

Regulation of Adipose Deposition in Lymphedema

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▲ Introduction

Lymphedema is a common and morbid condition that arises from the failure of the lympho-vascular system to adequately clear interstitial fluid. It is a chronic and progressive disease that begins with fluid accumulation, but over time the pathology transitions to one of fibroadipose accumulation. The deposition of adipose tissue is a key histologic and pathologic process in chronic lymphedema. However, despite the fact that this process is the event that makes lymphedema resistant to commonly used treatments, such as compression garments and massage, little is known about how adipose deposition in lymphedema is regulated. Our lab has recently shown that lymphatic injury results in activation of adipose differentiation genes resulting in hypertrophy and proliferation of adipocytes (1, 2). In fact, we have found that the adipose tissue that is deposited in lymphedema is histologically similar to that found in generalized obesity as evidenced by infiltration of chronic inflammatory cells (3, 4). These findings suggest that adipocytes and the process of adipose deposition may play a role in the pathology of lymphedema. This is a concept that is supported by the fact that adipose deposition is a key regulator of a variety of disease processes. One mechanism by which adipose tissues modulate pathology is through the elaboration of growth factors and cytokines including IL6. In the current study we chose to focus on IL-6 since previous studies have demonstrated that the expression of IL-6 is significantly increased in both primary and secondary animal models of lymphedema and that IL-6 is known to play a critical role in adipose tissue homeostasis (5, 6). What remains to be clearly delineated is what mechanisms may underlie this association between inflammation and adipose homeostasis in the setting of lymphedema.

In this study we sought to investigate the role of IL-6 in adipose deposition in the setting of lymphedema. We found that clinical biopsy specimens and serum from patients with lymphedema had significantly increased IL-6 levels in both serum and lymphedematous tissues. This expression of IL-6 was strongly associated with both adipose deposition and inflammation in our murine

Referat zu: IL-6 regulates adipose deposition and homeostasis in lymphedema.

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models of lymphedema. Interestingly, CD4+ cell inflammation appeared necessary for these processes and loss of IL-6 function lead to a dramatic increase in adipose deposition following lymphatic disruption.

Methods

Human lymphedema tissue and serum specimens: Human normal skin and lymphedema specimens were collected by Professor Waldemar Olszewski at the Polish Academy of Science under an IRB approved protocol.

Serum was obtained from the Stanford Center for Lymphatic and Venous Disorders under an IRB approved protocol. Serum samples were collected from 26 patients with post-mastectomy lymphedema (grades I-III) and 20 patients with a history of breast cancer, but without lymphedema. Using multiplex bead-based immunoassay, serum IL-6 levels were quantitated and represented as median fluorescent intensity (MFI). Patient demographics, including body mass index (BMI) and lymphedema grade, were collected for each patient. Lymphedema grade defined by the International Society of Lymphedema classification scheme (7).

Murine Models of Lymphedema: Female C57BL/6J, interleukin-6 deficient (IL-6KO) and CD4 knockout (CD4KO) mice were acquired from Jackson Laboratories. In this study, two mouse models of lymphatic injury were utilized to evaluate the outcomes of different degrees of adipose deposition on IL-6 expression. Our lab has previously described an axillary lymph node dissection (ALND) model that has been utilized to evaluate the effects of lymphatic injury in a setting of limited adipose deposition(4). Briefly, this model involves making a 1 cm incision in the axilla through which the axillary lymph node basin, including the perinodal tissue, is excised. Control animals are treated with a simple incision with no interruption of the lymphatic basins. The second model that was utilized is a well described tail model of lymphedema (1, 4, 8, 9). This model allows one to study the effects of lymphatic injury in the setting of significant adipose deposition. In short, this model involves removing a circumferential full thickness segment of skin approximately 2mm wide at the mid-portion

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of the mouse tail, followed by microsurgical ligation of the deep collecting lymphatics. Our previous studies have demonstrated that 6 weeks postoperatively, these mice develop sustained lymphedema with concomitant inflammation, adipose deposition, and fibrosis, all of which are histologic hallmarks of lymphedema (1, 4, 8, 9). Control animals were subjected to skin incision with no disruption of deep lymphatics.

