Pneumoperitoneum Modifies Serum and Tissue CCL2-CCL5 Expression in Mice

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ABSTRACT

Background and Objectives: Laparoscopy is the preferred method when operating in the abdomen. In this study, we evaluated systemic and morphological peritoneal cytokine modifications (RANTES/CCL5 and MCP-1/CCL2) due to CO₂ pneumoperitoneum in rats.

Methods: Twenty-five prepubertal Sprague-Dawley rats were randomized into three groups. Pneumoperitoneum lasting 30 minutes was induced with a flow of 0.5 L/min, in two groups (S1 and S2, n=10, P/CO₂ of 6 and 10 mm Hg, respectively. In the control group (C, n=5), only anesthesia was carried out. All animals were sacrificed after 24 hours. The serum of the rats was collected for ELISA, and the levels of the cytokines RANTES and MCP-1 were investigated. An immunohistochemical analysis of RANTES and MCP-1 was performed on samples of the peritoneum, and the morphological evaluation was conducted with a blinded evaluation by two independent, experienced pathologists by using a grading system (0, 1+, 2+, 3+: no, faint, moderate, and strong reactivity, respectively).

Results: RANTES mean levels were significantly different in the S1, S2, and C groups (70.3 ± 2.26, 58.23 ± 4.32, 29.66 ± 4.03, respectively, P = .0001). The levels of MCP-1 were 32.1 ± 1.63 in the S1 group, 27.0 ± 9.26 in the S2 group, and 16.4 ± 9.55 in the C group (P = .159). Normal control peritoneum showed little reactivity, whereas a moderate to strong cytoplasmic reaction to anti-CCL5/CCL2 antibodies was observed in mesothelial and inflammatory cells in the S1 and S2 groups.

Conclusion: CO₂ pneumoperitoneum evokes an inflammatory response by modifying plasma RANTES levels and peritoneal CCL5/CCL2 expression.

Key Words: Pneumoperitoneum; Laparoscopy; Inflammation; CCL5; CCL2.

INTRODUCTION

Laparoscopy is now considered a superb diagnostic tool and is the preferred method to explore and operate in the abdominal cavity. The gas used in videoendoscopic laparoscopic surgery to induce pneumoperitoneum (PN) is carbon dioxide (CO₂), which is inert, blood soluble, and not explosive.1 Many symptoms that are reported after laparoscopy are generally attributed to CO₂ insufflation into the peritoneal cavity. The peritoneum has a large capacity for absorption and reaction; it is one of the rich, vascularized organs with a surface lining that is coated by highly differentiated mesothelial cells. The peritoneum is able to synthesize chemokines, which are cytokines that are significant in driving different elements of inflammation.2 The creation of CO₂ PN induces morphological alterations in the mesothelium, which are microscopically apparent. We have previously described a peritoneal chronic inflammation in rats 24 hours after PN and consisting mostly of eosinophils, granulocytes, mastocytes, and macrophages.3 These modifications appear to be related to intraabdominal CO₂ pressure.4 Volz and Suematsu demonstrated ultrastructural changes after PN, such as swelling up of mesothelial and an enlargement of intercellular junctions.5,6 The observed uncovering of the mesothelium basement membrane may be dependent from macrophage-evoked changes of peritoneal immunity and could be assessed as the main cause for surface-
induced adherence. Bertram et al advised that injection and grafting of mesothelial cells into the abdomen was related to reduced development of adhesions. Thus, to decrease postinflammatory and surgical adhesions, it is important to maintain the integrity of the peritoneal surface. Leukocytes have a primary role when the peritoneal serosa is damaged. Activation of these cells and degranulation release active components, such as cytokines and growth factors. Among the cytokines, RANTES/CCL5 (Regulated upon Activation of Normal T cell Expressed and Secreted/Chemokine [C-C motif] ligand 5) and MCP-1/CCL2 (Monocyte Chemoattractant Protein-1/Chemokine [C-C motif] ligand 2) exert proinflammatory activities. These chemokines are secreted in infectious and inflammatory diseases and mediate the recruitment of immune cells. In particular, RANTES/CCL5 is produced by different cell types, such as T lymphocytes, macrophages, platelets, and eosinophils playing an active role in the migration of these cells. Nevertheless MCP-1/CCL2 mediates monocyte and macrophage recruitment and migration to the inflammation site.

Furthermore, laparoscopy causes intra-abdominal acidosis, perhaps evoked by the local decrease in partial oxygen pressure. This effect seems to have a beneficial influence on the peritoneal immune system. Following an open surgical procedure, there is a surge in proinflammatory cytokines, such as TNF-α and IL-1, that are attenuated by laparoscopy. Therefore, laparoscopic surgery changes not only peritoneal integrity but also its biological environment.

