Ph.D. Theses

Implications of lysyl oxidase and lysyl oxidase-like 2 enzyme expression for epithelial and neuroepithelial tumor progression

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1. Introduction

The critical importance of stroma in tumorigenesis has been confirmed by a series of experiments that, by modulating the stromal cell and/or extracellular matrix composition, have demonstrated either an inhibitory or enhanced effect on cancer growth. Among others, members of the lysyl oxidase enzyme family have come in focus of research investigating tumor-stromal interactions. Due to enzymatic activity of secreted lysyl oxidases, certain proteinaceous components of the extracellular matrix undergo oxidative deamination, and the newly formed aldehyde residues then interact spontaneously to form covalent cross-linkages. Extensive covalent cross-linking leads to a stiffer, more insoluble matrix, ultimately affecting cancer cell behavior. The expression pattern and essential function of lysyl oxidase activity in normal tissues is well described. Recently, in addition to the basic cross-linking activity, a growing number of studies reported novel roles for lysyl oxidases in various types of tumors. However, data we gained show quite controversy, and address a massive quantity of problems remain to be elucidated.

2. Objectives

In our research summarized in present thesis, we studied LOX and LOXL2 enzyme expression in various normal and malignantly transformed cell types and tissues. We explored possible

genetic and epigenetic mechanisms underlying the particular expression patterns. Subsequently, we identified functional consequences to LOX and LOXL2 expressions in those various types of cells and tissues, and investigated molecular mechanisms behind the altered phenotypes. Ultimately our studies were addressed to identify possible molecular targets for future cancer prevention and therapy.

Specific aim 1:
to characterize LOX expression pattern in normal tissues of the central nervous system and in neuroepithelial tumors.
Specific aim 2:
to investigate the effects of LOX expression in context of astrocytic tumor progression.
Specific aim 3:
to characterize LOXL2 expression pattern in the normal colon and esophagus, and in colon tumors and esophageal tumors.
Specific aim 4:
to investigate possible biological mechanisms controlling LOXL2 expression in the colon and the esophagus.
Specific aim 5:
to characterize LOXL2 expression pattern in the normal and malignant mammary epithelium.
Specific aim 6:
to investigate the effects of LOXL2 overexpression in normal mammary epithelial cells and breast adenocarcinoma cells.

Specific aim 7:

to investigate possible biological mechanisms controlling LOXL2 expression in the breast epithelium.

3. Materials and methods

For our experiments we used *in vitro* cell cultures of NHA, U87 MG, U251 MG, U343 MG-A, HCT-116, DLD-1, HCT-15, WHCO1, WHCO3, WHCO5, WHCO6, HMEC, MCF-10A, MCF-7, T-47D, MDA-MB-231 cell lines. For immunohistochemical studies, we used formalin-fixed paraffin-embedded tissue samples of normal brain and malignant astrocytoma, normal colon and colorectal adenocarcinoma, normal esophagus and esophageal squamous cell carcinoma, normal breast and infiltrating lobular breast carcinoma. To examine cell morphology we used phase contrast microscopy, to detect protein localization we utilized confocal laser microscopy. Overexpression of LOXL2 in mammary epithelial cells was achieved by Lentiviral transduction. Amounts of proteins expressed by cultured cells were monitored by Western blot and real time quantitative PCR analyses. Evaluation of *in vitro* cell migratory ability was tested using the MICS chamber system. Enzymatic activity of lysyl oxidases was confirmed by *in vitro* lysyl oxidase assay, based on the detection of hydrogen peroxide released as a side product of the amine oxidase chemical reaction. Promoter analysis of the *loxl2* gene was performed using online available databases and

programs NCBI Nucleotide Search and GrailEXP CpG Island Locator. Promoter demethylation (5-aza-dC) and histone deacetylase inhibiton (Trichostatin A) treatments were used to confirm potential epigenetic silencing mechanisms contributing to low levels of protein expression detected in certain cell types.

4. Results

We designed LOX and LOXL2 antibodies and confirmed their specificity, and subsequentally used them in immunocytochemical, immunohistochemical, and Western blot analyses.

Using tissue sections and cultured cell lines we detected LOX expression in normal and malignantly transformed human astrocytes. The intensity of LOX production positively correlated with astrocytic tumor grade. LOX produced by astrocytes proved to be catalytically active. Elevated LOX levels in high grade astrocytoma cells contributed to elevated cell migration, which could be inhibited by specific the LOX inhibitor beta-aminopropionitrile, and treatment with catalase. These results confirm that elevated migratoy ability of high grade astrocytoma cells is, at least partially, due to elevated LOX activity. Hydrogen peroxide released during oxidative deamination reactions catalyzed be LOX results in focal adhesion activation, increased phosphorylation of FAK(Tyr576) and paxillin(Tyr118), which could explain the more migratory phenotype of those cells.

Immunohistochemical analysis of colon and esophagus tissue sections revealed that LOXL2 is abundantly expressed in colon adenocarcinomas and esophageal squamous cell carcinomas, when compared with normal tissues. The increased LOXL2 expression was statistically significantly positively correlating with the tumor grade of colon adenocarcinomas. Loss of heterozygosity studies showed that in approximately one third of these tumor types, the loss of one copy of the *loxl2* gene occurs. We also identified promoter hypermethylation and histone deacetylation as epigenetic gene silencing mechanisms possibly contributing to the low level of LOXL2 production in normal and low grade colon and esophageal cancers tested.

