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Interleukin Expression in the Area damaged by the Development of Abdominal Cavity Adhesions

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Abstract

Background: This study sought to determine the dynamics of IL gene expression during serous membrane damage using an animal model of aseptic peritoneal injury.

Methods: In our study, we used 35 male Wistar rats. Macroscopic and microscopic studies were conducted between 6 hours and 30 days after peritoneal damage was induced. In the damaged peritoneal area, we assessed IL gene expression across the experimental timeframe.

Results: We found that the majority of the studied genes had three characteristic peaks in expression: at 6 hours, on day 3, and on day 14. These effects were observed for chemokine (CXC motif) ligands 1 and 3, IL1b, and IL6. Two peaks of increased expression (on days 3 and 14) were noted for CXCL1, CXCL5, INFγ, IL2, IL4, IL10, TNF, and CD40LG.

Conclusion: We hypothesize that the absence of attention to the changes that occur in the peritoneum after aseptic damage has prevented research from focusing on the important stage of the formation of the richly vascularized adhesions that are unable to regress. Based on the results of our study, we conclude that it is critically important to influence the last wave of IL expression activation (2 weeks after aseptic peritoneum damage) to effectively prevent adhesion formation.**(International Journal of Biomedicine. 2017;7(4):293-297.)**

Key Words: interleukins • adhesions • peritoneal cavity • chemokines

Abbreviations

CXCL, C-X-C motif chemokine ligand; **CD40LG**, CD40 ligand; **IFNγ**, interferon gamma; **IL**, interleukin; **TNF**, tumor necrosis factor.

Introduction

Postoperative abdominal adhesions and fibrosis are major complications of surgery and often result in infertility, abdominopelvic pain, and small bowel obstruction. Adhesions are likely the result of inflammatory responses to surgery-derived tissue trauma, bacterial infection, or foreign substances in the peritoneal cavity. However, the molecular mechanisms that underlie adhesion formation remain unknown.[1]

The major proinflammatory cytokines secreted due to peritoneal trauma are TNFα, IL1β, IL6 and IL8. TNFα and IL1β are the early regulators of the immune response and induce the release of secondary cytokines such as IL6 and IL8.[2,3] Currently, the data on the correlation between the concentration of cytokines in plasma and peritoneal fluid are contradictory. Most researchers have observed an increase in cytokine concentration in the zone of damage compared with the concentration of serum cytokines.[4] However, Florence Riché and colleagues did not find such a correlation in their study.[5] F. Fredriksson and colleagues established that the levels of IL-6, IL-1β, TNF-α in the peritoneal fluid increased 6 hours post-injury, whereas only the level of IL-6 increased in the plasma at this time point.[6]

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According to various researchers, the peak concentrations of proinflammatory cytokines were observed during the first 24 hours after peritoneal damage; these levels stabilized on the third day and decreased by the seventh day post-injury.[4,7-9] G.Wang and colleagues operated on the abdominal organs of patients and experimental models and found that the peritoneal fluid concentrations of IFN-γ and IL-17 increased 6-12 hours after surgery and reached their peak at this time, whereas TGF-β1 concentrations had two peaks at 2 and 72-96 hours after injury.[10]

The inconsistent data regarding cytokine dynamics after serous membrane damage increase the importance of studying cytokine gene expression. Receiving new knowledge on this issue will allow us to investigate the mechanisms involved in the formation of adhesions and develop methods to prevent their formation.

Objective: To study the dynamics of IL gene expression during serous membrane damage using the peritoneal aseptic inflammation model.

Materials and Methods

In our study, we used 35 nine-month-old male Wistar rats weighing 220-250 g. The rats were sedated using ketamine 50 mg/kg, droperidol 2.5 mg/kg and atropine 0.4 mg/kg. An aseptic inflammatory process in the abdominal cavity was simulated by opening the serous-muscular layer of the cecum with a 1cm incision, followed by closing the wound using Schmieden sutures and the scarification of the abdominal peritoneum of the right side channel in a 1.5 cm × 1.5 cm region.[11,12] The animals were housed in keeping with the rules for good laboratory practice. The experiments were performed in accordance with the norms for the humane treatment of animals, which are regulated by the International Guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the Irkutsk Scientific Center of Surgery and Traumatology. Animals were sacrificed and tissues were collected under ketamine anesthesia at 7 time points, ranging from 6 hours to 30 days. The severity of the adhesions was visually assessed in accordance with the developed protocol (Table 1).