CD4 Depletion Studies: Adult female C57BL/6J mice underwent the tail model of lymphatic disruption as described above. These animals were allowed to recover for 2 weeks and then were randomly assigned to a treatment group or control group (n=8-10 animals/group). The experimental group received intraperitoneal CD4 monoclonal neutralizing antibodies (10ug/g) every 5 days for a total of 4 weeks as previously described (4). Control animals received an isotype control antibody administered in the same fashion. Confirmation of CD4 depletion was demonstrated by flow cytometry of splenic cell populations.

STAT inhibition: To evaluate the effect of IL-6 blockade on adipose deposition, a small molecule inhibitor of JAK1/2 (AZD1480; Astra-Zeneca), the primary signaling pathway of IL-6(10), was utilized. Mice received oral doses of 60mg/kg AZD1480 for 6 weeks beginning in the immediate postoperative period. Control animals were treated with vehicle.

Histology: Tissue sections were fixed in 4% paraformaldehyde and decalcified using EDTA and then embedded in paraffin. Immunohistochemistry was performed for CD45⁺, CD4⁺, IL-6⁺, and STAT-3⁺ cells. Cell counts were performed on randomly chosen high-powered fields by 2 blinded reviewers. Hematoxylin and Eosin stains were also performed.

ELISA: IL-6 levels in tissue lysates and serum were evaluated using an enzyme linked immunoabsorbent assay.

Statistical Analysis: Student's T-test was used to compare differences between 2 groups and ANOVA was employed for multiple comparison analysis. Correlation between groups was determined by using a Pearson's coefficient. Finally, analysis of clinical lymphedema specimens was conducted using a Wilcoxon matched pair T-test. Data are presented as means +/- standard deviation unless otherwise noted with a p<0.05 considered significant.

Results

Local and systemic IL-6 expression is increased in patients with lymphedema: We found that patients with lymphedema had a significant increase in the number of IL-6⁺ cells as compared to matched controls in their lymphedematous tissues. Furthermore, we observed a

significant increase in pSTAT-3⁺ staining, a key intracellular downstream mediator of the IL-6 activation cascade, in the lymphedematous tissue. These immunohistochemical findings were further supported by the finding that patients with lymphedema also had significant elevations in serum levels of IL-6 when compared to matched control patients. When IL-6 levels were compared to lymphedema grade or BMI, no correlation was observed. The fact that no correlation between these variables existed was surprising, but may be related to the fact that the many of the subjects had BMI's less than 30.

IL-6 levels correlate with adipose deposition in the setting of lymphatic disruption: Histologic analysis of the mouse models of lymphatic injury revealed significant adipose deposition only in the lymphedematous tail sections and not in the ALND model system. Immunohistochemical analysis revealed a small but significant increase in pSTAT-3⁺ cells in the mouse forelimb of ALND mice. However, there was a marked and significant increase in the number of pSTAT3⁺ cells in the tail model and IL-6 tissue expression was 20-fold greater than control animals. These findings parallel our clinical findings and demonstrate that IL-6 expression in lymphedema is associated with adipose deposition.

Adipose deposition and IL-6 expression is dependent on CD4⁺ cell infiltration: Our lab has previously shown that mice deficient in CD4⁺ cells (CD4 knockout mice; CD4KO) do not develop tail lymphedema following lymphatic disruption (4). To explore the hypothesis that CD4⁺ cells are necessary for adipose deposition and IL-6 expression in the setting of lymphedema, we performed tail surgeries in wild-type and CD4KO mice as well as in wild-type mice depleted of CD4⁺ cells using neutralizing antibodies (α -CD4). We found that CD4KO and α -CD4 treated mice had significantly less adipose deposition following tail skin/lymphatic excision and that this phenotype was associated with a significant decrease in the number of pSTAT-3⁺ cells and IL-6 tissue expression. These findings suggest that CD4⁺ cell inflammation is necessary for the development of adipose tissue and IL-6 expression in the setting of lymphedema.

