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THE EFFECTS OF PEROPERATIVE PERITONEAL LAVAGE

mechanism and clinical implications

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The effects of peroperative peritoneal

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De effecten van peroperatief peritoneaal spoelen

mechanisme en klinische implicaties

(met een samenvatting in het Nederlands)

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A special achievement requires three conditions:

The credulity of all,
The complacency of a few,
And the tacit complicity of the main protagonist.

(ait: Camille Claudel)

Contents

Abbreviations

ANOVA	analysis of variance
BSA	bovine serum albumin
CAPD	continuous ambulatory peritoneal dialysis
CD	cluster of differentiation
CL	chemiluminescence
DCFH-DA	dichlorofluoresceine diacetate
EDTA	ethylenediaminetetraacetic
ELAM-1	endothelial cell adhesion molecule-1
ELISA	enzym linked immuno sorbent assay
FCS	fetal calf serum
HSA	human serum albumin
HBSS	Hanks' balanced salt solution
Ig	immunoglobulin
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IL	interleukin
IU	international units
kD	kilodalton
LCL	luminol chemiluminescence
LDH	lactate dehydrogenase
mAB	monoclonal antibody
M-199	medium 199
MC	mesothelial cells
MF	mean fluorescence
MFI	mean fluorescence intensity
NADH	nicotinamide adenine dinucleotide
NAC	N-acetylcysteine
NF-kB	nuclear factor kappa B
PAA	plasminogen activator activity
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocytes
PI	propidium iodide

PVP-I	polyvinylpyrrolidone iodine
ROS	reactive oxygen species
RPMI-1640	Rosewall Park Memorial Institute medium
SD	standard deviation
SEM	standard error of the mean
SPG	sucrose phosphate glutamine medium
TF	tissue factor
TGF	tissue growth factor
TNF	tumour necrosis factor
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
VCAM-1	vascular cell adhesion molecule-1

Preface

Most surgeons have accepted the philosophy that in case of contamination, the abdominal cavity should be 'drowned' in irrigation fluids. Currently, the use of peritoneal lavage is a custom in modern surgical practice, particularly in cases of peritonitis, colonic resection with faecal spilling, peritoneal adhesion formation and prevention of tumour recurrence.[1] The basic idea behind the use of peritoneal lavage is dilution and mechanical elimination of substances that have accumulated in the peritoneal cavity during the course of disease or operative procedures and after trauma. It is assumed that these substances exert important pathophysiological effects and that their removal from the peritoneal cavity may favourably influence the outcome for the patient. Irrigating the abdominal cavity is, however, more based upon habit than upon scientific evidence. Although the concept of mechanical peritoneal cleansing with solutions seems logical and cosmetically appealing, the supporting experimental and clinical data remain unconvincing.[2]

One aspect of the controversial role of peroperative lavage is the possible interference of exposure to solutions with the natural local defence mechanisms of the peritoneal cavity. Intra-abdominal surgical intervention disturbs the integrity of the peritoneal cavity, with alterations in host metabolic and immune homeostasis. For instance, exposure to foreign materials,[3,4] trauma to the serosa,[5,6] suturing technique[7], and exposure to gloves[8] during surgery has been implicated in the development of septic complications, adhesion formation and tumour metastasis. In recent literature it is highlighted that certain operative factors may contribute to postoperative immunomodulation and subsequently influence mobidity and mortality after surgical intervention.[9,10]

Over the past few years it has become increasingly clear that the peritoneal membrane, in particular the mesothelial lining, contributes significantly to important inflammatory processes occurring in the peritoneal cavity.[11,12] Therefore, with the routine use of peroperative peritoneal lavage we have focused our attention on both assessing the influence of the exposure to various solutions on modulation of peritoneal cell functions as well as development of a solution that will complement natural defence mechanisms.

This has resulted in the following objectives:

1. to elucidate the effects of peritoneal lavage solutions on peritoneal defence mechanisms *in vitro* (Chapters 1-3);
2. to assess the contribution of peroperative lavage on postoperative peritoneal responses *in vivo* (Chapters 4-5); and
3. to evaluate the modulation of biochemical parameters of mesothelial cells by oxygen radical scavengers (Chapter 6).

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General introduction

Peroperative lavage

The act of performing peroperative lavage is as old as that of abdominal operative procedures. Successful peritoneal lavage in the treatment of peritonitis was reported by Price[1] in 1905 and Torek[2] in 1906. In 1911, Morse reported the first successful closure of a perforated gastric ulcer in Great-Britain. The abdomen was irrigated with no less than 17 pints of hot water at a temperature of 60°C.[3] However, for more than 40 years the technique remained more or less dormant because of the belief expressed by Deaver in 1910, that irrigation would spread infection to uninvolved areas.[4] Its revival in the fifties is attributed to Burnett who produced experimental and clinical data that irrigation offers the patient with a contaminated peritoneal cavity a better prognosis.[5]

Experimental & clinical studies

The current surgical literature certainly recommends some type of peritoneal irrigation as the most effective and efficient way of removing all gross contamination and bacteria. During the last 25 years, a number of authors have presented both experimental and clinical data to demonstrate a decrease in mortality and morbidity after irrigation of the peritoneal cavity. The majority of these studies have been undertaken in animals and patients with established peritonitis[6], and some have considered the possible prophylactic role of lavage in routine surgery.[7-10]

The primary benefit of peroperative lavage is thought to be the physical removal of organisms and debris from the peritoneal cavity. A secondary benefit could result from the removal of toxins. Some authors consider saline alone for 'mechanical' lavage to be adequate. Hartmann, Ringer and physiological saline solutions have all been used with varying success. Hau induced peritonitis with a 100% mortality rate in rats.[11] Treatment with systemic antibiotics reduced this mortality to 70%. When peritoneal lavage with normal saline was added to the treatment the mortality dropped further to 25%. Tolhurst *et al.* also reported a reduction in mortality after peroperative lavage with Hartmann solution in rats with faecal peritonitis (66%).[12] However, they were also the first who observed effects of lavage on wound healing and adhesion formation. Their results showed that peritoneal lavage in the rat was associated with a delay in the healing of peritoneal wounds. One

explanation for this phenomenon was that lavage caused removal of the cells of the peritoneal fluid from the peritoneal cavity. These cells have been shown to have a role in the early stages of peritoneal wound healing. These observations were confirmed by histological studies. Growth factors or other substances important in normal wound repair were not studied in this setting.

Two clinical studies published in the early eighties have shown that additional peritoneal lavage with normal saline did not reduce the complication rates in patients with ongoing peritonitis.[7,13] Although the counts of bacteria in the peritoneal cavity may be reduced by saline lavage, the risk of postoperative sepsis is not.[7] Rosato and co-workers emphasized that lavage solutions do not reach all areas of the peritoneal cavity. They found large quantities of locular fluid at autopsy of rat peritoneal cavities.[14] Other explanations for persistent bacteremia after peritoneal lavage are, for instance, dispersal of organisms and microbial adherence to the serosal mesothelial surface.[15] Therefore, other authors recommend a wide range of different antiseptics to reduce the incidence of sepsis.[16] As early as 1923, addition of antiseptics was introduced. The abdomen of patients with sepsis were irrigated with an alcohol solution.[17] The advantage of using antiseptic lavage solutions for eliminating contamination and surgical spillage lies in their rapid bactericidal action and broad spectrum of activity.[18,19] The use of antiseptics was reappraised because of the increasing problem of antibiotic-resistant bacteria. The antiseptics most often used for the prevention or treatment of bacterial peritonitis are povidone-iodine (PVP-I) and the formaldehyde-releasing compounds, noxytiolin and Taurolin. Chlorhexidine and hydrogen peroxide have also been suggested as alternative treatments.

Polyvinylpyrrolidon-iodine (PVP-I): Several reports on the instillation of PVP-I into the peritoneal cavity have suggested that this may be of some benefit,[20-25] while others indicate that PVP-I damages the peritoneal cavity and interferes with peritoneal defences and in this way causes increased mortality.[13,22-24] The studies that do show a beneficial effect with intraperitoneal PVP-I demonstrate its efficacy only when it is used very early in the course of peritonitis.[25] One explanation for this phenomenon could be an early neutrophil influx in the peritoneal cavity. Abbuglosu observed that PVP-I induced a polymorphonuclear cells (PMN) influx, probably as a result from irritation of the peritoneum.[26] In addition, Hau and Dunn concluded that non-specific immuno-enhancing and PMN stimulating compounds are only

effective in experimental models, before exposure of the host to micro-organisms.[27,28] A rise in peritoneal PMN before initial infection may be protective. Unfortunately, most patients with peritonitis do not arrive in the operating room within an hour after onset of disease.

Taurolidine (Taurolin): Taurolidine is an antimicrobial chemotherapeutic agent with bactericidal,[29] anti-endotoxic,[30] and anti-adherent properties.[31] It consists of two molecules of taurine amide combined with three molecules of formaldehyde. Its advantage over noxytiolin, also a formaldehyde carrier, is that taurolidine is stable in solution.[32] When it exists in solution the formaldehyde groups denature lipopolysaccharide (LPS) components of bacterial cell walls. However, taurolidine is also presumed to function through the taurine component.[33] Taurine has been considered an amino acid with antioxidant properties. Taurolidine may have direct effects on immune functions and it has been hypothesized that intraperitoneal irrigation may affect regulation of local host defence mechanisms. Although Billing and co-authors found the neutrophil influx not diminished, they observed enhanced consumption of opsonins after intra-abdominal treatment with Taurolin.[34] Their explanation was that this was due to more severe bacterial contamination in the Taurolin treated group. However, reduced opsonic capacity could also result in less sufficient phagocytosis. Little *et al.* described prevention of the systemic postoperative cellular immunosuppression after laparotomy with intraperitoneal injection of taurolidine in the rat.[35] In addition, taurine was also shown to be protective against cytotoxicity to hepatocytes caused by different chemical compounds and may therefore also reduce cell death.[36] These properties may explain the favourable effect of intraperitoneally instilled Taurolin on adhesion formation described by Leaper[37] and the reduced morbidity in patients with established peritoneal sepsis found by Browne *et al.*[38] Both cellular injury and metabolism may be advanced in the presence of peritoneally instilled Taurolin, and may therefore prevent intra-abdominal complications.

Chlorhexidine: Limited clinical data are available on the use of peritoneal lavage with diluted chlorhexidine solutions to control peritoneal sepsis.[39] Platt and co-authors suggested that regular irrigation with chlorhexidine 0.02% could be beneficial in patients at risk from septic peritonitis.[40] Of the five antiseptics they tested, only chlorhexidine had a protective effect in mice. Diluted chlorhexidine 0.02% has also been successfully used

prophylactically at very low concentrations in peritoneal dialysis (CAPD) fluid.[41]

Dakin's solution: Sodium hypochlorite (NaOCl) has a long history of use as it was introduced as an antimicrobial agent for topical use in open wounds during World War I by Nobel Prize winner Alexis Carrel.[42] In the 1980's, reports began to accumulate suggesting significant toxicity of Dakin's to host cell populations.[43,44] However, the effects of peritoneal lavage with Dakin's solution has never been evaluated in any kind of clinical trial. Dakin's solution was experimental tested as an anti-tumour irrigant in both superficial wounds and the peritoneal cavity (see page 10/11).[45,46]

Hydrogen peroxide: Hydrogen peroxide in diluted solution was found to be useful in cleansing the abdomen of particulate matter.[47] Others also found it helpful, but these were subjective impressions and no experimental or controlled clinical data are available.

