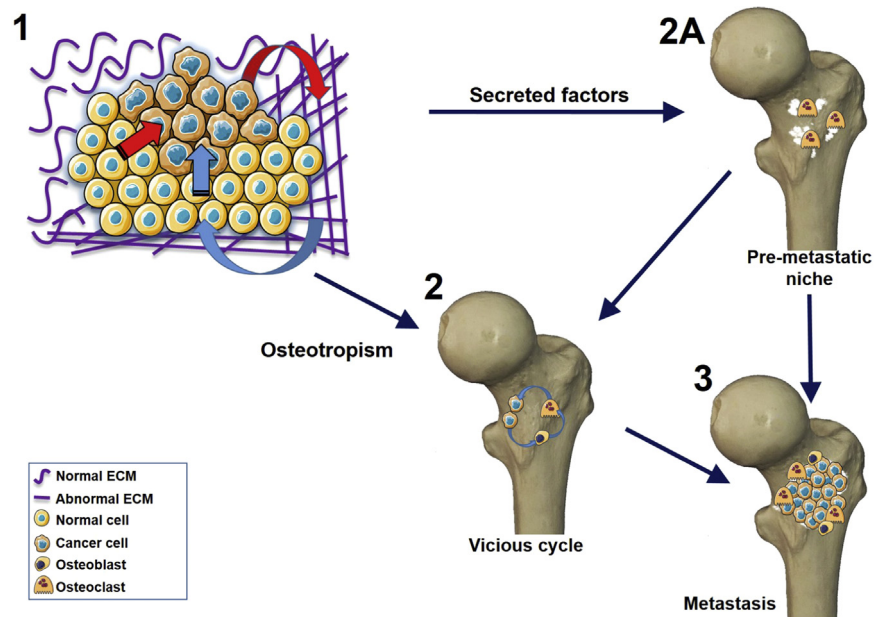


Fig. 1. ECM changes in bone metastasis.

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