Lysyl Oxidase Mediates Hypoxic Control of Metastasis

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Abstract
Hypoxic cancer cells pose a great challenge to the oncologist because they are especially aggressive, metastatic, and resistant to therapy. Recently, we showed that elevation of the extracellular matrix protein lysyl oxidase (LOX) correlates with metastatic disease and is essential for hypoxia-induced metastasis. In an orthotopic rodent model of breast cancer, a small-molecule or antibody inhibitor of LOX abolished metastasis, offering preclinical validation of this enzyme as a therapeutic target. (Cancer Res 2006; 66(21): 10238-41)

Introduction
Metastasis is a multistep process that is influenced by cell-cell and cell-matrix interactions as well as by tumor blood and lymph supply (1). Hypoxia, the condition of low oxygen tension that is present in all solid tumors (>1 cm³ due to inadequate blood supply, is clinically associated with metastasis and poor patient outcome, although the underlying processes remain unclear (1). Recently, gene microarray studies done in our laboratory revealed that expression of an extracellular matrix (ECM) protein, lysyl oxidase (LOX), is consistently overexpressed by hypoxic human tumor cells (2).

In the ECM, LOX initiates the covalent cross-linking of collagens and elastin, thereby increasing insoluble matrix deposition and tensile strength (3). LOX is synthesized in fibrogenic cells as a pro-enzyme that is cleaved and glycosylated before secretion (3). Once LOX is secreted, the proenzyme is then proteolytically processed by procollagen C-proteinase (bone morphogenic protein-1) releasing a 32-kDa biologically active mature form of the protein. LOX is induced by several growth factors and steroids, such as transforming growth factor-β, tumor necrosis factor-α, and IFN-γ (3). Four LOX-related proteins have been identified (LOXL1, LOXL2, LOXL3, and LOXL4) that all contain the 205 amino acid LOX catalytic domain in their COOH terminus (3). Nevertheless, it is clear that LOX has a unique role because LOX knockout mice die at parturition (3). Although the main activity of LOX is thought to be oxidation of specific lysine residues in collagen and elastin outside of the cell, recent reports indicate that it can induce chemotaxis and even act intracellularly by regulating gene expression (3). LOX elevation occurs in metastatic and/or invasive breast cancer cell lines and correlates with increased staging in renal cell carcinoma (4). In vitro invasion of breast cancer cells can be prevented by treatment with antisense oligonucleotides or with a small-molecule inhibitor of LOX activity called 3-aminopropionitrile (BAPN; ref. 4). Furthermore, increased LOX expression is associated with the early stromal reaction in breast cancer (5). We therefore investigated whether LOX was involved in the enhanced invasive and metastatic potential of hypoxic tumor cells, focusing on breast cancer.

LOX Is Essential for Hypoxia-Induced Metastasis
Induction of LOX by hypoxia is mediated by hypoxia-inducible factor. LOX possesses a canonical and functional hypoxia response element in its promoter. Hypoxia-increased LOX mRNA expression results in dramatically increased levels of secreted LOX protein and activity (measured in the conditioned medium of cells). Furthermore, there is a statistically significant association between LOX expression and hypoxia as assessed by pimonidazole staining in orthotopically grown breast cancer tumors and between LOX and hypoxia in patient array data.

LOX expression in cancer patients. Paradoxically, LOX was originally identified as a tumor suppressor in nontumorigenic revertants of ras-transformed fibroblasts and loss of LOX is associated with tumorigenesis in several cancer types (3). Several reports exist describing reduced LOX expression in human cancer patients, supporting the idea that LOX acts as a tumor suppressor. Other reports describe elevated LOX levels in patients with cancer. The majority of all these reports were unable to provide statistical analyses due to the small numbers of patients studied. Larger studies in patients with gastric (6), colon (7), and prostate (8) cancers have generated convincing data to show that a percentage of these tumors have reduced LOX expression due to methylation (gastric) or mutation (colon). These data raise the distinct possibility that intracellular LOX and extracellular LOX have opposite functions. Therefore, strategies to inhibit LOX to prevent metastases should be focused on the extracellular protein until the intracellular role of LOX is better characterized.

In our recent article, we did a retrospective study using a published microarray data set from 295 breast cancer patients (9), identifying a positive association between LOX expression and hypoxia. We additionally found an association between LOX expression and outcome of patients with estrogen receptor (ER)–negative tumors, such that patients with high LOX had shorter metastasis-free and overall survival. In a prospective study using a tissue array from head and neck cancer patients (n = 91), we also found a strong association between LOX expression and a marker of hypoxia (CA-IX, ref. 10). Furthermore, there was a similar correlation between LOX expression and survival, such that head and neck cancer patients staging positive for LOX had significantly reduced metastasis-free and overall survival.