Another adjuvant treatment that has been used to decrease the effects of peritonitis is intraperitoneal lavage with antibiotics. Topical antibiotic lavage seems to be logical in order to achieve higher antibiotic levels in the tissue fluid for optimal bacterial killing. The first report on the addition of antibiotics to the lavage solution is by Dees.[48] Since then, the pharmacokinetic activity in the peritoneal cavity has been extensively researched.[49,50] Apart from the fact that intraperitoneal instillation of antibiotics results in therapeutic serum levels, there is no evidence that the addition of an antibiotic solution improves the outcome in patients compared with those who already receive perioperatively systemic antibiotics. Lally *et al.* found no difference in survival or residual abscess formation when antibiotics were added to the lavage solutions.[51] In other studies the addition of antibiotics to the lavage was shown to improve the results.[52,53] However, antimicrobial peritoneal lavage in clinical practice appears to have little effect on serosally adherent bacteria.[15] A number of previous reports also describe effects of antibiotics on phagocytic cell functions.[54] Some antibiotics augmented the respiratory burst of neutrophils and may therefore interfere with the ability of the host to control microbial growth. Many authors warned for the adverse effects that can occur after peritoneal lavage with antibiotics, such as: irritation, formation of adhesions, allergy, antibiotic-induced endotoxin release, and increased catabolism.[19,55-58]

Prevention of adhesion formation

Peritoneal adhesion formation is the primary cause of small bowel obstruction and female infertility. According to Menzies and Ellis, adhesions develop with an incidence of more than 90% after laparotomy.[59] In addition to the anatomy of the peritoneum, knowledge of pathophysiology and peritoneal wound healing is necessary to intercede in the development of adhesions (see page 12). Theoretically there are several methods to prevent adhesions after surgery. In 1993, a survey in the United Kingdom demonstrated that 68% of the general surgeons questioned performed some kind of peritoneal lavage in an attempt to prevent adhesion formation.[60] The large number of drugs given topically has been reviewed by Holz,[61] who concluded that no fully successful means of preventing peritoneal adhesions has yet been discovered. Mechanical as well as enzymatic removal of the fibrin that had already formed was not effective in reducing the incidence of adhesions.[62,63] More viscous solutions have a greater ability to separate the injured surfaces and appear to prevent adhesion formation in this way.[64] Ringer's lactate instillation in rats was studied by several groups,[65,66] but to date this has not led to an unambiguous conclusion. Polyvinylpyrrolidone (PVP) has also been widely used to prevent adhesions.[67-69] Gilmore tested a new PVP-I/PVP solution in an experimental rat model, in which adhesions were induced by a standard peritoneal defect and suturing. He found highly significant anti-adhesive effects in rats treated with PVP-I/PVP peroperatively and attributed this effect to PVP by acting as a visceral lubricant.[18]

Investigators subsequently showed that the serosa itself possesses pronounced fibrinolytic activity,[70] while Whitaker *et al.*[71] demonstrated that pure cultures of mesothelial cells were capable of fibrinolysis (*figure 1*, page 24). Moreover, many studies have proven that various forms of experimental injury to the mesothelium greatly reduce its fibrinolytic potential.[72-74] It also appeared that keeping the serosa moist does not prevent a significant impairment of fibrinolytic function.[75] Antibody inhibition studies and antigenic immunoassays have shown clearly that tissue plasminogen activator (tPA) is the major plasminogen activator in human peritoneal biopsies.[76,77] Attempts were made to stimulate the lysis of fibrin and prevent serosal adhesions by instillation of plasminogen activators[78-80] or plasmin.[81] However, the loss of plasminogen-activating activity was particularly associated with a dramatic increase in peritoneal plasminogen activator inhibitor (PAI-1) levels during

the early period after surgery.[82] The identification of the cells that produce PAI has been performed by in situ mRNA hybridisation.[83] These studies confirmed that the mesothelium plays a critical role in the inhibition of peritoneal fibrinolysis following injury.

Fibrous adhesions especially result from denudation of peritoneal surfaces,[84] from the presence of intra-abdominal ischaemic tissue,[85] or as a reaction to a variety of foreign materials introduced into the peritoneum by the surgeon.[75,86,87] During these conditions, human peritoneum shows a marked loss of peritoneal plasminogen-activating activity.[76,84,88] Inflamed or injured peritoneum produces inflammatory cytokines, which stimulate mesothelial cells to produce PAI-1.[89] In this way it is clear that the inflammatory and fibrinolytic pathways are closely linked. Excessive inflammatory reaction during the postoperative phase may affect adhesion formation via two mechanisms. Firstly, enhanced procoagulant activity and increased production of the inhibitor of fibrinolysis (PAI-1) by mesothelial cells. Secondly, increased tissue injury to undamaged mesothelium as a consequence of increased neutrophil influx and activity. If normal fibrinolytic activity is preserved or recovers quickly, fibrous depositions are lysed and permanent adhesions are avoided. Prevention may be attempted at any stage in the pathological sequence, from minimising injury and inflammation via anticoagulation to fibrinolytic enhancement. Thus, although the use of various solutions to clean the abdominal cavity is a widespread procedure, its effects on the most active fibrinolytic layer, the mesothelial lining, has not been studied.

Oncological surgery

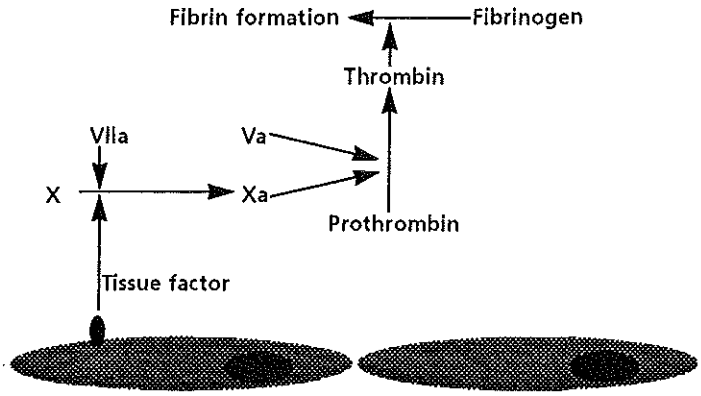
After elective colorectal operations, infections of the incisional wound and peritoneal cavity may originate from bacteria of the resected bowel. Peroperative lavage during colonic resection for a malignancy is not only a procedure to clear faecal contamination,[7] but is also considered as a procedure to eliminate tumour spilling. Peritoneal washings during colorectal surgery for cancer showed positive cytology in various studies.[90-93] By handling the tumour during operation, cancer cells may be disseminated and cause local peritoneal tumour recurrence. The hope for success of peroperative lavage lies in the supposition that malignant cells might be vulnerable to an instilled cytotoxic agent and that this may prevent loose cells to adhere to the peritoneum.

In search of tumouricidal agents that could be applied locally within the operative area Nash described the effectiveness of proflavine hemisulfate.[94] Distilled water,[95] hydrogen peroxide,[96] Dakin's (sodium hypochlorite) solution,[45,46] chlorhexidine,[97,98] and noxytiolin[99] are also suggested solutions in oncological abdominal surgery. The follow-up report by Arons and Smith of patients with or without operative wound washings, however, revealed a poor correlation between the finding of tumour cells in wound washings and subsequent local recurrence.[100] Also Murthy observed that increased cell delivery itself is not sufficient for tumour cell attachment.[101] Before free malignant cells can invade and grow into clinical recurrence, they must first attach to underlying tissue. Cell attachment is a complex process that involves special conditions for successful implantation and growth.[102,103] Clinical and experimental observations suggest that trauma induced by chemical,[104,105] mechanical[106,107] or surgical[108-110] means may promote tumour metastasis. This leads to the speculation that micro-environmental conditions and not local tumour cell contamination at the time of surgery and during the postoperative period are prognostically significant. The effects of exposure of the peritoneum to lavage solutions on microenvironmental conditions and local tumour adhesion and implantation is not known.

Local pathophysiologic responses

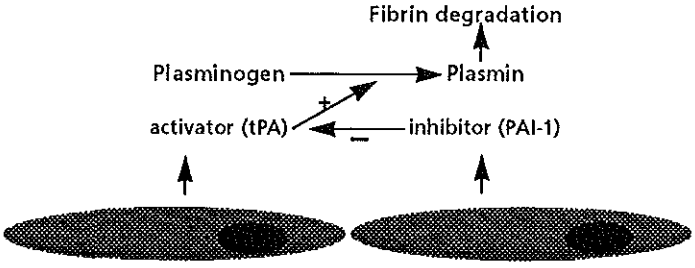
The peritoneum is a smooth layer of flat mesothelial cells resting on a basement membrane with a deeper layer of vascularized connective tissue. This layer covers the intestinal organs as the visceral peritoneum and the abdominal cavity as the parietal peritoneum. In adults, its surface measures about 1.7 m^2. Most of this surface behaves as a passive semipermeable membrane for the exchange of water and solutes of small molecular weight. The human peritoneal cavity exists as a potential space with no more than 50 ml of clear, sterile fluid with a low specific gravity (<1.016) and low protein content (usually <3 g/dl). Fibrinogen is not present and, therefore, serous fluid will not clot.[111] Cells contained in the peritoneal cavity, together with the mesothelium, play a crucial role in the maintenance of sterility of the peritoneal cavity and the host defence against intra-abdominal infections.

Figure 1. Extrinsic cascade of coagulation activated by mesothelial cells



X = factor X, Xa = activated factor X, VIIa = activated factor VII, Va = acitvated factor V.

Figure 2. Components of the fibrinolytic system and their effects upon fibrin



PAI-I = plasminogen activator, tPA = tissue type plasminogen activator.

The local inflammatory response of the peritoneum is similar to other tissues, but the peritoneal lining presents a large exudative and absorptive surface. At sites of irritation, there is an outpouring of fluid into the peritoneal cavity that, in contrast to normal serous fluid, has a high protein content (>3 g/dl) and contain many cells, primarily granulocytes, which phagocytize and kill micro-organisms. The exudate contains fibrinogen that polymerizes to solid fibrin by local tissue factor (*figure 2*). Plaques of fibrinous exudate

form on the inflamed peritoneal surfaces. This exudate glues adjacent bowel, mesentery, and omentum to each other. The omentum is particularly involved in this process of localizing the infection, which is further aided by inhibition of motility in involved intestinal loops. The inflammatory process also results in a marked depression of the fibrinolytic system of the peritoneal mesothelium. Fibrin formation is one of the peritoneal defences that has to control the local inflammatory process (*table 1*).[112,113]

Table 1. Peritoneal defence mechanisms against foreign material.

1. absorption into diaphragmatic lymphatics
2. cellular and humoral mechanisms
3. localization by fibrinous adhesions and fibrin formation

Normal peritoneal tissue has measurable levels of plasminogen, which can be converted to plasmin and is then able to rapidly lyse fibrin deposits in the peritoneal cavity (*figure 1*). Numerous factors, including infection, trauma, and sterile irritants reduce these tissue plasminogen levels to below detectable levels. It has been shown that this reduction in functional fibrinolytic activity is mainly mediated by induction of PAI-1 synthesis.[77] As a result of a disbalance in fibrinolytic enzymes, fibrin deposits may remain *in situ*.