Tissues were fixed with FineFix (Milestone S.r.l., Sorisole (BG), Italy) and then embedded in paraffin for histological investigation. We used Hematoxylin & Eosin staining and the Van Gisone method to detect collagen fibers. We used a Nikon 80i microscope to visualize the slides.

To study cytokine gene expression, tissue was taken from the damaged zone of the cecum and placed in RNAlater solution (Ambion, Canada, Cat #7020). After exposure to the solution at 4°C for 12 hours, the material was placed in storage at -20°C. The study of the serous-muscular layer of the cecum in intact animals served as a control (n=5). To isolate total RNA, the RNeasy Mini Kit was used (Qiagen GmbH, Germany, Cat. No.74104). For RNA DNase clearing, Rnase-Free DNase (Qiagen GmbH, Germany, Cat. No. 79254, Lot No.139294845) was used. After incubation, the samples were cleared using the RNeasy Mini Kit (Qiagen GmbH, Germany, Cat. No.74104). To obtain cDNA, a cDNA-RT2 First Strand Kit (Qiagen GmbH, Cat No. 330401, Lot No. DC08-8) was used. The gene expressions of the ILs were determined using the RT²-Profiler™ polymerase chain reaction (PCR) Array Rat Wound Healing Kit (Qiagen GmbH, Cat. No. 330503).

Statistical analyses of the results were performed using the provided online software obtained with the RT2_Profiler PCR Array® kit (http://www.qiagen.com/Products/Genes and Pathways/Data Analysis Center Overview Page/RT2 Profiler PCR Arrays Data Analysis Center).

Table 1.

The macroscopic scale for assessing severity of adhesions in the abdominal cavity

Score	Number of commissures	Morphology	Extension	Intestinal deformation
0	Absent	NA	NA	NA
1	solitaire	membranous	1 anatomic region	Light, w/o luminal narrowing
2	2 commissures (interviscerale or viscera-parietal)	Loose, non-vascularized	1 abdomen level	Medium deformation w/o luminal narrowing
3	>2 commissures	Dense, non-vascularized	2 abdomen levels	Deformations and luminal narrowing up to ½
4	Conglomerate	Dense, vascularized	Totally	Severe deformations and luminal narrowing >½

Results

Marked peritoneal hyperemia with visible fibrin overlays was observed 6 hours after simulating peritoneal damage under aseptic conditions. A microscopic examination showed hemorrhage, moderate neutrophilic infiltration of the submucosal layer, areas not covered by mesothelium, thickening and neutrophilic infiltration of the peritoneum in the areas adjacent to the damaged area, and neutrophilic infiltration in the sutured intestinal tract. Within 24 hours of injury, the animals showed moderate hyperemia of the peritoneum and fibrinous deposits on the peritoneum.

Formations of intestine-omentum, intestine-abdominal wall, and intestine-intestine adhesions were observed in 80% of the cases. Significant neutrophilic infiltration of the peritoneum and subserous layers was observed in the region of adhesion formation and the zone of the sutured intestinal tract.

Three days after injury, the mild hyperemia of the peritoneum remained, and the fibrinous deposits persisted. The formation of adhesions was observable in 80% of the cases. A microscopic analysis of the adhesion sites showed a proliferation of granulation tissue, characterized by a large area of adhesions, increased density in the connective tissue, and significant inflammation surrounding this junction. On day 7, all of the animals had formed adhesions, with 80% having

formed a conglomerate of adhesions and intestine-intestine type adhesions. All cases showed multiple adhesions, with 3-4 recorded adhesions per animal on average. A microscopic analysis revealed long, indurated adhesions without significant vascularization; the formation of a capsule surrounding the sutures was also noted. On day 14, the adhesive process was observed in all of the animals, and multiple intestine-intestine, intestine-omentum, and intestine-abdominal wall adhesions were observed. These adhesions led to severe deformity and narrowing of the intestinal tube as well as swelling of the superjacent segments in 60% of all cases. Microscopy revealed thick, vascularized adhesions and a proliferation of connective tissue surrounding the sutures.

