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Research Article

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Effect of Neutrophil Gelatinase-Associated Lipocalin (NGAL) on the Activation of MAPK and Smad3 Signaling Pathways in Human Peritoneal Mesothelial Cells Under Peritonitis

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Abstract

Background and Objectives: Peritoneal Dialysis-Related peritonitis (PD-related peritonitis) remains a leading cause of technical failure and technique discontinuation in End-Stage Renal Disease (ESRD) patients. Neutrophil Gelatinase-Associated Lipocalin (NGAL) has emerged as a highly sensitive biomarker that escalates during acute episodes of peritonitis; however, its precise direct role in governing local inflammatory cascades and fibrotic progressions within Human Peritoneal Mesothelial Cells (HPMCs) remains unclear. This study aims to evaluate clinical effluent NGAL alterations and systematically investigate whether NGAL acts as a direct driver or a synergistic enhancer of pro-inflammatory Mitogen-Activated Protein Kinase (MAPK) and pro-fibrotic Smad3 signaling pathways in HPMCs.

Methods: A clinical cross-sectional evaluation was conducted on 30 Continuous Ambulatory Peritoneal Dialysis (CAPD) patients, divided into a peritonitis group ($n=16$) and a stable control group (n=14). Effluent NGAL, White Blood Cell (WBC) counts, and serum C-Reactive Protein (CRP) were measured. In vitro, primary cultured HPMCs were subjected to 24-hour serum starvation before being divided into four treatment conditions: a Control group, an NGAL group a TGF-beta1 group, and an NGAL+TGF-beta1 combination group. Western blot analysis was performed across multiple time intervals (0, 5 minutes, 4 hours, and 24 hours) to monitor the protein and phosphorylation levels of P38, ERK1/2, JNK, and total Smad3.

Results: Clinically, NGAL levels in the peritoneal fluid of peritonitis patients exhibited an explosive surge. Effluent NGAL values demonstrated tight linear positive correlations with local effluent WBC counts and systemic serum CRP concentrations. Conversely, molecular biological assays yielded prominent negative traits at the cellular level. While the positive control (TGF-beta1) stimulated expected time-dependent downstream signaling, the independent administration of high-concentration recombinant NGAL provoked no statistically significant modifications in the phosphorylation bands of p-P38, p-ERK1/2, or p-JNK, nor did it alter Smad3 expression compared to the control group ($P>0.05$). Furthermore, adding NGAL to TGF-beta1 demonstrated no synergistic or inhibitory impacts on any evaluated pathways.

Conclusions: Although NGAL spikes drastically during acute peritonitis and serves as a highly robust and "pure" diagnostic marker for local injury and inflammatory load, it does not directly trigger or synergistically amplify the key MAPK and Smad3 pro-inflammatory and pro-fibrotic pathways within human peritoneal mesothelial cells. These findings clarify that NGAL acts primarily as a protective or defensive acute-phase "indicator" rather than an active pathological "driver" of the inflammatory cascade during peritonitis. Therapeutic interventions aiming to halt long-term peritoneal fibrosis should pivot away from neutralizing NGAL alone, focusing instead on blocking its upstream inciting triggers or downstream TGF-$\beta1$ pathways.

Keywords: Peritoneal dialysis-related peritonitis, Neutrophil gelatinase-associated lipocalin (NGAL), Human peritoneal mesothelial cells (HPMCs), MAPK signaling pathway, Smad3, Peritoneal fibrosis