First line cellular defence

In the early phase after tissue injury or contamination non-specific defence mechanisms plays a primary role.[114] Major intraperitoneal tissue injury is commonly followed by widespread activation of the white cell population. Whithin 24 hours a rapid influx of PMN occurs in response to a variety of stimuli, including products of bacterial growth and several inflammatory mediators.[115] The peritoneum usually contains fewer than 300 cells per mm^3, mostly macrophages plus some desquamated mesothelial cells and lymphocytes. Abdominal surgery, even in the absence of gross contamination or infection, elicits a rapid and transient influx of PMN, which may raise their numbers[116] to more than 3000 per mm^3. Following the PMN response, the peritoneal cavity is invaded by mononuclear cells, which scavenge the cavity by removing any remaining cellular debris. Degranulation of peritoneal mast cells releases vasoactive substances and leads to influx of complement and opsonins, which coat bacteria and promote phagocytosis.

and killing of micro-organisms by neutrophils is essential for host survival after any serious contamination of the peritoneal cavity. However, the neutrophil has acting also paradoxical effect on the clinical outcome in inflammation. Neutrophil enzymes are capable of digesting all living tissue. Autodigestion by these enzymes has been shown by Ohlsson, who found large amounts of neutrophil collagenase and elastase in the peritoneal fluid of patients with diffuse peritonitis.[117] This process is responsible for the release of massively increased quantities of several inflammatory mediators that may spill over into the general circulation to exert systemic effects. Large quantities of neutrophils undergo stimulation by complement split products, cytokines and bacteria with a consecutive release of both lysosomal proteinases and oxygen metabolites. Under these circumstances normal inflammatory defences become detrimental to the host. Increased accumulation of neutrophils via chemotactic factors and a consequent release of proteases might propagate the inflammation and injury of tissue. This model has also been suggested in other focal infections such as pulmonary inflammation[118] and meningitis[119].

Coincidentally with the appearance of large numbers of PMN, changes in mesothelial cells are observed.[120] The presence of PMN within the exudate over an extended period in the peritoneal cavity, and prolonged release of cytotoxic products may cause continuing injury to mesothelium.[121] Products of neutrophils, such as oxygen free radicals and proteases, have been shown to induce endothelial cell lysis and detachment *in vitro*.[122] It is known that PMN exert similar effects on cultured mesothelial cells.[123,124] Activated PMN firmly adhere to mesothelial cells and this adhesion is associated with observed mesothelial injury.[125]

Interaction between peritoneal cells

A large number of studies on chemically induced peritonitis have shown that mesothelial cells become rounded and separated from each other, allowing neutrophils and macrophages to penetrate the mesothelial intercellular space between lining cells.[126] In addition, peritoneal cells appear as aggregated cells and a large numbers of neutrophils and macrophages adhere to the mesothelium. The molecules that facilitate interactions between cells and between cells and tissue matrix are known as cell adhesion molecules. If cells are to interact with one another, two complementary molecules are required, the

adhesion molecule and its ligand. Under unstimulated conditions, neutrophils do not attach to other cells. In response to chemotactic factors and cytokines released in the local environment, leucocyte adhesion molecules are activated for subsequent interactions with mesothelial ligands.[127,128]

In the field of surgery a variety of pathological conditions exist that are associated with specific function or dysfunction of adhesion molecules. These are inflammatory processes, ischaemia-reperfusion, transplant rejection, arteriosclerosis and thrombosis, and tumour progression and metastasis.[129] Leucocyte-endothelial cell adhesion molecules have been studied extensively during the last years.[130] However, the molecules that mediate subsequent leucocyte entry into non-vascular spaces, such as the abdominal cavity during states of peritoneal inflammation, have not been identified. Because the peritoneal mesothelial lining represents the final barrier to leucocyte migration into the abdomen, it is likely that adhesion molecules expressed by mesothelial cells are involved in this process (*table 2*).[127,129,131,132]

Table 2. Adhesion molecules associated with the peritoneum [127-131]

Adhesion molecule	Cells expressing adhesion molecule	ligand	cells expressing ligand
ICAM-1 (CD54)	MC, leucocytes	Mac-1 (CD11b/CD18)	leucocytes
VCAM-1 (CD106)	MC, leucocytes	VLA-4 (CD29/CD49d)	leucocytes
CD44	MC, PMN	hyaluronate, collagen	extracell. matrix
VLA-4 (CD29/CD49d)	monocytes	VCAM-1, fibronectin	MC,extracell. matrix

MC= mesothelial cell; PMN= polymorphonuclear neutrophil; CD= cell determinant; ICAM= intercellular adhesion molecule; VCAM= vascular cell adhesion molecule; VLA= very late antigen.

Two main groups of surface molecules that are involved in this leucocyte interaction have been identified. The first group is the integrin superfamily, of which the most important leucocyte adhesion molecule involved is the CD11/CD18 adhesion molecule complex on leucocytes.[133,134] One ligand for

CD11/CD18 mediated adhesion is the intercellular adhesion molecule-1 (ICAM-1), also located on mesothelial cells.[135,136] However, studies have shown that there is CD11/CD18, ICAM-1 independent interaction between PMN and mesothelial cells.[137] Another ligand of this superfamily that is known to mediate leucocyte attachment and transmigration through activated mesothelium is vascular cell adhesion molecule-1 (VCAM-1). It is still unclear whether VCAM-1 expression on mesothelial cells is constitutive or only can be induced after stimulation.[126,127,135,138] A second group of cell surface molecules are called selectins in which the endothelial cell adhesion molecule-1 (E-selectin) is thought to be involved in leucocyte attachment to endothelium during acute inflammation.[139] Other authors previously demonstrated that selectins are not present on mesothelial cells, and could not be induced by stimulation with tumour necrosis factor (TNF).[135,140]

Humoral defences: the role of cytokines

Surgical injury and infection stimulate the production of a variety of endogenous mediators.[141-143] These mediators initiate immune, haematologic and metabolic alterations that are integral to the response of the host to injury. Complement, cytokines and interferons (INF) mediate, via both paracrine and autocrine mechanisms, the non-specific host defence.[144,145] Cytokines are only one component of the complex inflammatory cascade, but an important and proximal one. They comprise a diverse group of proteins, which share a number of characteristic properties (*table 3*). The biologic activities and synergistic actions of these proteins in peritoneal repair has only partly been elucidated. In moderate quantities, these proteins confer beneficial effects important in combatting the results of injury. Exaggerated or prolonged production of cytokines, however, may produce detrimental effects and lead to a poor clinical outcome. A certain cytokine can even be either inhibitory or stimulatory, depending on concentration, the presence of other factors, and the receptor distribution on the cell membrane of the effector cell.

Badia[146] and Tsukada[147] detected the pro-inflammatory cytokines TNF, interleukin-1β (IL-1β) and interleukin-6 (IL-6) within the peritoneal exudate in response to laparotomy, whereas INF-γ was not detected. Local production of cytokines far exceeds the concentration measured in plasma, indicating a local inflammatory process and suggesting that cytokines do not

Table 3. Characteristic features of cytokines [144]

* Protein hormones active at very low concentrations (pg/ml).
* Produced during the effector stages of natural and specific immunity.
* Like other polypeptide hormones, they initiate action by binding to specific recetors on the surface of target cells, where cell division is sometimes regulated (i.e. function as growth factors)
* Produced by a diverse range of cells, at multiple sites and often have overlapping actions.
* Act on a wide range of different cells and often influence the synthesis of other-cytokines.
* Serve to mediate and regulate immune and inflammatory responses and have important autocrine, paracrine and endocrine actions.
* Invoke sometimes slow cellular responses (4-6 hours) involving new protein synthesis.

indicating a local inflammatory process and suggesting that cytokines do not equilibrate between the peritoneal cavity and plasma.[148] The discrepancy between the cytokine concentrations in plasma and peritoneal fluid suggests that this response originates within the peritoneal cavity. These mediators (TNF, IL-1 and IL-6) are produced by peritoneal cells and induce the production of IL-8.[149,150] It has also been shown recently that when mesothelial cells are stimulated, their release of IL-8 is polarized and more to the apical side of the cell (intra-abdominal) than to the basal side.[150] This cytokine contributes to the influx of neutrophils by increasing their adhesion to endothelial and mesothelial cells and by acting as a potent chemotactic factor.[151]

Local intraperitoneal cytokine production thus modulates the intraperitoneal inflammatory response, including neutrophil chemotaxis, activation of macrophages, initiation of processes for tissue repair and regeneration and may have important effects on the control of peritoneal coagulation and fibrinolysis.[152]

By far the most common causes of intraperitoneal adhesion formation are surgical intervention[153] and peritonitis of infectious origin[154,155]. The mechanisms by which these conditions induce adhesions have not been fully clarified. It is hypothesized that IL-1 and TNF are increased and exert local effects on the mesothelial lining. For instance, the production of the fibrinolytic activator (tPA) may decrease and the production of the fibrinolytic inhibitor (PAI-1) may increase, which will result in decrease of the fibrinolytic

activity in the peritoneal cavity. In this way, cytokines may influence adhesion formation.[156] Kaidi *et al.* observed that intravenous combined anti-TNF and IL-1 treatment decreased adhesion formation in a rat model.[157]

The duration and intensity of the inflammatory response are critical to carefully control tissue damage and facilitate the repair mechanisms involved in wound healing. For instance, transforming growth factor (TGF)-β, which displays many immunosuppressive activities, plays an important role in limiting the inflammatory response.[158] However, some authors found that TGF-β stimulated cultured mesothelial cells and also promoted adhesion formation *in vivo*.[159,160] Several factors play a regulatory role in the production of inflammatory cytokines, and more generally in immune response and cytotoxicity. Imbalance in synthesis and degradation of these proteins may result in a more complicated postoperative clinical course.

Factors detrimental to peritoneal defences

The host defence mechanisms are particularly important because of their role in the control of infections and tumours, both of which are apt to cause early or late complications following intra-abdominal surgery. The efficiency of the host defence mechanisms of the peritoneal cavity will be influenced by many factors, some of which will be generalized and will have effect on the whole organism, such as the response to shock or trauma. Others will be due to local factors within the peritoneum. For instance trauma, foreign body products (e.g. talc), haemoglobin, or bile are all known to have profound effects on peritoneal host defences. The adjuvant effect of haemoglobin on peritoneal infection was first considered by Davies and Yull.[8] Only free haemoglobin but not intact red blood cells produced this effect.[162] Intraperitoneal haemoglobin markedly reduced the migration of PMN in the peritoneal cavity.[163] It is also well known to surgeons that some patients can tolerate an intra-abdominal bile collection with minimal disturbance, whereas other patients will become rapidly and seriously ill. It is felt that subtle differences in the constitution of the bile have an important effect on the peritoneal response and can cause mild chemical peritonitis.[164] Bilirubin itself is not toxic and neither is cholesterol or lecithin. Bile acids, however, are toxic and have haemolytic activity.