The study was conducted to examine the effect of CO2 PN on the serum levels of inflammatory cytokines and the corresponding tissue immunohistochemical analysis and expression.

**MATERIALS AND METHODS**

**Animals**

The experiment was conducted as previously reported. Twenty-five prepubertal Sprague-Dawley rats were in the object of the protocol. Animals were housed following the protocol suggested by Institutional Animal Care and Use Committee of the University of Campania “Luigi Vanvitelli,” Faculty of Medicine, in agreement with the recommendations of National Institutes of Health. The rats were housed in a stable arrangement (12 hours of daylight at a temperature of 22°C) with food and water ad libitum.

**Experimental Protocol**

On the day of the study, the rats were randomized as follows: PN was evoked in two groups with CO2 (S1 and S2, n = 20) with a flow rate of 0.5 L/minute. In the last group (C, n = 5), only general anesthesia was executed and acted as control. The designated pressures were chosen according to our previous protocol and other studies.

Anesthesia was induced by an intramuscular administration in the legs of the animal with a dose of 5 mg/kg tiletamine-zolazepam hydrochloride at a 1:1 ratio and a dose of 0.2 mg/kg and xylazine at a dose of 5 mg/kg into the leg of the animal. Fifteen minutes after, the abdominal wall was shaved and cleaned; a Veress needle was inserted in the abdominal cavity, and PN was created with an inflator (Storz Electronic CO2 Endoflator, Karl Storz GmbH & Co., Tuttingen, Germany) that delivered medical CO2 at 37°C and an outflow of 0.5 L/minute for 30 minutes at different pressures (6 and 10 mm Hg). All rats were sacrificed after 24 hours from surgery with a lethal administration of tiletamine-zolazepam hydrochloride.

Cytokines in rats with PN at 6 mm Hg and 10 mm Hg and in control rats were measured. At 24 hours after PN, the serum was collected and conserved at −20°C until cytokine evaluation, and then, an enzyme-linked immunosorbent assay (ELISA) was performed. In particular, cytokine levels (IL-6, IL-1β, IL-1α, TNF-α, MIP IFNβ, MCP-1, and RANTES) were quantified in serum samples, which were optimally diluted.

A multiplex assay kit manufactured by Signosis (Cat. N. EA-1201; Santa Clara, CA, USA) was used for the contemporaneous measurement of the mentioned cytokines.

All optical densities were measured at 450 nm. The levels of cytokines are expressed as ng/mL.

**Histological Examination**

Immunohistochemical analysis was performed on paraffin-embedded sections following a well-established method. Primary antibodies included CCL5/RANTES ABfinity recombinant rabbit oligoclonal antibody (catalog no. 710001) diluted 1:500 in PBS, rabbit polyclonal MCP-1 (ab9669, AbCam, Cambridge, UK) diluted 1:1000 in PBS. Slides were evaluated independently by two experienced pathologists (SP and OP), with good agreement (Cohen’s κ, P < .001). The κ-value between the two sets of scores was 0.82, indicating good concordance. The expression of CCL5/RANTES and MCP-1 was semi quantitatively evaluated according to the intensity of cytoplasmic tissue stain
from 0 to 3 as follows: (0, 1+, 2+, 3+: no, faint, moderate, and strong reactivity, respectively). To identify the predominant lymphocyte subtype, the labeled immune cells number was determined for each slide and each antibody in all groups and is expressed as the mean of at least 10 fields at 40× magnification.

**Statistical Analysis**

Variables were tested for normality and were log transformed to achieve normal distribution as appropriate. Differences between different treatments were evaluated by ANOVA to analyze the effect of each cytokine. The results are expressed as the mean ± SD. A value of $P < .05$ was considered statistically significant.

**RESULTS**

The rats showed no clinically relevant symptoms. To understand the effects of PN treatment on inflammatory response mediators, a probable pattern of cytokines that are involved in immunological mechanisms was examined.

The rats insufflated with 10 mm Hg and 6 mm Hg PN showed higher plasma TNF-α levels (0.32 ± 0.1 and 0.26 ± 0.04, respectively) compared to those of the control group (0.23 ± 0.01), with the 10 mm Hg PN group presenting higher levels compared to those of the 6 mm Hg PN rats with no statistical significance ($P = .38$) (Figure 1). Similarly, the three groups had different plasma IL-6 levels, although the observed difference was not significant (10 mm Hg PN, 1.11 ± 0.26; 6 mm Hg PN, 0.93 ± 0.28; controls, 0.73 ± 0.18; $P = .293$) (Figure 2).