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Background

Peritoneal Dialysis (PD), as one of the primary renal replacement therapies for patients with End-Stage Renal Disease (ESRD), is widely utilized due to its capacity to better preserve residual renal function, provide hemodynamic stability, and grant patients a higher quality of life and autonomy [1]. However, the long-term success of peritoneal dialysis is challenged by various complications, among which peritoneal dialysis-related peritonitis (PD-related peritonitis) is one of the most severe and common acute complications [2]. Peritonitis is not only the primary cause leading to the technical failure of peritoneal dialysis and subsequent transition to hemodialysis, but it is also a key factor increasing patient hospitalization and mortality rates [3]. During the occurrence of peritonitis, the peritoneum functions as a semipermeable membrane, and its structure and function undergo drastic changes [4]. Acute inflammatory assault leads to the damage and shedding of Human Peritoneal Mesothelial Cells (HPMCs), triggering a complex inflammatory cascade reaction. Long-term repeated episodes of inflammation further induce peritoneal fibrosis, neovascularization, and ultrafiltration failure, ultimately causing the peritoneum to lose its ability to clear toxins and water [5]. Therefore, searching for biomarkers that can achieve early diagnosis of peritonitis and reflect the severity of peritoneal damage, as well as exploring the molecular mechanisms of inflammatory signal transduction, is of great clinical significance for intervention.

Neutrophil Gelatinase-Associated Lipocalin (NGAL), also known as Lipocalin-2 or 24p3, is a glycoprotein with a relative molecular weight of 25 kDa. NGAL was initially discovered in the specific granules of human neutrophils, but subsequent studies have demonstrated that under conditions of ischemia, infection, or toxic injury, various epithelial cells (such as renal tubular epithelial cells, hepatocytes, alveolar epithelial cells, etc.) also express and secrete NGAL in large quantities. As an acute-phase reactant protein, NGAL plays a central role in innate immunity; it can restrict bacterial growth by chelating bacterial siderophores, thereby exerting an antibacterial effect. In the field of nephrology, urinary or blood NGAL has been widely recognized as an early sensitive biomarker for Acute Kidney Injury (AKI). In recent years, researchers have begun to focus on its manifestation in Peritoneal Dialysis Effluent (PDE), suggesting that it may reflect the local intraperitoneal inflammatory load and the stress state of mesothelial cells. The Mitogen-Activated Protein Kinase (MAPK) signaling pathway is an important transduction system for eukaryotic cells to respond to extracellular stimuli and convert them into intracellular biological effects. The typical MAPK family includes extracellular signal-regulated kinase (ERK1/2), P38 MAPK, and c-Jun N-Terminal Kinase (JNK). These pathways play pivotal roles in regulating the production of inflammatory cytokines, cell apoptosis, and Mesothelial-to-Mesenchymal Transition (MMT). Furthermore, Transforming Growth Factor-β1 (TGF-β1) acts as a core molecule promoting fibrosis, inducing the progression of peritoneal fibrosis through classic signaling pathways such as Smad3. Although NGAL is significantly elevated during peritonitis, detailed experimental evidence is still lacking regarding whether it directly activates these pro-inflammatory or pro-fibrotic pathways. This study aims to systematically evaluate the expression of NGAL in peritonitis and its impact on HPMC signaling pathways through clinical sample analysis and in vitro cell experiments.

Clinical Study Objects and Methods

This study first conducted a clinical cross-sectional evaluation, enrolling a total of 30 Continuous Ambulatory Peritoneal Dialysis (CAPD) patients. Among them, 16 patients were diagnosed with peritoneal dialysis-related peritonitis (peritonitis group). The diagnostic criteria strictly followed the guidelines of the International Society for Peritoneal Dialysis (ISPD), requiring the fulfillment of at least two of the following three criteria: abdominal pain or cloudy effluent; effluent White Blood Cell (WBC) count > 100/mL with a neutrophil proportion > 50%; positive effluent pathogen culture. The remaining 14 cases were stable dialysis patients without manifestations of peritonitis (control group). To deeply explore the role of NGAL at the cellular level, this study utilized primary cultured Human Peritoneal Mesothelial Cells (HPMCs). HPMCs were isolated from omental tissue, digested with trypsin, centrifuged, and then seeded in M199 culture medium containing 10% fetal bovine serum. The cells were cultured at 37°C in a 5% CO_2 environment. Upon reaching 80%–90% confluence, the cells underwent 24 hours of serum starvation to enter a quiescent stage, thereby ruling out the interference of basal serum components on colorimetry and signaling pathways.