Local factors that initiate and suppress peritoneal cell activation may prove to be important determinants of neutrophil function and local

immunity. Several studies have documented the effects of various antiseptic and antimicrobial agents on the cellular influx and cytotoxic reaction.[56,165-167] Abbuglosu reported that both PVP-I and saline induced a peritoneal PMN influx. The authors suggested that this increase in neutrophils probably resulted from irritation of the peritoneum by the lavage.[26] Hoda observed that in response to intraperitoneal injections of saline in rats the serosal cells over the spleen were focally denuded.[168] Occasional multilayering of serosal cells was observed, especially in the sections of omentum, spleen and intestine. Irritants caused a widespread denudation of surface cells with fibrinous and fibrous adhesions and foreign body granuloma formation. The cellular response was mainly PMN in the early stages, but after eight hours an alteration in ratio of PMN to mononuclear cells was observed. Sayek and co-au-thors described that saline did not alter the peritoneal bactericidal activity, phagocytic activity, chemotaxis and total cell counts at the end of four hours.[169] Topley and co-workers extensively studied the effect of continuous ambulatory peritoneal dialysis (CAPD) fluids on the function of peritoneal cells, neutrophils as well as mesothelial cells. They reported impaired cellular host defences in dialysis cycles as a result of the depressive effect of sodium lactate on resident neutrophils.[170] Mesothelial cells exposed to unused CAPD fluids became activated and induced IL-6 secretion. These effects were attributed to unphysiological composition. Their current data demonstrate improved mesothelial cell and PMN function (most functions were restored to control values) following exposure to bicarbonate fluids when compared to lactate buffered fluids.[171] Some authors reported that irrigants left in the peritoneal cavity inhibited phagocytosis.[28,113] Billing *et al.* observed that in peritoneal exudate bacterial opsonisation is poor and complement and immunoglobulins have been destroyed by proteinases. They studied the effect of local serum application in patients and found the peritoneal bacterial opsonization and elimination improved by restoration of the physiologic intra-abdominal milieu.[172,173] Thus, the vicious cycle of events during peritoneal inflammation may be interrupted by lavage of the peritoneum to remove harmful substances, inflammatory mediators, neutrophils and their proteolytic enzymes. At the same time this procedure may interfere with some of the natural defence mechanisms, in particular regulatory functions of peritoneal cells.

Ways of controlling host responses: immuno-modulation

Based on the pathophysiology there are multiple potential sites at which intervention can modulate the local system in favour of the host. In addition, diminishing overproduction of cytokines in response to a stimulus and inhibition of the recruitment of white blood cells into the peritoneal cavity may reduce the morbidity and mortality of surgical intra-abdominal interventions.[174] In this respect, the mesothelial cell appears to have the potential to amplify the inflammatory response by inducing IL-8 secretion to an even harmful extent.[175] The *in vitro* effects of IL-8 on neutrophils include trans-endothelial migration, adhesion molecule expression, chemotaxis, degranulation, and induction of a respiratory burst.[176-180] Neutralization of IL-8 with antibodies against IL-8 largely inhibited the neutrophil influx induced by endotoxin *in vivo*.[181] Because IL-8 is produced *de novo* in the presence of an inflammatory stimulus and not released from pre-existing stores, modulation of IL-8 production may provide a more promising pharmacologic target than manipulation of other chemoattractants. DeForge and co-workers observed IL-8 synthesis regulated by oxygen radicals and found oxidant radical scavengers to inhibit IL-8 expression at the level of transcription.[182,183] *In vitro* studies also demonstrated pentoxifylline to be a selective modulator of cytokine production. Production of TNF, IL-2 and IL-8 are inhibited by pentoxyfylline, while the production of IL-6 appears to be stimulated.

A correlation between generation of oxygen radicals in tissues following injury or inflammation and the subsequent infiltration of neutrophils that release more damaging products has already been observed.[184] Adjuvant therapeutic therapies should be directed at preventing non-specific tissue injury as a consequence of excessive inflammation. On the one hand, PMN-induced mesothelial injury is associated with PMN adhesion and this injury can be prevented by blocking the CD11/CD18 adhesion molecule complex located on activated PMN.[185] Definition of the molecules involved in the adhesive interactions between neutrophils and mesothelial cells provides insight into therapeutic approaches to regulate the neutrophil influx. On the other hand, the response to a hostile environment, although multifactorial, seems to evolve through a common pathway.[186] Mesothelial cell activation may initiate intracellular signaling events that converge upon a common pathway. One model recently described is the presumed involvement of reactive oxygen intermediates in the activation of an intracellular nuclear factor, the nuclear

factor-kappa B (NF-kB). This transcriptional factor activate the expression of genes involved in inflammatory, immune and acute phase responses.[187] A characteristic of NF-kB is that many different agents can induce its activity. Among these are viruses, bacterial LPS, protein synthesis inhibitors, oxygen radicals, and selected cytokines (TNF, IL-1).[188] The peritoneal mesothelium displays a considerable reactivity to infectious and tumour processes.[189] The expression and secretion of leukocyte-directed cytokines by mesothelial cells may represent a non-specific response to tissue injury. Augmentation of this response via, for instance, oxidative stress and the subsequent activation of NF-kB may intensify the inflammatory reaction. Because inflammatory reactions require *de novo* synthesis of many proteins, an agent that inhibits transcription or protein synthesis may have an anti-inflammatory effect.

The ideal lavage solution

In the postoperative course the overall result of the physiological defence systems and therapeutic manoeuvres determines the patient's situation. It recently has become clear that cytokines and other mediators produced by the host, are responsible for inflammation, sepsis and other complications following intra-abdominal infection.[143] Peroperative restoration of the physiologic intra-abdominal environment may be of more importance than bacterial elimination and can improve a favourable clinical course after abdominal surgery.[172,190,191] Also the finding described by Lores that normal saline solution compared with other lavage regimens (antiseptic as well as antimicrobial) was quite effective in decreasing bacterial colony counts suggests that the mechanical and dilutional factors of the lavage might be as important as the antibacterial action.[192]

Given the multi-faceted nature of peritoneal inflammation and in particular adhesion formation, it is obvious that a sequential approach is required to optimize the result of peritoneal repair and subsequently reduce adverse pathophysiological consequences that occur after intra-abdominal surgical intervention. Modulation of principal steps of the local inflammatory, coagulant and fibrinolytic cascade may be accomplished by administration of an ideal solution. Much effort has been put into research on

biocompatibility of peritoneal dialysis (CAPD) solutions. Nonphysiological composition, in particular the combined effects of low pH, lactate, hyperosmolality and high glucose concentration were found to adversely affect peritoneal cell viability and function.[193] Four main aspects have to be taken into account in the search for a solution for intra-abdominal instillation during surgical intervention.[194] It is hypothesized that this ideal solution should;

a) not be cytotoxic

b) limit the initial inflammatory response,

c) inhibit local coagulation, and

d) promote local fibrinolysis.

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Influence of peroperative lavage solutions on peritoneal defence mechanisms *in vitro*

1

Abstract

Irrigation of the abdominal cavity with peroperative lavage solutions may interfere with peritoneal host defence mechanisms. We examined the *in vitro* reaction of mesothelial regulatory function and PMN oxidative defence mechanism to incubation with seven commonly used lavage solutions. After exposure to diluted solutions, the following reactions were determined for mesothelial cell (MC) monolayers: *1.* toxicity (microscopy and LDH release) *2.* integrity (inulin passage) and *3.* activation (IL-8 production). PMN were studied for toxicity and superoxide anion release.

Incubation of MC monolayers with non-toxic concentrations of standard solutions increased the permeability of the monolayers to inulin and induced MC to release IL-8 (range 1.9 - 24.6 ng). Furthermore, changes in superoxide anion release by stimulated PMN after exposure to PVP-I, Taurolin and Dakin's solution in diluted concentrations indicated that the oxygen metabolism of these cells was modulated. We conclude that lavage solutions both poison and stimulate MC and neutrophils, and some solutions are more potent than others.

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Introduction

"Peritoneal toilet" is considered mandatory in the treatment of peritoneal contamination, while it diminishes debris, bacteria and toxins. The possibility that irrigation of the abdominal cavity interferes with peritoneal host defence mechanisms has been considered.[1-5] Peritoneal response to intra-abdominal contamination or injury involves the priming and activation of neutrophils and mononuclear phagocytes with the subsequent release of proinflammatory mediators. Recent studies have shown that the MC, lining the peritoneal cavity, contribute themselves significantly to pro-inflammatory events[6] and actively participate in the recruitment of neutrophils (PMN) into the peritoneal cavity.[7] *In vitro* experiments have shown that peritoneal MC synthesize interleukin-8 (IL-8).[6,8,9] The primary biological activity of IL-8 appears to be the induction of neutrophil chemotaxis[10], which is evident at very low concentrations. In addition, higher concentrations of IL-8 cause the neutrophil respiratory burst and degranulation.[11,12]

Peritoneal defence mechanisms may be induced by activation of the peritoneal membrane through chemical agents. Release of mediators such as IL-8 in response to irrigation of the peritoneal cavity with lavage solutions may contribute to neutrophil influx. In addition, an overshoot of the neutrophil influx, with high amounts of toxic neutrophil products, is associated with pathological changes seen in inflammation[13] and may in part determine the severity of peritonitis.[14]

An ideal lavage solution, therefore, should be non-cytotoxic, should not interfere with monolayer integrity and should not stimulate MC to synthesize the pro-inflammatory cytokine IL-8. Other functional properties of MC, such as contribution to local coagulation and fibrinolysis also should not be affected by lavage solutions. In this study we examined the effects of various lavage solutions on the viability and activation of human MC monolayers and PMN *in vitro*.

Materials and methods

Culture and identification of omental mesothelial cells. MC were obtained from the omental tissue of patients undergoing elective abdominal surgery who had given informed consent. The MC were isolated according to techniques modified from Nicholson *et al.*[15] and Wu *et al.*[16]. The omentum was transferred to fluid containing 0.05% trypsin-0.02% EDTA (Gibco, Paisley,

Scotland). After 15 min the detached MC were pelleted by centrifugation at 800 xg for 5 min and were resuspended in M-199 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Gibco), gentamicin (10 µg/ml; Merck, Darmstadt, FRG), vancomycin (25 µg/ml; Lederle, Etten-Leur, The Netherlands), amphotericin B (4 µg/ml; Bristol-Myers, Woerden,The Netherlands) and L-glutamine (2 mM; Gibco). MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (80 cm^2; Nunc, Roskilde, Denmark) precoated with fibronectin (gift of the Central Laboratory of the Netherlands Red Cross (CLB), Amsterdam, The Netherlands). The identity of MC was demonstrated by the absence of von Willebrand factor staining[17] and the presence of intracellular cytokeratins via immunofluorescence with monoclonal antibodies (Dakopatt, Glosstrup, Denmark).[18]

PMN isolation. Blood was obtained from healthy volunteers. Granulocytes were purified from a buffy coat by centrifugation over a Ficoll-Histopaque (Sigma Chemical Co, St Louis, MO) layer, then washed with RPMI-1640 medium with 0.5 % human serum albumin (HSA) at 4°C. This was followed by hypotonic lysis with sterile aqua bidest to remove erythrocytes. PMN were resuspended in RPMI-1640 medium with 0.5 % HSA to a final concentration of 10^6 cells per ml.