LOX and proliferation. Previous reports have shown that genetic inhibition of LOX increases cell proliferation, again supporting the idea that LOX acts as a tumor suppressor. Mechanistically, LOX is thought to affect fibroblast proliferation through elevated cyclin D1, as antisense down-regulation of LOX in rat kidney fibroblasts increases cyclin D1 expression through β-catenin signaling (11). Recently, it was reported that it is the COOH-terminal propeptide region of LOX that acts intracellularly to suppress transformation of fibroblast by Ras (12). This could

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explain why chemical inhibition of LOX enzymatic activity (extracellularly) does not affect proliferation. In breast tumor cells, genetic inhibition of LOX through short hairpin RNA (shRNA) expression did not have a significant effect on cell growth, either in vitro or in vivo, or on cell cycle distribution. These studies further raise the importance of understanding the intracellular and extracellular roles of LOX.

**LOX and invasion.** What is clear and consistent is that LOX inhibition by genetic or chemical means prevents in vitro invasion of breast, melanoma, and pancreatic cancer cells (4). We found a role for LOX in the enhanced invasiveness of hypoxic tumor cells, providing a mechanism of how oxygen deprivation enhances in vitro invasion of cancer cells (1). Hypoxia-induced invasion can be prevented by treatment with LOX antisense oligonucleotides, shRNA expression, or small-molecule inhibitors in breast, cervical, head and neck, pancreatic, renal, lung, colon, and melanoma, human cancer cells. The decreased invasive activity of LOX shRNA-expressing breast cancer cells could be rescued by overexpression of a mature form of LOX but not by a mutant form lacking its catalytic domain. Invasion of LOX shRNA-expressing cells could also be increased by the addition of conditioned medium from control cells, supporting a role for secreted LOX.

We additionally examined the phenotypic growth of control and shRNA cells in collagen, in the presence or absence of hypoxia. Control cells formed branch-like invasive structures particularly under hypoxia, whereas shRNA cells remained spherical and noninvasive in both aerobic and oxygen-deprived conditions. When we stained these structures for LOX, we found little expression in the shRNA cells and in the aerobic control cells. Staining was dramatically increased by exposure to hypoxia, resulting in high levels expressed on the outside of the cell with the most intense staining at the leading edge of pseudopodia extending along hair-like fibers into the surrounding ECM. These experiments suggest that LOX is involved in the matrix remodeling that occurs during invasive migration that allows cell movement.

Invasive migration is a complex process involving a series of adhesion and detachment interactions with the ECM, primarily collagen (13). We showed that hypoxic LOX expression results in increased actin polymerization, focal adhesion formation, cell-matrix adhesion, focal adhesion kinase (FAK) activation, cell movement, and cell migration. We found that these events, all of which are essential for invasive migration, could be abolished by LOX shRNA and restored through overexpression of mature LOX (and not mutant LOX lacking a catalytic domain). FAK activation could be further inhibited by addition of small-molecule inhibitors to LOX or treatment with a β-1 integrin blocker, suggesting that LOX enzymatic modification of collagen increases availability of this β-1 integrin ligand-stimulating activation. Addition of catalase could further reduce FAK activation, supporting a recent report that showed a role for aerobic LOX in FAK activation and cell movement through $H_2O_2$ production and Src activation (14).

LOX inhibition in aerobic cells has been reported to result in increased actin polymerization and focal adhesion formation through regulation of the p130Cas/Crk/DOCK180 signaling pathway, preventing cell migration and motility (15). Although these results are contradictory to our own, several factors can explain the experimental differences. First, in their experiments, cells were grown on fibronectin and not on collagen. Second, we used genetic, pharmacologic, and antibody methods to inhibit LOX. Lastly, actin polymerization and focal adhesion formation are indicative of increased adhesion interactions that have been reported to prevent migration in some situations and to increase migration in others, such as epithelial-mesenchymal transition that is observed in cancer dissemination, and in conditions such as oxygen deprivation (13).