The experimental design divided the cells into four treatment groups:

a) Control group: Given serum-free M199 culture medium.

b) NGAL group: Recombinant human NGAL protein was added to the culture medium at a concentration of 0.5 μg/mL. This concentration was established based on the average level measured in the effluent of clinical peritonitis patients, ensuring high biological relevance.

c) TGF-β1 group: Recombinant human TGF-β1 was added at 10 ng/mL, serving as a positive control group to induce cell activation and fibrotic responses.

d) NGAL + TGF-β1 combination group: Simultaneously treated with 0.5 mg/mL NGAL and 10 ng/mL TGF-β1, aiming to observe whether NGAL exerts a synergistic enhancing or inhibitory effect on TGF-β1-mediated pathways. Cell samples were collected at multiple time points (0, 5 minutes, 4 hours, and 24 hours) in this study. Western blot was employed to detect the protein expression levels of P38 MAPK, phosphorylated P38 MAPK (p-P38), ERK1/2, phosphorylated ERK1/2 (p-ERK1/2),

JNK MAPK, phosphorylated JNK MAPK (p-JNK), and total Smad3. For each protein indicator, GAPDH was used as an internal control for standardization.

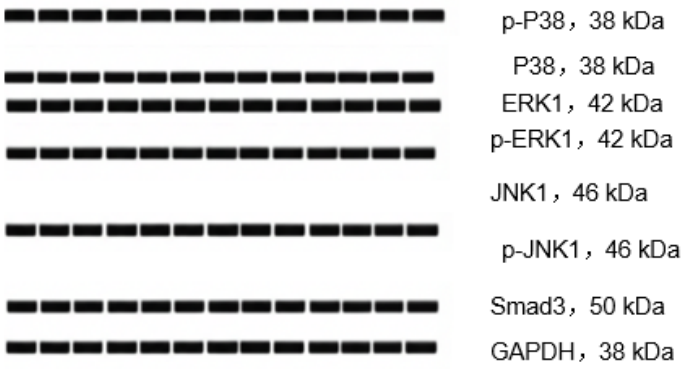
Results

Clinical test results revealed that effluent NGAL levels in peritonitis patients exhibited an explosive increase, with the median concentration significantly higher than that in the non-peritonitis group. This elevation was detectable in the early stage of infection, demonstrating extremely high diagnostic sensitivity. Further linear correlation analysis indicated a significant positive correlation between effluent NGAL levels and effluent white blood cell counts (correlation coefficient R ranging between 0.73 and 0.89), reflecting the severity of local inflammatory infiltration. Since NGAL is primarily released by activated neutrophils, this result aligns with pathophysiological logic. Concurrently, local NGAL levels were also positively correlated with serum C-reactive protein (CRP) levels (R^2 = 0.704), indicating that local intraperitoneal infection triggers a distinct systemic immune response. See table1. Following detailed molecular biological analysis, the experimental results did not present prominent characteristics. In the positive control group (TGF-β1 group), HPMCs exhibited the expected signal activation pattern: after 30 minutes to 1 hour of stimulation, the expression levels of p-P38, p-ERK1/2, and p-JNK showed no significant increase, and the activation of Smad3 demonstrated a distinct time-dependency, which is consistent with literature reports of TGF-β1 acting as a potent pro-inflammatory/pro-fibrotic factor. However, in the treatment group independently administered with 0.5 mg/mL NGAL, the results demonstrated prominent negative characteristics. The specific data are as follows (See table 2).

Western blot images showed that the phosphorylated protein bands in the NGAL-treated group had no statistical difference compared with the control group at all observed time points. Detection at the RT-PCR level also confirmed this, as NGAL failed to upregulate the mRNA levels of these signaling molecules. In the NGAL + TGF-β1 co-treatment group, the activation intensity of the signaling pathways was basically identical to that of the TGF-β1 alone group. The addition of NGAL failed to further enhance the phosphorylation levels of P38, ERK1/2, or JNK induced by TGF-β1. This indicates that in human peritoneal mesothelial cells, NGAL is neither a direct activator of the MAPK pathway nor a synergistic enhancer of TGF β1 signal transduction. This finding holds significant theoretical value, distinguishing the distinct roles of NGAL as an inflammatory "indicator" versus an inflammatory "driver". See Figure1 (Table 1&2).