Exposure of cells to lavage solutions. Seven lavage solutions were studied, viz. NaCl 0.9%, Hartmann solution (Ca^{2+} 1.8 mmol/l, K^+ 3.2 mmol/l, Na^+ 146.5 mmol/l, lactate 3.2 mmol/l), Betadine 1% (povidone-iodine 100 g/ml diluted in NaCl 0.9%; Asta Medica BV, Diemen, The Netherlands), Dakin's solution 0.5% (NaOCl 5 mg/ml; OPG Farma, Utrecht, The Netherlands), Taurolin 0.5% (5 mg/ml taurolidine, 12.5 mg/ml povidone diluted in Ringer-solution, Geistlich-pharma, Wolhusen, Switzerland), chlorhexidine 0.02% (Vifor Medical SA, Huizen, The Netherlands) and hydrogen peroxide solution 1% (diluted in aqua dest; Merck, Darmstadt, Germany). Serial dilutions of the lavage solutions were prepared in M-199 for optimal pH (7.2-7.4) and osmolarity (280-300 mosmol/kg). Diluted antiseptic solutions were checked for minimal bactericidal concentration for *S. aureus* and *E. coli.*[19]

MC were seeded into 24-well culture plates (Costar, Cambridge, UK), precoated with fibronectin, and allowed to grow to confluency before use.

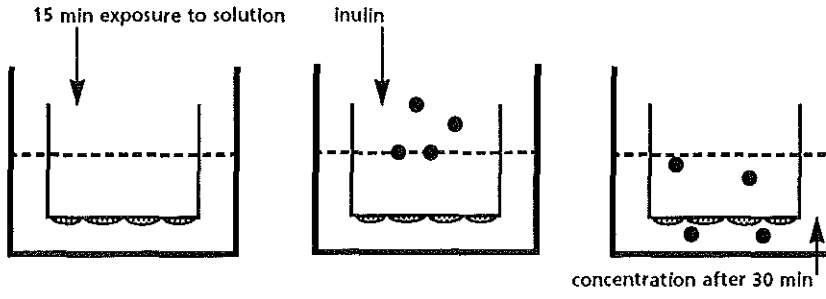
Medium was removed from the wells and replaced by 500 µl of lavage solutions. After 15 min of incubation at 37°C and 5% CO_2, fresh supplemented medium (500 µl) was added to replace the lavage solutions. Supernatants were collected at 24h, centrifuged (800 x*g* for 5 min) and stored at -70°C for quantitation of synthezised IL-8 by an enzyme-linked immunosorbent assay (CLB, Amsterdam, The Netherlands). All experiments were performed in triplicate. Interleukin-1β (2 ng/ml; R&D systems, Abingdon, UK) is known to stimulate the production of IL-8 by MC, and served in all experiments as a positive control. Lavage solutions and reagents used were free of detectable amounts of endotoxin, as was determined with an endotoxin assay (Sigma).

Assessment of mesothelial cell injury. MC toxicity was assessed by examining the monolayers under phase-contrast microscopy and by measurement of lactate dehydrogenase (LDH) release.[20] MC were seeded into 24-well culture plates and grown to confluent monolayers. Thereafter, the medium was removed, and the cells were incubated for 15 min with the various lavage solutions. After incubation, fresh medium without fetal calf serum (FCS) but with 1% bovine serum albumin (BSA) was added to the cells. The mesothelial monolayers were incubated with the medium for one hour, whereafter the medium was collected and centrifuged (800 x*g*) to remove dead cells. LDH activity in this medium (50 µl aliquots) and in cell lysate (5 µl aliquots) was assayed spectrophotometrically (Perkin-Elmer Lambda 2). The assay was performed according to Bergmeyer *et al.*[21] The LDH release was expressed as percentage of total (100%) cellular enzyme activity liberated from the cells by 1% (v/v) Triton-X100. The accuracy of LDH leakage as an indicator of lethal cell injury was confirmed by comparison with viability determinations based on trypan blue exclusion.[22] All experiments were performed in triplicate.

Assessment of mesothelial integrity. Permeability of ^{3}H-labeled inulin (Amersham, Amersham, USA) across MC monolayers was studied on inverted mesothelial monolayers, in the physiologically relevant direction (submesothelial tissue to abdominal cavity). MC were subcultured to confluent monolayers hanging on polycarbonate membranes (0.4-µm pore size, 24.5 mm diameter) in Transwell cell culture chambers (Costar, Cambridge, UK) according to Parkos *et al.*[23] with modifications.[24] MC were added to the inverted inserts and allowed to attach overnight. Thereafter, the inserts were

placed upright into 6-well culture dishes. The cultures were maintained for 5 days to reach confluency, as determined by phase-contrast microscopy and microscopy of May-Grünwald/Giemsa stained filters. Inverted monolayers were preincubated for 15 min with various lavage solutions (diluted concentrations) added to the lower compartment. Media in both compartments were removed and replaced with M-199 containing 0.5% BSA. After 60 min, cell culture media (1.5 ml M-199 with 0.5% BSA) containing radiolabeled inulin (0.2 µl/ml) was added to the upper compartment of the Transwell system and incubated for 30 min at 37°C in a 5% CO_2 atmosphere (*figure 1*). Aliquots of upper (1.5 ml) and lower (2.5 ml) compartments were sampled. The amount of radioactivity present in the fluids as well as that of the filter, cut out of its cylindric container, were determined in a gamma counter. Recovery was always > 92%. Transwell filters coated with fibronectin without MC monolayers served as control. Data are expressed in terms of percentage inulin (of total 100%) at the apical side of the monolayer.

Figure 1. Inverted mesothelial monolayers in a Transwell system



Mesothelial monlayers were incubated for 15 min with various solutions. Thereafter, solutions were removed and the radiolabeled inulin was added to the upper compartment and incubated for 30 min. Aliquots were sampled of upper and lower compartments to determine the amount of radioactivity present.

Assessment of PMN viability and activity. Experiments were performed to investigate the effect of lavage solutions on the survival of PMN leukocytes and their potency to produce oxygen radicals. To determine PMN viability we used propidium iodide (PI).[25] PMN suspensions (100 µl; 10^6 cells/ml) were

incubated for 15 min at 37°C in 0.5 ml of lavage solution. PI (10 µl; final concentration 6 mg/l) was then added. The percentage of viable cells after exposure to lavage solutions was determined by flow cytometry (Becton Dickinson). Nonviable cells will take up PI and exhibit fluorescence. The percentage of nonviable cells was calculated electronically.

The chemiluminescence assay described by Mills *et al.*[26] was modified and was used to measure the oxidative metabolic responses of human PMN. Isolated PMN, phorbol myristrate acetate (PMA;50 ng/ml) and luminol (0.03 mmol/ml) were all diluted in Hanks' balanced salt solution containing 1% gelatin (G/HBSS). PMN suspensions (100 µl; 10^6 cells/ml) were added to 900 µl of diluted lavage solutions and were incubated for 15 min at 37°C. Hundred µl of this cell suspension were diluted in 700 µl of G/HBSS containing 100 µl of luminol. After stimulation with 100 µl of PMA, counts were obtained every two min for 30 min at 37°C. PMN suspensions not stimulated with PMA served as controls for each experiment. PMN luminol-enhanced chemiluminescence (LCL) was expressed as the percentage of mean luminescence relative to PMA-stimulated PMN not incubated with lavage solutions (= 100%). All assays were performed in triplicate at three different occasions.

Statistical analysis. Results were expressed as mean and standard deviation (SD). The data were submitted to statistical analysis with the Student-*t* test where groups were compared with the control, $p < 0.05$ was considered significant.

Results

I. Viability and activation of mesothelial monolayers after exposure to various lavage solutions.

Mesothelial viability. MC monolayers rapidly respond to a variety of stimuli by contraction, rounding up and separation from neighbouring cells and from the underlaying extracellular matrix (*figure 2*). All antiseptic solutions in standard concentration (recommended by the manufacturers) were 100% lethal for MC monolayers *in vitro*. Serial dilutions were studied for all solutions. A diluted lavage solution was defined as "non-toxic" when it left a MC monolayer intact as judged by phase-contrast microscopy and a percentage LDH activity comparable with monolayers exposed to medium without serum (*table 2*). After exposure to lavage solutions in non-toxic concentrations, a

characteristic morphologic change in cell shape was seen in light microscopy. MC appeared as loosely aggregated cells with a filamentous meshwork between the cells (*figure 3*). Usually, non-toxic dilutions still showed bactericidal activity (*table 1*).

Table 1. Standard solutions and dilutions

name	pH	osmolarity	highest dilution tested	MBC
	of the standard solution			
NaCl 0.9%	7.3	302	1	-
Hartmann solution	6.7	304	1	-
Povidone-iodine 1%	5.4	352	1:100	1:250
Dakin's solution 0.5%	9.2	458	1:200	1:50
Taurolin 0.5%	8.0	252	1:10	1:50
chlorhexidine 0.02%	5.6	252	1:10	1:250
hydrogen peroxide 1%	4.2	1002	1:100,000	1:15,000

Serial dilution of the standard lavage solutions was performed with medium (M-199). After dilution, all solutions had optimal pH (7.2-7.4) and osmolarity (280-300 mosmol/kg). An intact MC monolayer in phase-contrast microscopy was the criterion for the highest dilution tested. Minimal bactericidal concentration (MBC) for diluted solutions was determined with *S. aureus* and with *E. coli*.

MC monolayers exposed to culture medium supplemented with 10% serum suffered less injury (*tabel 2*; 5.3% release). Exposure to culture medium without serum increased the percentage of LDH release significantly (13.6%). MC monolayers were injured even by exposure to diluted lavage solutions as indicated by release of the cytosolic enzyme LDH from the cells. The physiological salt solutions caused about 20% cell death. To create more buffer capacity, Na-bicarbonate or HEPES was added. However, no benificial effects on LDH release were noted. Addition of 1% BSA to NaCl 0.9% or Hartmann solution reduced LDH release slightly (data not shown). PVP-I in diluted concentrations was less irritating and did not affect LDH significantly compared with supplemented culture medium (10% FCS).

Table 2. Mesothelial viability (%LDH) and monolayer integrity (% inulin passage)

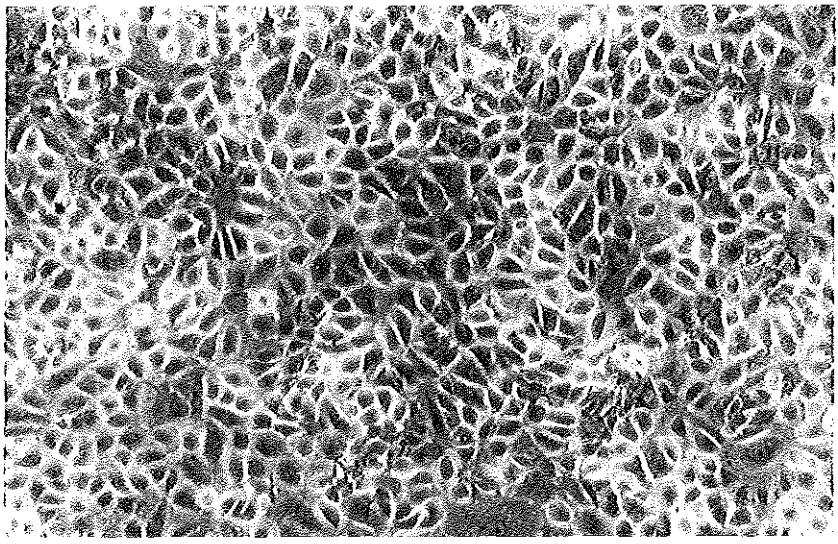
	% LDH (SD)		% inulin (SD)	
culture medium	5.3	(0.9)	17.6	(2.2)
without serum	13.6*	(1.0)	21.4	(2.9)
bare filter	--		35.1	(2.3)
NaCl 0.9%	22.2*	(4.1)	21.4	(7.3)
Hartmann	17.0*	(5.1)	20.6	(5.9)
PVP-I 1:100	13.1*	(1.0)	22.7*	(4.4)
Dakin's 1:200	14.9*	(2.1)	22.1	(6.5)
Taurolin 1:10	20.0*	(2.8)	24.6*	(2.5)
chlorhex 1:10	12.7*	(0.9)	23.7*	(3.7)
H_2O_2 1:100,000	16.6*	(1.8)	22.6*	(3.9)

Release of LDH from MC and inulin permeability of MC monolayers exposed to culture medium or to different kinds of (diluted) lavage solutions for 15 min. Dilutions were made with medium M-199. The data are presented as the percentage LDH released divided by total LDH (100%) and as percentage inulin after 30 min at the apical side of the monolayer. Every result of %LDH is the mean and (SD) of three different measurements in triplicate. Results of %inulin are the mean and (SD) of four separate experiments. *Indicates statistically significant difference ($p< 0.05$) compared to supplemented medium.