Interestingly, PN groups showed higher RANTES levels compared to controls, and the differences were statistically significant. In particular, the mean RANTES level in the 6 mm Hg PN samples was 70.3 ± 2.26 vs 29.66 ± 4.03 in the controls ($P = .0000$). Similarly, the 10 mm Hg PN group had significantly higher RANTES levels than controls (58.23 ± 4.32 vs 29.66 ± 4.03, $P = .0001$) (Figure 3). In addition, the levels of MCP-1 were more increased in the 6 mm Hg PN group (32.1 ± 1.63) than in the 10 mm Hg PN group (27.0 ± 9.26) compared with those of the control group (16.4 ± 9.55). However, the values between groups were not significantly different ($P = .159$) (Figure 4). Interestingly, both RANTES and MCP-1 levels showed a trend toward higher inflammatory cytokine values in low respect to high pressure group, but they were not significant.

No differences were reported regarding IL-1α and MIP levels among the three groups. IL-1β value did not exceed the assay limit; however, samples from the 6 mm Hg PN group showed IL-1β concentrations that were 4-fold higher than those of the 10 mm Hg PN group.

Finally, IFN-γ levels were slightly increased in PN rats compared with those of control rats.
Histological Results

Normal control peritoneum shows absent to faint reactivity to the anti-CCL5/RANTES antibody (Figure 5, A and B). The visceral peritoneum of the 6 mm Hg PN group showed faint to moderate reactivity of mesothelial cells to the anti-CCL5/RANTES antibody (Figure 6, A and B), whereas at 10 mm Hg, PN, there was strong cytoplasmic reactivity of mesothelial cells to the anti-CCL5/RANTES antibody (Figure 7A [arrows]), and strong immunoreactivity was observed in omentum adipose tissue (Figure 7B [arrows]) and was associated with inflammatory cells Figure 7B [arrow heads]). Faint reactivity to the anti-MCP-1 antibody was also observed in the control peritoneum (Figure 8A, [arrows]). Adipose tissue and the inflammatory infiltrates showed moderate to strong immunoreactivity to the anti-MCP-1 antibody in 6 mm Hg PN group (Figure 8B [arrows]). Strong immunoreactivity to the MCP-1 antibody was observed both in omentum adipose tissue and inflammatory infiltrates (Figure 8C).

DISCUSSION

Laparoscopy is widely used and well incorporated in clinical practice as the preferred, minimally invasive technique for operating in the abdominal cavity. The induced PN modifies the peritoneal environment. The gas that is usually employed to lead to PN is CO2. When the abdomen is distended by CO2, it exerts a direct pressure on peritoneal cells and reacts with the H2O found in peritoneal liquid. This phenomenon decreases the pH of abdominal cavity and determines an environment that changes the defense mechanisms of the abdominal cavity,17,18 which is an element in reducing the inflammatory response.11

Vascularized tissue, such as peritoneum, replies to chemical or physical insult through an inflammatory reaction. Soluble molecules such as chemokines have been demonstrated to stimulate diverse inflammatory factors, such as leukocyte influx.9

The peritoneum has a great capacity for absorption and action through mesothelial cells, which synthesize cytokines and chemokines that are primary factors in tissue inflammation.

In previous works, we showed that PN causes peritoneal inflammation and the activation of inflammatory cells that release substances responsible for the increase in vascular permeability, complement, and opsin.3,4 To understand the effects of laparoscopy with CO2 PN on mediators of inflammatory responses, the cytokines involved in immunological responses were examined. Several papers have confirmed a considerable increase in some cytokines.19,20 IL-6 represents the most important cytokine determined postoperatively, followed by TNF-α. In our experimental protocol, serum levels of IL-6, TNF-α, IL-1α, IFN-γ, MCP-
1/CCL2, IL-1β, RANTES/CCL5, and MIP were assessed after inducing PN at two different pressures and in the control group. We showed that PN modifies serum concentrations and tissue expression of the chemokines CCL5 and CCL2. RANTES/CCL5 showed the most dramatic increase.

RANTES/CCL5 controls the activation and recruitment of inflammatory cells. Moreover, since chemokine receptors are also expressed on other cells, they may also play a role in other regions of the organism, as well as in mechanisms of tissue repair and scarring.21 There are many studies that establish a role of CCL5 in disease activity and in leading

Figure 6. Omentum and visceral peritoneum. A and B, CO₂ 6 mm Hg PN showing faint to moderate reactivity to the anti-CCL5/RANTES antibody of mesothelial cells (arrows). Haematoxylin counterstaining. PN, pneumoperitoneum. Original magnification, A, 20×; and B, 40×.

Figure 7. Omentum and visceral peritoneum. A, 10 mm Hg PN showing strong cytoplasmic reactivity to the anti-CCL5/RANTES antibody of mesothelial cells (arrows). B, A strong immunoreactivity is observed in omentum adipose tissue (arrows), also associated to inflammatory cells (head arrows). Haematoxylin counterstaining. PN, pneumoperitoneum. Original magnification, A, 20×; and B, 40×.