In another set of experiments we show, that LOXL2 expressed by mammary epithelial cells is catalytically active, and that overexpression of LOXL2 in those cells only facilitates cell migration in already transformed mammary epithelial cells, but not in normal mammary epithelial cells. To identify possible molecular mechanisms underlying the differential effects of LOXL2 overexpression in normal and transformed mammary epithelium, we found that in normal mammary epithelial cells a previously unknown 50 kDa LOXL2 fragment is present bound to the plasma membrane. The 50 kDa LOXL2 is probably due to extracellular enzymatic processing of the full length LOXL2, supported by our experimental results. Furthermore, to explain why LOXL2 expression is lost in non-invasive mammary adenocarcinomas, we again identified the two epigenetic gene silencing mechanisms discussed above.

5. Conclusions

In conclusion, results of present thesis on individual roles of LOX and LOXL2 in astrocytic-, colon-, esophageal-, and mammary tumors can be summarized as follows:

- LOX expression positively correlates with astrocytic tumor grade
- LOX activity induces cell migration through focal adhesion activation by means of hydrogen peroxide release
- LOXL2 expression is elevated in colon adenocarcinomas and esophageal squamous cell carcinomas which is in positive correlation with the tumor grade in colon adenocarcinomas
- loss of heterozygosity affecting the *loxl2* locus can be detected in approximately one third of colon adenocarcinoma and esophageal squamous cell carcinoma tumors
- decreased LOXL2 expression in poorly-invasive breast carcinoma cells is due to active epigenetic gene silencing mechanisms
- elevated LOXL2 expression promotes cell migration in malignantly transformed mammary epithelial cells, but not in normal mammary epithelial cells, possibly due to altered proteolytic LOXL2 processing

6. List of publications

Articles discussed in the thesis:

Hollosi P, Yakushiji J, Fong KS, Csiszar K, Fong SFT
Lysyl oxidase-like 2 promotes migration in non-invasive breast cancer cells but not in normal breast epithelial cells
International Journal of Cancer 2009; accepted, in press

Laczko R, Szauter KM, Jansen MK, **Hollosi P**, Molnar J, Muranyi M, Fong KS, Hinek A, Csiszar K
Lysyl oxidase (LOX) promotes FAK/paxillin activation and migration in invasive astrocytes
Neuropathol Appl Neurobiol. 2007 Dec;33(6):631-43.

Fong SF, Dietzsch E, Fong KS, **Hollosi P**, Asuncion L, He Q, Parker MI, Csiszar K
Lysyl oxidase-like 2 expression is increased in colon and esophageal tumors and associated with less differentiated colon tumors
Genes Chromosomes Cancer 2007 Jul;46(7):644-55

Articles not discussed in the thesis:

Peterfia B, **Hollosi P**, Szilak L, Timar F, Paku S, Jeney A, Kovalszky I.
Role of syndecan-1 proteoglycan in the invasiveness of HT-1080 fibrosarcoma
Magy Onkol. 2006;50(2):115-20. Hungarian.

Kovalszky I, Dudas J, Gallai M, **Hollosi P**, Tatrai P, Tatrai E, Schaff Z
Proteoglycans in the liver
Magy Onkol. 2004; 48(3):207-13. Hungarian.

Kiss AL, Botos E, Turi A, Mullner N, **Hollosi P**, Kovalszky I
Ocadaic acid treatment alters the intracellular localization of caveolin-1
and caveolin-2 in HepG2 cells human isolated coronary arteries
Acta Biol Szeged 2003; 47:11-17

Poster presentations related to the topic of the thesis

Fong SFT, Fogelgren B, Szauter-Molnarne K, Moon C, **Hollosi P**, Weaver V, Kirschmann DA, Csiszar K
Lysyl oxidase (LOX) is expressed in the microenvironment of breast tumor cells and promotes epithelial plasticity, invasive properties and metastasis.
American Association for Cancer Research, April 16-20, 2005, Anaheim, CA, USA

Fong SFT, Fogelgren B, Szauter-Molnarne K, Moon C, **Hollosi P**, Weaver V, Kirschmann DA, Csiszar K
Lysyl oxidase (LOX) is re-expressed in the microenvironment of breast tumor cells and promotes epithelial plasicity, invasive properties and metastasis
Second National Meeting of the American Society for Matrix Biology, 2004, San Diego, CA, USA

Fong SFT, Moon C, Fogelgren B, Szauter K, **Hollosi P**, Kirschmann DA, Chun M, Csiszar K
Lysyl oxidase induces epithelial-mesenchymal transition and invasive phenotype in breast cancer cells.
XIXth meeting of the Federation of the European Connective Tissue Societies, July 9-13, 2004, Taormina-Giardini Naxos, Italy

Fong SFT, He QP, Fong KSK, Dietzsch E, Asuncion L, **Hollosi P**, Parker MI, Csiszar K
Increased Lysyl Oxidase-Like Expression in Colon and Esophageal Tumors are Assiociated with Markers of Poor Prognosis
Biomedical Sciences Symposium, 2004, Honolulu, HI, USA