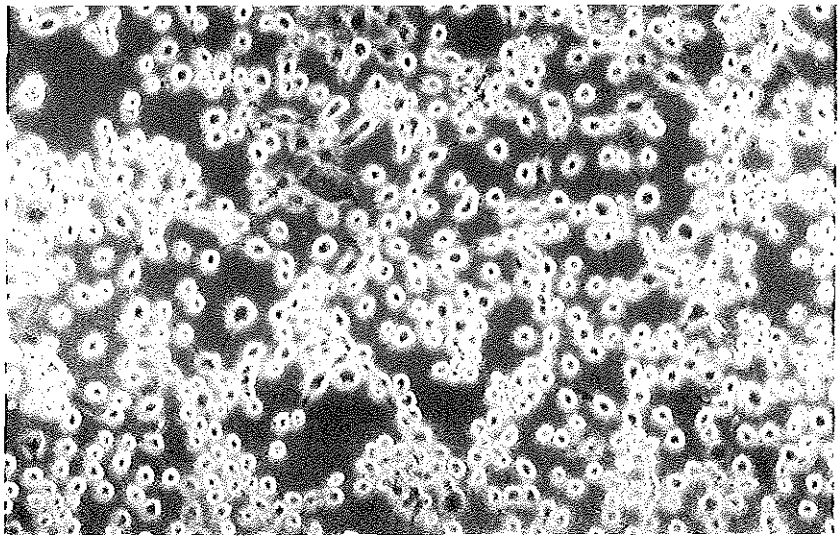
Monolayer integrity. Table 2 also illustrates the permeability of inulin across the micropore filter in the absence or in the presence of pretreated MC monolayers. A bare filter (precoated with fibronectin but without cells) allows unrestricted passage of inulin, which after 30 min amounts to 35% (of total 37.5%). Intact MC monolayers exposed to medium supplemented with serum restrict the passage of inulin to 17.6%. Although non-toxic dilutions were used, permeability of the macromolecule inulin as an index of tight junction integrity was increased by all lavage solutions. Noteworthy, the salt solutions (NaCl 0.9% and Hartmann) which caused relatively high amounts of LDH release, did not induce significant increase in the permeability of inulin.

Figure 2.

a.

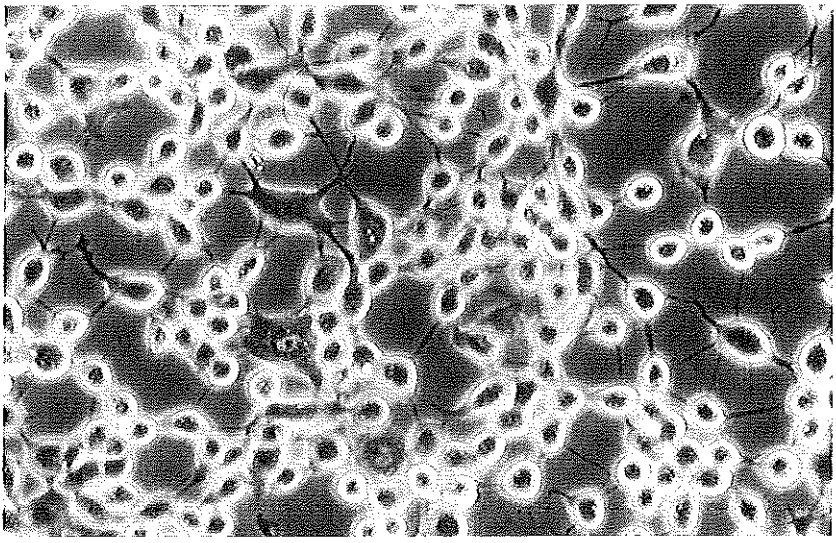


b.



(a) Phasecontrast microscopy of adherent MC in control medium (x 200) and *(b)* after exposure to hydrogen peroxide 1% (1:1000). MC react with contraction, rounding up, and separation from neighbouring cells and from the underlaying matrix. (x 200).

Figure 3.

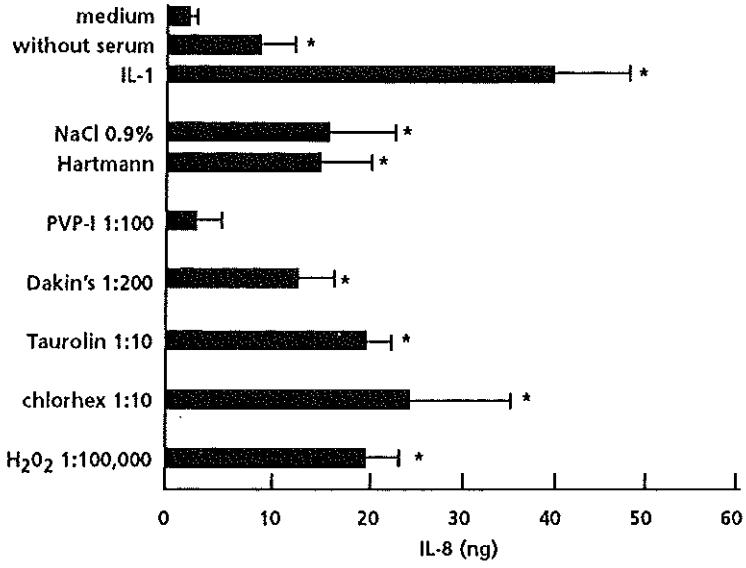


Phasecontrast microscopy of an MC monolayer after 1h exposed to Hartmann solution for 15 min (x 400). Note the cell shape change and filamentous network formation between cells.

IL-8 release. Unstimulated cultured mesothelial monolayers released small amounts of IL-8 (0.8 ±0.4 ng) during 24h incubation with supplemented medium (10% fetal calf serum). IL-8 release of MC after exposure to medium without serum was significantly enhanced (7.3 ±4.4 ng). Stimulation of MC monolayers with IL-1β (2 ng/ml) induced the release of 38.3 ±9.7 ng of IL-8. The release of IL-8 in response to diluted lavage solutions rose significantly ($p < 0.05$) above background levels (*figure 4*). However, these solutions failed to increase IL-8 release significantly compared to medium without serum.

It was notable that after PVP-I (1:100) incubation, the IL-8 concentration was less enhanced compared with the other solutions. Hydrogen peroxide in micromolar concentrations still induced IL-8 release. To exclude neutralization or interference of the test solution with IL-8 measurement, an IL-8 standard concentration was incubated for one hour with the test solutions. This treatment had no effect on the amount of IL-8 measured.

Figure 4. Secretion of IL-8 by confluent monolayers of MC



Dose-effect of various lavage solutions on the 24h release of IL-8. In each well, about 100,000 MC cells were present in a volume of 0.5 ml. Stimulation with IL-1β (2 ng/ml) strongly induced IL-8 release (38.3 ng). The data presented are the mean and (SD) from three separate experiments in triplo with MC prepared from separate omental specimens. *Indicates statistical significant difference ($p < 0.05$) versus the medium with serum (10%) value.

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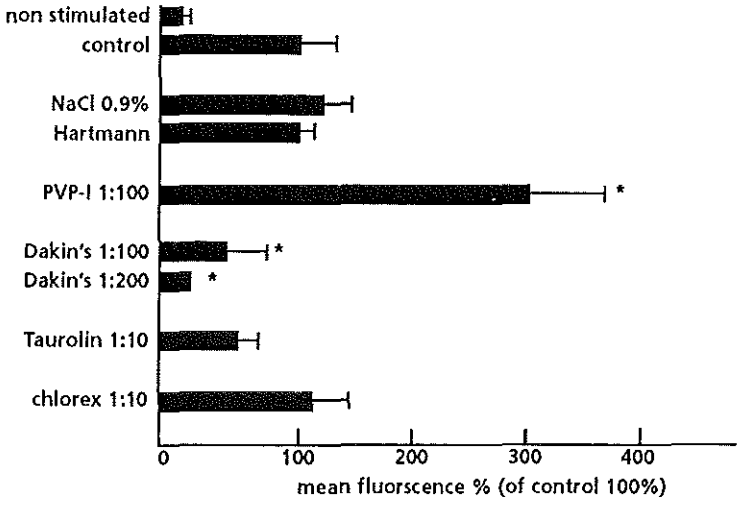
II. Influence of lavage solutions on viability of PMN and on activation of the respiratory burst.

PMN were resistent to higher concentrations of all lavage solutions than were MC monolayers. The use of PI and flow cytometry showed that PVP-I (standard 1% and 1:10) caused over 95% PMN death. Analysis after exposure to modified Dakin's solution 0.5% (1:10) showed that more than 40% of the PMN were non viable. Most PMN incubated in undiluted Taurolin (0.5%) and chlorhexidine (0.02%) exhibited loss of normal forward and side scatter by flow cytometry, indicating changes in cell shape and surface. Only concentrations in which PMN were viable (> 95%) and had a normal scatter pattern, were tested for their effect on for superoxide anion responses. Hydrogen peroxide was not tested.

Figure 5. Effect of lavage solutions on PMN respiratory burst



Human PMN were incubated with various solutions and were stimulated with PMA. The respiratory burst was assayed by luminol-dependent chemiluminescence. PMN stimulated with PMA exposed to Hanks' balanced salt solution served as a negative control in each experiment. Results are expressed as percentage mean fluorescence (MF) relative to PMA-stimulated PMN not incubated with lavage solutions (control = 100%). Data are derived from four separate experiments (SD). *Statistical significant difference ($p < 0.05$) compared to the control value.

The results of the PMN assay for oxidative burst activity are given in Figure 5. Mean fluorescence (MF) of PMN stimulated with PMA in the presence of various concentrations of each agent was compared with the MF of control, i.e. PMA-stimulated PMN. Pre-exposure to NaCl 0.9% or Hartmann solution did not significantly affect PMA-induced CL. Preincubation with diluted PVP-I resulted in increased PMA-induced, luminol-dependent CL ($p < 0.05$). To investigate the effect of PVP-I on luminol itself, we studied the effect in the absence of cells. PVP-I did not affect LCL. To test whether PVP-I itself has direct effect on the PMN oxidative burst, we studied the effect on PMN CL in the absence of PMA. PVP-I did not stimulate PMN. Thus, PVP-I seems to augment PMA-induced stimulation of the PMN oxidative burst, but has no PMN-stimulating properties by itself. Any significant effect of PVP-I on the CL of a non-cellular superoxide generating system (xanthine/xanthine-oxidase) was not found. Diluted chlorhexidine also

enhanced LCL, but this increase was not significant. In contrast, Taurolin caused a dose-dependent decrease in PMA-induced, luminol-dependent PMN CL (p= 0.069, NS). Dakin's solution also inhibited luminol-dependent PMN CL. Unexpectedly, at lower concentrations of Dakin's solution (1:100) the luminol CL of viable cells was more depressed than at higher concentrations of Dakin's (1:50). Addition of Dakin's solution (0.5%) to xanthine/xanthine-oxidase enhanced luminol CL. This effect was significant at Dakin's 1:10, while no effect on the CL was found at Dakin's 1:100.