NGAL group TGF-β1group NGAL+TGF-β1group

C 0 5m4h24h 0 5m 4h 24h0 5m 4h 24h (Figure 1).



Note*: Expression of p-P38, p-ERK, p-JNK, Smad3 in different groups. A P-value less than 0.05 is considered statistically significant.

Figure1: Expression of MAPK and Smad3 in different groups.

Table 1: Clinical Indicators Comparison.

Clinical Indicators	Peritonitis Group (N=16)	Non-Peritonitis Group (N=14)	Statistical Difference (P-Value)
Effluent NGAL (ng/mL)	1245.8±342.1	42.5±18.3	< 0.001*
Effluent WBC (/mL)	1850.0±720.5	8.2±4.5	< 0.001*
Serum C-reactive protein (mg/L)	56.4±22.8	3.2±1.4	< 0.01*
Age (years)	54.2±12.5	56.8±10.2	> 0.05

Note*: NGAL levels in peritonitis patients. A P-value less than 0.05 is considered statistically

significant*: of clinical significance compared to Non-peritonitis group.

Table 2: Signaling Pathway Activation in NGAL vs. Control Group.

Detection Indicator (Protein Level)	Stimulation Time	Ngal Group Vs. Control Group	Statistical Significance (P-Value)
p-P38/P38	5 min - 24 h	No significant change	> 0.05
p-ERK/ERK	5 min - 24 h	No significant change	> 0.05
p-JNK/JNK	5 min - 24 h	No significant change	> 0.05
Smad3	5 min - 24 h	No significant change	> 0.05

Note*: Expression of p-P38, p-ERK, p-JNK, Smad3 in different groups. A P-value less than 0.05 is considered statistically significant.

Discussion

The experimental results of this study reveal a seemingly paradoxical phenomenon: clinically, NGAL is highly expressed in the effluent of peritonitis patients and is closely correlated with the severity of inflammation; however, in in vitro cell experiments, the direct application of NGAL fails to activate the major inflammation- and fibrosis-related signaling pathways of HPMCs. This phenomenon of "high clinical expression but no cellular activation" can be thoroughly discussed from the following dimensions. First, the biological nature of NGAL and its position in the inflammatory chain need to be re-examined. In the pathological process of peritonitis, bacterial products (such as lipopolysaccharide, LPS) first activate Toll-like receptors (especially TLR4 or TLR2) in peritoneal tissues, thereby initiating the NF-κB and MAPK signaling pathways to produce large amounts of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6. The production of NGAL is often the "consequence" rather than the "cause" of the activation of these pathways [6]. Multiple studies have shown that IL-1β is a potent stimulus inducing epithelial and mesenchymal cells to express NGAL, and its activation mechanism depends on the MAPK/ERK pathway [7]. Therefore, the extremely high level of NGAL in peritoneal dialysis effluent is actually a sensitive "product" of the extensive inflammatory response within the peritoneal cavity, reflecting the activation of neutrophils and the acute stress state of mesothelial cells after injury [8]. Previous studies have shown that NGAL is a highly promising biomarker for peritonitis, which can reflect changes in the peritoneal microenvironment more sensitively than traditional WBC counts [9]. Furthermore, follow-up studies have revealed that in patients with effective treatment, NGAL levels rapidly decline as inflammation is controlled; conversely, if NGAL levels remain elevated after 5 days of treatment, it indicates a poor prognosis, including a significantly increased risk of technical failure or the need for peritoneal catheter removal [10].