Figure 8. Omentum and visceral peritoneum. A, A faint reactivity to the anti-MCP1 antibody is observed in normal control peritoneum (arrows). B, CO₂ 6 mm Hg PN: adipose tissue and the inflammatory infiltrate showing moderate to strong immunoreactivity to the anti-MCP1 antibody (arrows). C, CO₂ 10 mm Hg PN: a strong immunoreactivity to MCP1 antibody is observed both in omentum adipose tissue and inflammatory infiltrate. Haematoxylin counterstain. Original magnification, A, 20×; and B and C, 40×.
to leukocyte enrollment, neovascularization, and formation of fibrous tissue. In our study, we showed that there was not only a significant modification of the serum levels but also strong tissue expression, suggesting the influence of this chemokine in the development of inflammation. Kawashima et al. showed that during laparotomy, a large amount of CCL5 was produced, and they postulated that this increase may be indicative of mesothelial cell injury. They noted that in the presence of lipopolysaccharide, there was no significant production of CCL5 by exudate cells. Our study showed significant modifications in RANTES/CCL5 serum levels in the groups insufflated with CO2 compared with those of the control group. This chemokine, together with CCL2, were also strongly expressed in peritoneal tissues, as revealed by histological examination. Therefore, we may speculate that these modifications are directly indicative of mesothelial cellular injury.

Interestingly, we found a trend toward higher inflammatory cytokine levels in the low-pressure group. In our opinion, these results could be related to the experimental model and its variables. However, we might speculate that low pressure is associated with higher CO2 absorption and mesothelial oxidative stress that evoked a different cytokine response between normal, infected, or previously inflamed peritoneum clearly more studies are necessary to understand how cytokines relate each other and how we could modify this clinical status in order to add a benefit for the patient.

Cytokines have been identified as important mediators in both acute and chronic human inflammatory disorders. The role of inflammatory mediators is not completely understood, especially in morbidity and mortality of intra-abdominal sepsis, and there is no consensus on the clinical use of mediators in diagnosing or managing this problem. However, recently, many researchers have tried to evaluate cytokines and chemokines in order to define a diagnostic and a therapeutic role. In Pediatric appendicitis, through an analysis of inflammatory mediators, the assessment of previously identified mediators such as cytokines to leukocyte enrollment, neovascularization, and formation of fibrous tissue. In our study, we showed that there was not only a significant modification of the serum levels but also strong tissue expression, suggesting the influence of this chemokine in the development of inflammation. Kawashima et al. showed that during laparotomy, a large amount of CCL5 was produced, and they postulated that this increase may be indicative of mesothelial cell injury. They noted that in the presence of lipopolysaccharide, there was no significant production of CCL5 by exudate cells. Our study showed significant modifications in RANTES/CCL5 serum levels in the groups insufflated with CO2 compared with those of the control group. This chemokine, together with CCL2, were also strongly expressed in peritoneal tissues, as revealed by histological examination. Therefore, we may speculate that these modifications are directly indicative of mesothelial cellular injury.

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could not evaluate the differences in cytokine response in infection and or neoplastic conditions; nonetheless, when a safe mesothelial environment is present, obtaining less inflammatory cytokine response in the postoperative period as in clean surgery could be considered beneficial to the patient, but to date, targeted therapies remain experimental. We acknowledge that systemic consequences of peritoneal treatments should be investigated.

As reported above, the use of CO₂ for PN, the flow rate and the selected intra-abdominal pressure can cause biochemical and morphological modifications, hypoxia, and dehydration to mesothelium.3,4,28,29 The development of laparoscopic surgical techniques, the use of modern devices and image magnification support less invasive surgery that can avoid the insult of mesothelial cells and bleeding, which are the origins of adhesions. To avoid these complications, some researchers have tried to change the physical qualities of CO₂ such as humidifying, raising the temperature and the altering composition of the gas used for insufflation.2,30 Although more studies are needed looking at the potential role of RANTES and MCP-1, we suggest that targeting these chemokines could be beneficial in preventing postoperative peritoneal modifications such inflammation and adhesions. In terms of benefits for the patient, less pain, and fewer postoperative complications could be associated with minimally invasive surgery.

CONCLUSION

In conclusion, we have demonstrated in this study that PN modifies the serum and the corresponding tissue expression of the chemokines CCL5 and CCL2, which are involved in tissue inflammation and response to insults exerted by chemical and physical CO₂ action on the abdominal cavity. Targeting these factors appears to be an important means of providing an additional benefit to laparoscopy by modifying peritoneal inflammation and potential subsequent peritoneal adhesions. More studies are necessary to clarify these mechanisms and their possible influence in humans.

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