Discussion

Despite its widespread use, neither efficacy nor safety of peroperative lavage with electrolyte or antiseptic solutions has been established.[27-29] Although lavage is used under the old adagium "dilution is the solution to the pollution" and may reduce the number of bacteria in the abdominal cavity,[30] this procedure does not remove bacteria adherent to the mesothelial surface.[31] In addition, it has been suggested that the outcome of intra-abdominal infection depends more on the host response than on the pathogens involved.[32,33] Postoperative restoration of physiological local defence systems seems to be a major determinant for the clinical outcome. Toxicity of some solutions for tissue cells and interference of solutions with the defence mechanisms of the peritoneal cavity may delay restoration of normal physiology in the abdominal cavity and thus contribute to infection-like reactions. Recent studies have shown that some components of the immune system are activated after operation, which correlates with the risk for sepsis.[34] Intra-abdominal injury and infection initiate a cascade of events that results in major alterations in the immunological and hematological host response. Cytokines have beneficial properties in injury at low concentrations. However, an exaggerated or prolonged secretion of these mediators may be detrimental to the host. Release of pro-inflammatory cytokines in response to peritoneal lavage solutions may thus lead to decreased resistance to infection.

During the initial stage of peritonitis the peritoneum, in particular the mesothelial layer, acts as a barrier to infection. Evidence is accumulating that the mesothelium itself plays an active role in pathologic conditions of the peritoneum, such as inflammation, fibrous adhesion and tumour implantation. MC participate in local immunologic responses,[6,9] coagulation[16] and fibrinolytic homeostasis[35,36]. In response to peritoneal trauma the

mesothelium is actively involved in the process of transmesothelial migration of neutrophils.[37] Enhanced PMN activation can be detrimental to MC and can activate MC to synthesize IL-8.[38] Chemotactically stimulated PMN may also amplify the recruitment process of PMN to the inflammatory site by releasing IL-8 themselves. Wakefield *et al.*[39] demonstrated that in patients destined to develop postsurgical sepsis there is both phenotypic and functional evidence of neutrophil activation within 24h of surgery. So, factors that contribute to limiting peroperative immunoactivation seem to be crucial for an uneventful postoperative course.

The experiments in the present study demonstrate that human omental MC undergo important changes in function and physiology after interaction with various peritoneal lavage solutions *in vitro*. Firstly, the solutions used for intraperitoneal lavages are directly toxic to MC monolayers as observed by phase-contrast microscopy and quantified by LDH release. Commonly used NaCl 0.9% and Hartmann solution also induced a relatively high percentage of cellular injury. Addition of serum or albumin should reduce LDH release significantly. Secondly, MC monolayer integrity was also affected. Increased inulin permeability induced after exposure to lavage solutions was not due to cell loss, because non-toxic concentrations of these solutions (i.e. leaving the MC monolayer microscopically intact) also induced increased inulin permeability. Inulin passes a MC monolayer only intercellularly, and permeability is enhanced if there is any alteration in tight junctional permeability.[40] Tight junctions are structures involved in cellular apical-basal polarity, and thereby maintain an optimal cytokine gradient.[41] *In vitro* studies also indicate that neutrophil diapedesis and transport of macromolecules occur through tight junctions.[42,43] Changes in the shape and cytoskeleton of injured cells may destroy the functional integrity of monolayers of cells and may cause increased influx of intraperitoneal fluid and PMN.[44]

Thirdly, our data show that lavage solutions can directly stimulate human MC to release IL-8 *in vitro*. A network of mediators is operational in inflammatory reactions and their production is interregulated. IL-8 synthesis by peritoneal MC may be induced in response to specific stimulation, while other agents may have anti-inflammatory effects by inhibition of cytokine synthesis.[45,46] Billing *et al.*[47] demonstrated that intra-abdominal serum application restores the host defence in patients. Low concentrations of hydrogen peroxide are toxic for MC and stimulate the release of IL-8. Serum is known

to inactivate hydrogen peroxide. *In vitro* exposure of MC to control medium supplemented with serum (10%) was less stimulating for MC than medium without serum.

Changes in superoxide anion release by stimulated PMN indicate that the various lavage solutions modulate the oxygen metabolism of PMN. This effect varies with the specific solutions. Diluted PVP-I highly stimulates superoxide release of activated PMN, whereas Taurolin and Dakin's solution inhibit this release. Similar observations were found when superoxide anion release was measured by ferricytochrome *c* (data not shown). Hansbrough and colleagues[25] found similar effects for modified Dakin's solution on the PMN respiratory burst. Although there is a direct effect on the dye, and the superoxide anion release of PMN after exposure to Dakin's solution is the sum of two different effects, we hypothese that all of the dilute concentrations of Dakin's are, in fact, inhibitory to PMN oxidative burst activity. The mechanisms governing these effects have not yet been defined and such effects could be either advantageous or deleterious to the host response. However, additional factors e.g. exposure to an intraperitoneally instilled solution that initiates or suppresses priming of neutrophils could also be important determinants of neutrophil function and local immunity.

These results are in accordance with earlier *in vivo* observations which demonstrated that after peritoneal lavage with Hartmann's solution serosal healing was delayed.[48] Tolhurst and coworkers observed morphologic changes of the MC layer in histological studies. Lavage with an electrolyte solution was also thought to be responsible for increase in the formation of adhesions.[49] Similar responses as we demonstrated *in vitro* may occur *in vivo* and account for modulation of postoperative local immunoactivity. If the early inflammatory response is excessive, inappropriate persistence of anti-inflammatory compensation will result in later immunosuppression. Therefore, dilution of the pollution may be not the solution, but a confusion to the abdominal defence system.

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Expression of cell adhesion molecules by cultured mesothelial cells after exposure to various peroperative lavage solutions

2

Abstract

Peroperative intraperitoneal lavage could cause injury to and activation of mesothelium, with disturbance of its normal regulatory functions. MC, lining the peritoneal cavity, contribute to local inflammation and cell adhesion processes. *In vivo* studies have shown enhanced expression of cell-associated molecules to play a major role in the pathophysiology of abdominal inflammatory processes. In this study, the expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on cultured MC was studied by flow cytometry 6h and 20h after short exposure to various lavage solutions. We observed significantly increased ICAM-1 expression on the MC membrane 6h after incubation with Taurolin and Dakin's solution in non-toxic dilutions. No changes in VCAM-1 expression were found after exposure to all tested lavage solutions. Irrigation of the peritoneal cavity thus disturbs mesothelial regulatory functions and may contribute to neutrophil-mediated damaging conditions and tumour cell adhesion.

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Introduction

Peroperative lavage has become a standard surgical procedure to clean the contaminated or potentially contaminated abdominal cavity. Peroperative lavage is applied to reduce the quantity of bacteria and to remove concurrent substances. In this way lavage is supposed to assist host defence mechanisms. However, toxicity of some solutions for tissue cells and interference of solutions with local defence mechanisms[1-4] may delay restoration of normal physiology in the abdominal cavity and thus contribute to infection-like reactions.

Because of the position peritoneal MC occupy in the body, they play an important role in peritoneal inflammation and the corresponding cell-cell interactions. MC contribute directly to pro-inflammatory events and are actively involved in the recruitment of neutrophils into the peritoneal cavity.[5,6] Recruitment of neutrophils to sites of inflammation occurs in several steps. Leucocyte migration towards inflammatory tissue is initially guided by locally produced chemoattractants. In previous studies we have shown that human MC undergo important changes in function and physiology after exposure to various lavage solutions.[4,7] Lavage solutions can stimulate human MC to synthesize IL-8. This pro-inflammatory cytokine is suggested to be a promotor of inflammation by functioning as a potent neutrophil chemoattractant and activator.[8] The second step, transmigration of leucocytes into the adjacent tissue is regulated through several specific adhesion molecules. This process involves a family of cell surface glycoproteins, the integrins, which are regulated by different cytokines[9-11] and induce changes of cell shape and cytoskeletal organization. Adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are known to mediate leukocyte attachment and transmigration through activated mesothelium.[12,13] In addition, the aim of the present study is to elucidate the profiles of adhesion molecule expression on MC after exposure to lavage solutions, in order to investigate the functional changes and the assumed contribution to neutrophil migration in more detail.

Materials and methods

Culture and identification of omental mesothelial cells. MC were obtained from the omental tissue of patients undergoing elective abdominal surgery who had given informed consent. The MC were isolated according to techniques

modified from Nicholson *et al.*[14] and Wu *et al.*[15] and cultured as described previously. MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (80 cm^2 Nunc, Roskilde, Denmark) precoated with fibronectin (gift of the Central Laboratory of the Netherlands Red Cross (CLB), Amsterdam, The Netherlands). The identity of MC was demonstrated by the absence of von Willebrand factor staining[16] and the presence of intracellular cytokeratins via immunofluorescence with monoclonal antibodies (Dakopatt, Denmark).[17]

Exposure of cells to lavage solutions. Seven (diluted) lavage solutions were studied: viz. NaCl 0.9%, Hartmann solution, Betadine 1% diluted 1:100 (Povidone-iodine 100 g/ml, Asta Medica BV, Diemen, The Netherlands), Dakin's solution 0.5% diluted 1:200 (NaClO 5 mg/ml, OPG Farma, Utrecht, The Netherlands), Taurolin 0.5% diluted 1:10 (5 mg/ml taurolidine, Geistlich-pharma, Wolhusen, Switzerland) chlorhexidine 0.02% diluted 1:10 (Vifor Medical SA, Huizen, The Netherlands) and hydrogen peroxide solution 1% diluted 1:100,000 (Merck, Darmstadt, Germany). Dilutions of lavage solutions were prepared in M-199 for optimal pH (7.2-7.4) and osmolarity (280-300 mosmol/kg).

MC were seeded into 24-well culture plates (Costar, Cambridge, UK), precoated with fibronectin, and allowed to grow to confluence before use. Medium was removed from the wells and replaced by 500 µl of lavage solutions. After 15 min of incubation at 37°C and 5% CO_2, fresh supplemented medium (500 µl) was added to replace the lavage solutions. Supernatants were collected at 24h, centrifuged (800 *xg*/5 min) and stored at -70°C.