Second, the restriction of receptor expression may be the key reason why NGAL cannot directly activate signaling pathways. The physiological effects of NGAL depend on the mediation of its specific receptor (NGALR/24p3R) [11]. Under normal physiological conditions, the expression level of NGALR on human peritoneal mesothelial cells might be extremely low. Existing studies on mesangial cells have observed that the expression of NGALR requires induction by inflammatory factors such as IL-1β to increase significantly. The present experiment utilized relatively "quiescent" primary mesothelial cells; if the cell surface lacks sufficient NGALR, even the presence of high concentrations of NGAL protein in the environment cannot trigger the downstream kinase cascade [12]. This explains why the independent addition of NGAL failed to show phosphorylation of P38 or ERK. Furthermore, the primary function of NGAL in the peritoneal microenvironment may not be signal transduction. As a siderophore-binding protein, the core mission of NGAL is to participate in innate immune defense. By depriving bacteria of the iron elements required for growth, NGAL plays a role in inhibiting the spread of pathogenic microorganisms during the early stages of peritoneal infection [9]. Additionally, NGAL possesses protective mechanisms; for instance, it can stabilize matrix metalloproteinase-9 (MMP-9) by forming a complex, preventing its degradation, thereby playing a role in tissue repair and remodeling. This implies that the value of NGAL in peritonitis is reflected more in its metabolic regulation and antibacterial activity, rather than acting as a cytokine-like molecule to directly regulate gene transcription pathways.

Through comparison with the TGF-β1 group, this study further clarifies the drivers of peritoneal fibrosis. Although NGAL is significantly elevated during peritonitis, it does not possess the capacity to directly induce phenotypic transitions in mesothelial cells like TGF-β1 does [13]. This indicates that long-term peritoneal structural damage (such as thickening and fibrosis) caused by peritonitis may be attributed more to other pro-inflammatory factors (such as IL-1β, TNF-α) accompanying NGAL elevation and the subsequently triggered TGF-β1 autocrine loop, rather than the NGAL protein itself [13]. This conclusion provides guidance for the development of targeted drugs: to prevent peritoneal fibrosis, neutralizing NGAL alone may yield little effect, whereas blocking its upstream inflammatory inducing factors or downstream TGF-β1 pathways is more critical. The negative results of this study do not negate the clinical value of NGAL. On the contrary, it proves that NGAL is a highly "pure" marker of injury. It can faithfully reflect the external insult and inflammatory load sustained by the peritoneum, without generating complex positive feedback mechanisms to further worsen the disease like certain cytokines do. In clinical practice, utilizing the high sensitivity and specificity of NGAL in diagnosing peritonitis can assist physicians in achieving "precision diagnosis". In the future, if Point-of-Care Testing (POCT) devices based on effluent NGAL can be developed, it will significantly shorten the diagnostic cycle of peritonitis, winning valuable time for protecting the patient's peritoneal function.

Conclusions

Through clinical sample testing and in vitro molecular experiments, this study systematically elaborates on the relationship between NGAL and peritoneal dialysis-related peritonitis. The study found that effluent NGAL levels are significantly elevated when peritonitis occurs, and demonstrate a good linear positive correlation with WBC count and serum CRP, highlighting its excellent performance as an inflammatory diagnostic indicator. At the cellular and molecular levels, although NGAL is widely present in the peritonitis environment, it does not directly activate P38 MAPK, ERK1/2 MAPK, JNK MAPK, or classic Smad3 signaling pathways in human peritoneal mesothelial cells. This result excludes the possibility of NGAL serving as a direct ligand for the aforementioned pro-inflammatory and pro-fibrotic pathways. The role of NGAL leans more toward being a defensive acute-phase protein produced due to the stimulation of inflammatory factors, rather than an active driver of the inflammatory cascade. Future research directions should further investigate the dynamic changes in NGAL receptor expression on HPMCs under various pathological conditions (such as high-glucose environments and repeated inflammatory assaults), and evaluate whether NGAL participates in peritoneal protection and repair through non-MAPK-dependent pathways (such as iron metabolism regulatory pathways or anti-apoptotic pathways). Concurrently, multi-center, large-sample clinical trials should be carried out to establish standard thresholds of effluent NGAL for guiding clinical antibiotic use and prognosis determination, thereby comprehensively improving the management level of peritoneal dialysis.

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Conflict of Interest

None.

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