**LOX and metastasis.** Although evidence existed that LOX is involved in invasion, there was no direct evidence to indicate that LOX played a role in metastases. In an orthotopic model of breast cancer, we showed that LOX shRNA expression significantly reduced formation of lung metastases and liver metastases. To test the therapeutic usefulness of LOX inhibition, we treated mice bearing control orthotopic tumors with a small-molecule inhibitor of LOX, BAPN. As BAPN is not specific to LOX, we also treated mice with a specific antibody to LOX, which we showed inhibited LOX activity. Both small-molecule inhibition and antibody treatment eliminated the formation of metastases and were also effective in controlling metastases when given at a later time point after metastases had already formed.

These data suggested a role for LOX in controlling metastatic growth and dissemination. Indeed, we found that tail vein injection of LOX shRNA-expressing tumor cells into nude mice reduced formation of lung foci compared with injection of control cells. Although the cloning efficiency of both cell types was the same, the shRNA cells grew poorly in Matrigel compared with control cells that flourished especially under hypoxia. The LOX shRNA cell growth was identical to that seen in soft agar where no ECM components are provided, indicating that the shRNA cells were unable to interact with the ECM components provided by the Matrigel, resulting in growth inhibition. This in vitro finding was reflected by the reduction in size of the metastatic lesions formed in mice bearing LOX orthotopic tumors. In summary, we found that LOX was involved in both early and later stages of metastasis, suggesting that this enzyme may be an excellent therapeutic target for the treatment and prevention of metastatic disease.

**Implications**

It has long been known that tumor hypoxia is associated with increased invasion and metastasis and decreased survival (1). Attempts to explain this phenomenon have uncovered a role for a variety of proteins (16, 17). It has also long been known that the ECM can influence the invasive and metastatic capacities of cells (18). Our recent work shows an essential role for a hypoxia-regulated ECM protein, LOX, in the enhanced invasive and metastatic potentials of oxygen-deprived cells. Because there are very few treatment options for patients with metastatic disease, particularly those with breast cancer, uncovering a novel therapeutic target that can eliminate both early and late stages of metastasis has important clinical implications.

How does one protein have such an important role in both invasion and metastasis? Our data suggested that hypoxia increases the amount of enzymatically active secreted LOX that acts on collagen fibers outside of the cell increasing integrin activity resulting in cell movement, leaving behind remodeled matrix tracks that create a route through which others cells may travel, providing a "highway to metastasis." We showed that these events occur as a result of the role of LOX in cell-matrix adhesion and FAK activation.

How does LOX influence later stages of metastasis? The role of LOX in cell-matrix adhesion and migration is certain to additionally affect later stages of metastasis when cells must adhere to vessel walls, extravasate, and migrate to colonize secondary organs (Fig. 1). We further showed impaired metastatic
growth of cells with reduced LOX expression. We propose that inhibition of LOX prevents formation of a mature ECM required for correct survival signaling. Further studies are needed to investigate why LOX expression is essential for metastatic and not primary tumor growth. In concert with our findings, Payne et al. (14) showed elevated LOX expression in breast cancer metastases compared with that in primary tumor cells.

We also bring forth the hypothesis that LOX may be a tumor-secreted factor required for premetastatic niche formation [described by Kaplan et al. (19)], possibly through the recruitment of bone marrow–derived cells (BMDC). This hypothesis is supported by several observations. First, our own observation that the metastatic lesions formed in mice bearing control orthotopic tumors were composed of inflammatory cells. Second, it has been shown that LOX is a chemoattractant for monocytes stimulating their migration (3). Third, the premetastatic niches have highly elevated fibronectin expression, which can interact with LOX and be regulated by FAK (20). The influence of increased LOX expression on metastasis is predicted to adversely affect survival. This is reflected in the analysis of cancer patient samples, where elevated LOX expression was associated with decreased survival. The implication of these studies is that inhibition of LOX should increase survival of cancer patients that already harbor metastatic disease. We have found that LOX inhibition can significantly extend the survival of mice with metastatic disease. Furthermore, determination of LOX expression levels may be used in the clinic as a prognostic and diagnostic marker.

Our results provide strong evidence that LOX is a good therapeutic target for the prevention of metastasis in breast cancer and, potentially, other solid tumors. Targeting secreted LOX presents an attractive mechanism to control all stages of metastasis. Although small-molecule inhibitors of LOX would affect both intracellular and extracellular LOX, antibody-based inhibition of LOX would inhibit only extracellular LOX and has proven to be highly effective in preclinical studies in controlling metastases and increasing survival. As LOX inhibition had minimal effects on the growth of the primary tumor, we anticipate that combination of anti-LOX therapy with other agents, such as chemotherapy and radiotherapy, would act in concert to target both primary and metastatic growth.

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