On day 30, the adhesions in the abdominal cavity of the animals were most severe. In all cases, multiple adhesions were formed, including the most prognostically unfavorable type, the intestine-intestine adhesion. These adhesions led to severe deformity and the narrowing of the intestinal tube and the swelling of the superjacent segments in 60% of all cases. Microscopic examination revealed extensive adhesions with a high density of collagen fibers and rich vascularization. The scoring system for estimating the severity of adhesions [12] revealed that the severity of adhesions was moderate between 6 hours and 1 day. Further observation showed that the intensity of the adhesion process progressed with time, reaching its maximum at 30 days post-operation (Figure 1).

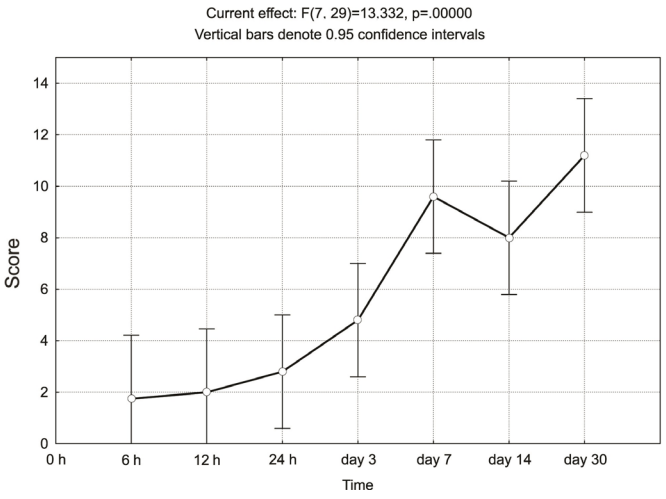

Fig. 1. *Intensity of the adhesion process in the abdominal cavity.*

We then evaluated the expression of interleukin genes in the serous-muscular layer of the damaged cecums. The results were compared with the gene expression in intact animals.

We found an increased expression of proinflammatory ILs 6 hours after the peritoneal injury (Figures 2 and 3). Specifically, increased expressions of CXCL1 (26.7-fold increase), CXCL3 (93.1-fold increase), IL1b (9.0-fold increase) and IL6 (25.7-fold increase), one of the major mediators of the acute phase of inflammation, were observed. The level of IL6 was significantly higher than in intact animals (*P=0.035*).

The maximum expression of CXCL1 (59.3-fold increase) as well as significant increases in CXCL3 (59.6-fold increase), CXCL5 (21.6-fold increase), IFNγ (121.4-fold increase), IL2 (108.1-fold increase), IL4 (26.7-fold increase), IL6 (11.3-fold increase), IL10 (12.6-fold increase), TNF (11.1-fold increase), and CD40LG (15.3-fold increase) were observed on the third day.