Loss of IL-6 expression is associated with adipose deposition: We next used the tail model to investigate the role of IL-6 in lymphedema. Wild-type and IL-6 knockout mice (IL-6KO) underwent tail skin and lymphatic excision. In separate experiments, wild-type mice underwent tail skin/lymphatic excision and were treated with either vehicle control or a small molecule inhibitor of JAK 1/2 to confirm our IL-6 KO mouse studies. We found that the loss of IL-6, either in IL-6 KO mice or by inhibition of JAK 1/2 pathway, was associated with a significant increase in adipose tissue in the setting of

lymphedema. Histologic analysis of IL-6 KO and JAK inhibited mice revealed a significant decrease in pSTAT-3⁺ cells therefore confirming the suppression of IL-6 activation. Furthermore, the loss of IL-6 resulted in a decrease in inflammatory infiltrates including CD4⁺ cells suggesting that IL-6 expression may be involved in decreasing adipose deposition and inflammation in the setting of lymphedema.

Discussion

The molecular and cellular mechanisms that govern the pathogenesis of lymphedema remain poorly understood and this gap in our knowledge is a significant barrier to the development of accurate means of predicting or treating this disease. This study sought to answer one part of this puzzle; how is adipose tissue deposition regulated in lymphedema.

The evaluation of clinical specimens revealed that patients with lymphedema had significantly elevated tissue and serum levels of IL-6 expression and its downstream pathways compared to matched controls. This finding is significant and may suggest that serum IL-6 levels could serve as a biomarker for aiding in the diagnosis and management of lymphedema. Currently there is no modality that can reliably predict the development of lymphedema. Furthermore, current means to follow clinical response to therapy consist of subjective measures such as volume or arm circumference. Therefore, the development of an objective measure that is reliable and quantifiable, such as IL-6 levels, would provide a more accurate method for predicting and following response to therapy.

To date, several clinical staging systems for lymphedema have been devised. However, all of them rely on subjective interpretations of the clinical condition. They are limited in scope and fail to take into account the underlying histopathologic changes that are associated with the evolution and progression of lymphedema. In this study we did not find a correlation between lymphedema grade and IL-6 expression. Although the study may have lacked statistical power to detect minor differences between the groups, the inability to discern a difference is likely a result of the shortcomings of the clinical staging schemes available today. A staging system that includes the histopathologic progression of lymphedema, including adipose deposition and fibrosis, may therefore be a more accurate means of categorizing lymphedema. Lymphedema is a progressive disease with a variable onset, progression and severity that differs greatly from patient to patient. This fact alone makes lymphedema difficult to study in humans. We have therefore relied on mouse models to explore the cellular and molecular mechanisms of this disease. The ALND model allows us

to evaluate the effect of lymphatic stasis in the absence of significant adipose deposition, whereas our tail model of lymphedema involves a more dramatic disruption of the lymphatics system with significant adipose deposition. When comparing these two models, we found that IL-6 expression, activation of its downstream cascade, and serum changes in IL-6 concentration occur to a greater degree in the tail model of lymphedema when compared to the ALND model. This suggests that the degree of lymphatic injury, and resultant adipose deposition, is a critical factor in regulating IL-6 expression. This hypothesis is supported by previous studies that demonstrate that adipose tissue is a major source of IL-6 (11, 12) and that IL-6 may function as an adipolytic hormone (13-18).

