National Institute of Allergy and Infectious Diseases

ROCKY MOUNTAIN LABORATORIES
Laboratory of Neurological Infections and Immunity
Innate Immunity and Pathogenesis Section

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Report for the doctoral dissertation of:

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Overview:

Prolidase (PEPD) is a ubiquitously expressed protease, yet it is unique in that its enzymatic activity is highly specific for dipeptides containing proline (Pro) or hydroxyproline (HyP). PEPD is recognized as having important roles in cellular homeostasis, and genetic polymorphisms are causative for human disease clinically recognized by imidodipeptiduria, chronic skin wounds particularly of the lower extremities, and impacts of immunity including increased susceptibility to bacterial infections. Specific defects in cellular function and collagen metabolism associated with loss of PEPD expression have been identified, but critical questions remain, including the role(s) of secreted PEPD, as well as enzyme-dependent vs -independent functions of PEPD in cell signaling.

The current Thesis explores the hypothesis that extracellular PEPD interaction with epidermal growth factor (EGFR) is an important determinant of cell proliferation and migration, and therefore is a direct driver of wound repair. Major findings include:

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a) Under conditions of mechanical cellular damage and IL-1b-dependent stimulation in the skin, HaCaT immortalized keratinocyte cell culture was used to determine the role for extracellular treatment with PEPD. After scratching of the cell monolayer, proliferation of the keratinocyte model was increased with addition of PEPD, although not in a dose-dependent manner.
b) The PI3K inhibitor LY294002, inhibited the PEPD-induced upregulation of cell proliferation markers, PI3K, Akt and mTOR, and reduced cell proliferation, demonstrating that PI3K is a downstream effector of secreted PEPD-dependent signaling.
c) PEPD simulated collagen biosynthesis in a concentration dependent manner in non-wounded cells, but this effect was increased in the wound model. Interestingly, treatment with extracellular PEPD decreased basal expression of NF-kB which is a known inhibitor of collagen biosynthesis.
d) Interestingly, treatment with extracellular PEPD did not affect HaCaT cell proliferation under basal conditions, but did have an impact under conditions of IL-1b treatment. The impact on cell proliferation and cell cycle proteins was explored, and found that PEPD treatment induced phosphorylation of EGFR, Akt and STAT3.
e) While cell proliferation was impacted under conditions of inflammation, cell migration seemed to be the most consistent phenotype. This included downregulation of E-cadherin and metalloprotease expression, with concomitant upregulation of HIF-1, TGFBR, COX-2 and N-Cadherin.
f) Mutants of PEPD were examined in the context of cell proliferation. The mutation with the most pronounced phenotype was recombinant PEPD containing G448R mutation which did not induce proliferation of HaCaT cells even in the presence of IL-1b.

Assessment:

The work is largely well completed and the interpretations are clear and highlight interesting roles for PEPD in proliferation of keratinocytes as the major epithelial barrier cell under conditions of inflammation. The thesis includes 1 review article and 2 peer reviewed papers for which the authors should be complemented. The current findings suggest avenues for future work, including how IL-1b augments the positive roles of extracellular PEPD in cellular responses, and how PEPD-dependent signaling integrates with additional pathways including HIF1a-dependent responses.

Recommendation:

The thesis is acceptable with minor revisions as suggested below:

Specific comments and clarifications for the authors consideration:

Text:

- The use of IL-1b has been used throughout but the justification for this experimental condition is not clear. Please provide a stronger justification of IL1b as a condition that should be studied in the

context of wound healing, with references on page 24. LPS is added as an additional stimulus on page 25; please also add references for why this stimulus was chosen.

- Page 26: it is not clear why the inhibitor gefitinib was used – please justify.
- Page 29, first line: The statement that gefitinib abolished PEPD-dependent EGFR signaling is not completely accurate. It appears that EGFR and AKT are potentially upregulated even in the presence of the inhibitor, suggesting that a statement reflecting the reduction in response may be more appropriate.

Figures:

Page 24/25: effect of PEPD addition (plus IL-1b and/or LPS), on HaCaT cell viability and IL-6 production should be shown as a fundamental validation of the model.

Figure 5: does the author have images of the wound closing or cell proliferation model? This would be illustrative here.

Figure 6 and Figure 10: the increases in specific markers should be quantified over multiple experiments using densitometry and included as graphs for these figures.

Figure 15: Treatment of cells with recombinant PEPD containing the G448R mutation does not induce proliferation of cells, but it is stated that this and other mutants stimulate activation/phosphorylation of signaling proteins downstream of EGFR. However, no control of unstimulated cells is included to judge the degree of activation. In addition, how it is possible that signaling cascades are intact, but the cell proliferation is seemingly divorced from this signal transduction following extracellular treatment with PEPD as a stimulus? The data for cellular proliferation in response to stimulation with the recombinant PEPD wild-type and mutants should be shown.

The above conclusions authorize me to apply to the College of Medical Sciences of the Medical University of Bialystok for admission of Magdalena Nizioł to further stages of the doctoral dissertation.

Signed,

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