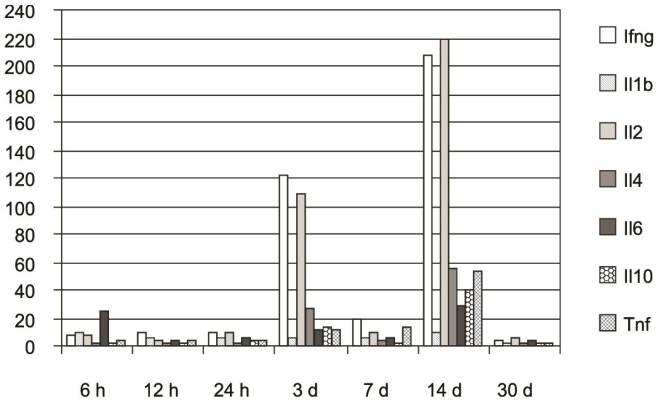

Fig. 2. *Change in IL gene expression in the zone of damage.*

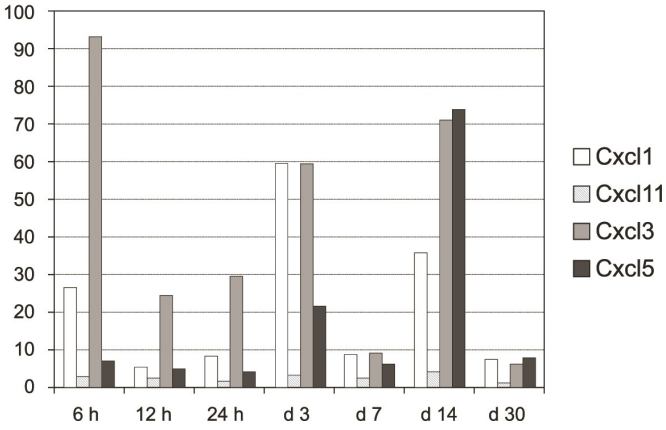

Fig. 3. *Change in chemokine gene expression in the zone of damage.*

By day 7, the expression level of the majority of the ILs had decreased. By day 14, however, repeated increases in the expressions of CXCL1 (35.8-fold increase), CXC3 (71.0-fold increase), CXCL5 (74.2-fold increase), IFNγ (207.0-fold increase), IL10 (39.6-fold increase), IL2 (219.3-fold increase) IL4 (55.7-fold increase), IL6 (29.0-fold increase), and CD40LG (29.1-fold increase) were observed. TNF reached its maximum level (52.8-fold increase) at this time point, as did CXC5, IFNγ, IL10, IL2, IL4, and CD40LG.

By day 30, the expression levels of the IL genes approached those of intact animals.

Discussion

We found that the majority of the studied IL genes were characterized by the presence of three peaks of increased activity: at 6 hours, on day 3 and on day 14 (for CXCL1, CXCL3, IL1b, and IL6). Two peaks of increased expression

(on day 3 and on day 14) were noted for CXCL11, CXCL5, IFNγ, IL2, IL4, IL10, and CD40LG (Table 2). We believe that the first peak at 6 hours was associated with the initiation of the inflammatory response that is naturally accompanied by the increased expression of proinflammatory ILs.[2,3] This observation coincides with the opinion of other authors concerning the key role that injury plays in the production of proinflammatory cytokines during the early period after peritoneal damage.[6,10]

Table 2.

Expression of interleukines genes in the zone of damage in 6 hours, on day 3 and day 14 of the pathological process (degree of increase compared to intact animals)

Time	6 hours	3 days	14 days
Cxcl1	26.7	59.3	35.8
Cxcl11	2.6	3.2	4.2
Cxcl3	93.1	59.6	71.0
Cxcl5	6.8	21.6	74.2
Ifng	8.0	121.4	207.0
IL10	1.9	12.6	39.6
Il1b	9.0	5.1	9.5
Il2	8.0	108.1	219.3
Il4	2.3	26.7	55.7
Il6	25.7	11.3	29.0
Il6st	0.3	0.7	1.9
Tnf	3.1	11.1	52.8
Cd40lg	0.6	15.3	29.1

The second peak most likely reflects the work of the key elements of the reparative process; specifically, day 3 is the start of the fibroblastic phase of inflammation during aseptic wound healing.

The most unusual peak (in terms of both time and maximum intensity) was the late peak of cytokine expression on the 14th day. This peak appears to reflect the intensity of the proliferative process during aseptic peritoneal damage and the restructuring of tissue associated with this proliferation. This peak was preceded by the maximum rise in intensity of the adhesion process in the abdominal cavity based on the observed morphological changes between the 14th and 30th day post-injury. We believe that the increase in CXCL5, which stimulated angiogenesis on the 14th day, is an appropriate response to peritoneal injury and indirectly reflects the intensity of the adhesion formation process and the formation of richly vascularized adhesions.

Almost all of the previous research on the dynamics of the concentration of cytokines after peritoneal injuries has been limited to observations on the first, third, and seventh days after surgery. The absence of attention to the changes in the peritoneum that occur long after aseptic damage precludes researching the important stage of the adhesion process, when richly vascularized adhesions incapable of involution are formed. Based on the results of our study, we believe that influencing the later wave of IL expression and activation (i.e., 2 weeks after the aseptic abdominal injury) is particularly important to prevent adhesions. This is especially important because it has been demonstrated that the neutralization of IFNγ, IL-17 and TGF-β1 during the maximum concentration of these cytokines significantly reduces the formation of adhesions in the abdominal cavity,[10] and the risk of adhesions re-forming after adhesiolysis is correlated with high levels of IL-6 and IL-1 in the abdominal cavity.[13]

At the same time, peritoneal fibroblasts should be viewed as a target to influence the adhesion formation process because they are the most significant pool of cells in the locus of adhesion formation. Previous studies have shown that fibroblasts isolated from the region of adhesions have significantly higher levels of IL-6, IL-10 and TNF-α mRNA than those from the abdominal cavity isolated outside of the adhesion zone.[14,15]

Competing interests

The authors declare that they have no competing interests.

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