As previously mentioned, inflammation is a key histopathologic hallmark of lymphedema. Our previous studies have demonstrated that both the ALND and tail model of lymphedema are strongly associated with a significant increase in both CD45⁺ and CD4⁺ cellular infiltration. Our lab has also demonstrated that chronic inflammation in the form of CD4⁺ cells is critical for regulating the pathology of lymphedema (3, 4). In this study we found that the inflammatory infiltrate was intimately associated with the accumulated adipose tissue. This finding suggests that this association may be bi-directional with inflammation promoting adipose deposition and vice versa. Furthermore, when CD4KO mice or α -CD4 depleted mice underwent lymphatic disruption in the tail, there was a dramatic diminution of adipose deposition. Taken together these findings suggest that chronic CD4⁺ cell infiltration is necessary for adipose deposition.

It is well known that inflammation is associated with an upregulation of inflammatory cytokines, one of which is IL-6. It is a pleiotropic cytokine that is produced by a number of inflammatory cells including macrophages and T lymphocytes (19). In this study we found that chronic inflammation is an important regulator of IL-6 expression in the setting of lymphedema. We have shown that loss of CD4⁺ cells was associated with a significant decrease in IL-6 expression and activation of its downstream mediators. When IL-6 function was lost or inhibited we observed a significant increase in adipose deposition and a concomitant decrease in the degree of inflammation. This finding implies that IL-6 expression in the setting of lymphedema may act to maintain both adipose homeostasis, by decreasing its deposition, and foster chronic inflammation. These findings are supported by recent data that suggests that IL-6 is a pleiotropic cytokine with not only a prominent role in inflammation (19), but a significant role in regulating adipocyte function (20, 21). Our findings suggest that in the setting of lymphedema, an increase in IL-6 expres-

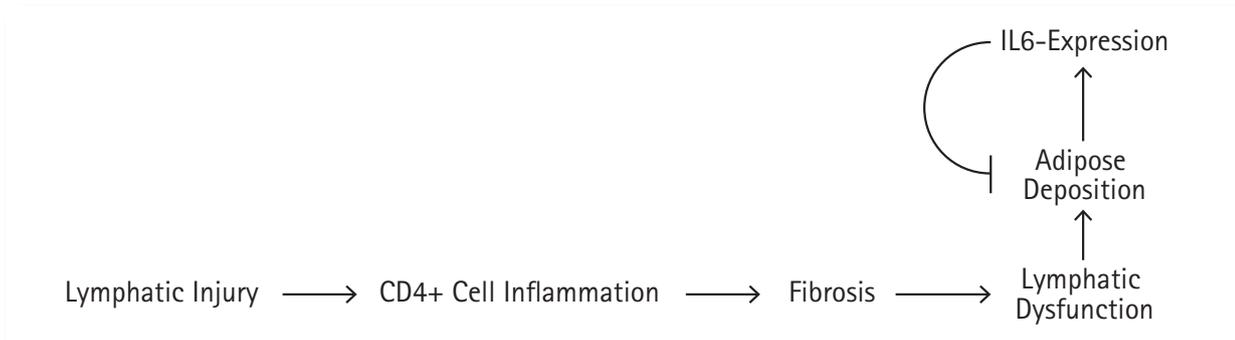


Fig. 1: This figure describes our central hypothesis. Lymphatic injury is characterized by chronic inflammation, with a strong CD4+ T cell component. This chronic inflammatory state drives fibrosis and eventual lymphatic dysfunction. In the setting of established lymphedema, adipose hyperplasia and hypertrophy are observed. IL-6 levels are elevated in this state and appear to play a homeostatic role by decreasing or maintaining adipose deposition.

sion may be a compensatory homeostatic response directed at limiting adipose deposition and modulating inflammation.

To conclude, this study demonstrates that patients with lymphedema display not only elevations in tissue, but also serum levels of IL-6, which may serve as a potential biomarker of lymphedema onset, progression and response to therapy. Furthermore, this elevation in IL-6 expression and activation were concordant with the findings in our mouse models. Chronic CD4⁺ cell inflammation is an important regulator of IL-6 expression and adipose deposition in lymphedema. Taken together, these findings suggest that IL-6 may play a critical role in

maintaining adipose homeostasis by limiting its deposition in the setting of lymphedema (Fig. 1).

References by the authors.

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