*Determination of surface antigen expression.*The expression of the adhesion molecules ICAM-1 (CD54) and VCAM-1 (CD106) by MC upon activation was measured by indirect immunofluorescent stainung employing primairy monoclonal antibodies (mAb) to ICAM-1 and VCAM-1, both purchased from Sanbios (Uden, The Netherlands). MC were subcultured to confluent monolayers in 6-well culture dishes (Costar). The MC were activated by incubation with human recombinant interleukin-1β (10 ng/ml), or exposed to various lavage solutions for 15 min as described above. After 6 and 20h, the MC were non-enzymatically detached by incubation with PBS/EDTA (2 mM) for 20 min at 37°C. MC in suspension were washed twice in ice-cold

PBS and were subsequently incubated with the primary mAbs for 30 min at 4°C. Thereafter, the cells were washed twice in ice-cold PBS, containing 0.5% (v/v) bovine serum albumin (BSA), and the procedure was repeated with R-phycoerytherine (RPE) labeled goat-anti-mouse-Ig (ITK diagnostics BV, Uithoorn, The Netherlands) for another 30 min at 4°C. After two washes, mAb binding was quantified for 10,000 cells with a FACScan (575 nm; Becton Dickinson, Mountain View, CA) and expressed as mean fluorescence intensity (MFI). Non-specific mAb binding was measured by the same assay procedure, with an antibody not expressed by MC. This value was substracted from all other results.

Statistical analysis. Results are expressed as mean and standard deviation (SEM). Data were evaluated using the paired Student-*t* test. A p-value ≤ 0.05 was considered significant.

Results

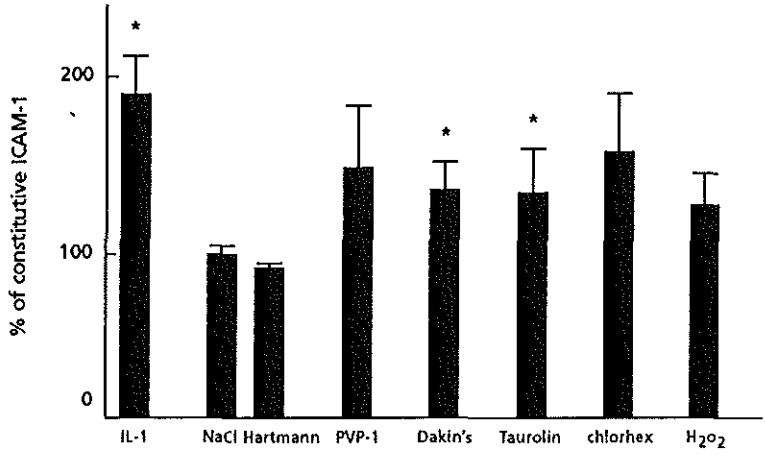
ICAM-1 and VCAM-1 are present on the surface of non-stimulated MC in significant quantities. After stimulation of the MC with IL-1β (10 ng/ml), the expression of both adhesion proteins increased with a maximum for both proteins 6h after stimulation. Figure 1 shows an 1.9-fold increase of ICAM-1 expression 6 hours after incubation with IL-1β ($p< 0.05$). All diluted solutions increased the expression of ICAM-1 on the MC membrane, only significantly for Dakin's (1:200) and Taurolin (1:10). Compared to control medium (M-199 without additions) NaCl 0.9% and Hartmann induced no change in ICAM-1 expression.

VCAM-1 expression was about 10% of total constitutive ICAM-1 expression. Figure 2 shows no significant changes of VCAM-1 expression on the MC membrane after stimulation with IL-1β or (diluted) lavage solutions. After 20h, the expression of both proteins, ICAM-1 as well as VCAM-1, on the MC membrane had almost returned to baseline levels (data not shown). E-selectin was not present on either resting or stimulated mesothelial membranes (data not shown).

Discussion

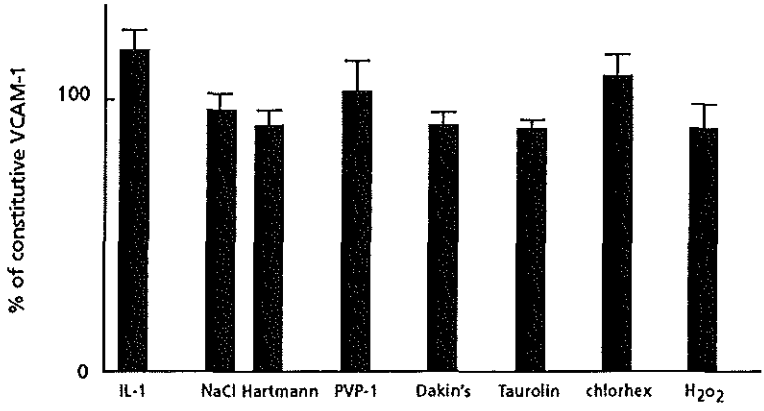
The evidence to date supports an effector role of MC in the acute inflammatory response and in repair of peritoneal injury. MC control the migration of

Figure 1.



ICAM-1 expression by MC 6h after incubation with (diluted) lavage solutions for 15 min. Values are given as percentage of the constitutive ICAM-1 expression in culture medium (mean ± SD). Data from flow cytometry analysis (n=4). *$p \leq 0.05$; paired *t*- test.

Figure 2.



VCAM-1 expression by MC 6h after incubation with (diluted) lavage solutions for 15 min. Values are given as percentage of the constitutive VCAM-1 expression after exposure to culture medium (mean ± SD). Data from flow cytometry analysis (n=4).

leukocytes, a feature which they have in common with the vascular endothelium.[10,19] Disturbance of mesothelial function has been associated with intra-abdominal complications presumably due to induced inflammatory responses.[20,21] Peritoneal irrigation of the abdominal cavity as performed prophylactically by many surgeons may damage and activate the mesothelial layer. In addition, migration of neutrophils towards the peritoneal cavity as a result of an increased peritoneal IL-8 level will be assisted by adherence of neutrophils on the MC membrane by expression of adhesion molecules.

Our results about ICAM-1 and VCAM-1 expression on unstimulated cultured MC correspond to those of Jonjic *et al.*[5] and Klein *et al.*[11]. Basal VCAM-1 expression on cultured MC from freshly obtained human omental species was about 10% of constitutive ICAM-1 expression (data not shown). IL-1 increases the expression of ICAM-1 significantly, and of VCAM-1 only slightly. The present study shows that various solutions stimulate the expression of leukocyte adhesion molecule ICAM-1 by MC, whereas the VCAM-1 induction was not affected. Recently, Zeillemaker *et al.* reported that activation of a MC monolayer modulates to a large extent the PMN adherence to and migration across these cell layers, but has little influence on the monocyte adherence and migration.[22] This may be explained by the relative insensitiveness of mesothelial VCAM-1 upon stimulation, because ICAM-1 mediates PMN attachment, spreading, and migration, whereas VCAM-1 preferentially contributes to monocyte adhesion.[23,24]

Neutrophils adhering closely to the mesothelium may create a microenvironment, in which the accumulation of oxidants and enzymes exceeds that of cellular defence antioxidant mechanisms, allowing mesothelial damage to occur. Peritoneal inflammation and adhesions are serious complications after infection, ischemia, mechanical trauma and surgery. Adhesion molecules, in particular ICAM-1, may also promote tumour progression and metastasis.[25,26]

In conclusion, irrigation of the peritoneal cavity, with the purpose to clean the abdomen, disturbs mesothelial regulatory functions, and in this way may contribute to neutrophil-mediated damaging conditions and increased tumour cell adhesion.

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The effect of peroperative lavage solutions on coagulant and fibrinolytic properties of mesothelial cells

3

Abstract.

In vivo studies have shown enhanced expression of cell-associated procoagulants and inhibitors of fibrinolysis to play a major role in the pathophysiology of adhesion and abscess formation after abdominal surgery. Peroperative lavage with the purpose to clean the abdominal cavity may cause injury to and activation of the mesothelium with disturbance of its normal regulatory functions.

MC monolayers cultured from human omental tissue were exposed for 15 min to various dilutions of clinically used standard lavage solutions. Cell injury (LDH activity), plasminogen activator inhibitor (PAI)-1 and tissue-type plasminogen activator (tPA) release were studied in the 24h supernatant. Procoagulant activity of human MC is due to tissue factor (TF) expression and was determined 6h after exposure to the solutions.

Changes in the balance of tPA and PAI-1 release, along with induction of PAI-1 activity (50% to 180%, $p< 0.05$) by MC monolayers exposed to most lavage solutions were found. Induction of pro-coagulant activity of MC monolayers irrigated with lavage solutions (70% to 130%, $p< 0.05$) was observed. In conclusion, exposure of cultured MC to lavage solutions modifies release and activity of an important inhibitor of the fibrinolytic cascade (PAI-1) and affects procoagulant activation on the mesothelial surface.

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Introduction

Peroperative lavage has become a standard surgical procedure to clean the contaminated or potentially contaminated abdominal cavity. Peroperative lavage is applied to dilute and mechanically eliminate substances that have accumulated in the peritoneal cavity during the course of disease or operative procedure. Although peritoneal cleansing with solutions seems logical and cosmetically appealing during colorectal surgery, the efficacy of such lavage in clinical studies has not been well documented.[1,2] In fact, it has previously been observed both in mice and rats that irrigation with solutions generally regarded as physiologically acceptable, combined with intra-abdominal manipulation, caused more adhesions than no irrigation at all.[3,4] Putman *et al.* also suggested that the technique rather than the disease process may be responsible for complications. Lavage was used only in a group of patients with perforated appendicitis with generalized or extensive peritonitis and compared with the same group of patients who had no lavage.[5]

Because of the position MC occupy in the peritoneal cavity they form an active permeability barrier regulating the passage of fluid and solutes across the peritoneal membrane.[6] They also contribute directly to pro-inflammatory events[7] and play an important role in peritoneal fibrinolytic and coagulant activity.[8,9] Peritoneal injury during operation leads to the formation of a transient fibrous exudate.[10] TF will be expressed by injured cells[11], and this initiates the extrinsic pathway of the coagulation cascade (directly activating factor VII, which in turn activates factor X), leading to thrombin generation and fibrin formation.[12] Under normal circumstances, there is a balance between coagulation and fibrinolysis in the abdominal cavity. If coagulation is activated, the fibrin clot formed is susceptible to lysis by tPA. The process of fibrinolysis is dampened when tPA is blocked by PAI-1. The factors by which the synthesis of these various proteins is regulated in the peritoneal cavity are only poorly understood. However, the reduction in peritoneal fibrinolytic activity, as occurs after injury or inflammation, is associated with adhesions.[13] Intra-abdominal operation alters inflammatory responses and disturbs fibrinolytic homeostasis.[14,15] It is therefore not surprising that by far the most common cause of intraperitoneal adhesions is previous surgical treatment.[16-18] In addition, peroperative irrigation of the abdominal cavity may delay postoperative restoration of the physiological balance between coagulation and fibrinolysis, with even more induction of adhesion

of peritoneal surfaces. This study was performed to determine the effects of various clinically used lavage solutions on the fibrinolytic (tPA and PAI-1) and coagulant (TF) activity of the mesothelium.

Materials and methods

Culture and identification of omental mesothelial cells. MC were obtained from the omental tissue of patients undergoing elective abdominal surgery who had given informed consent. The MC were isolated according to techniques modified from Nicholson *et al.*[19] and Wu *et al.*[20] and cultured as described previously. MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (80 cm^2 Nunc, Roskilde, Denmark) precoated with fibronectin (gift of the Central Laboratory of the Netherlands Red Cross (CLB), Amsterdam, The Netherlands). The identity of MC was demonstrated by the absence of von Willebrand factor staining[9] and the presence of intracellular cytokeratins via immunofluorescence with monoclonal antibodies (Dakopatt, Denmark).[21]

Exposure of cells to lavage solutions. Six lavage solutions were studied: viz. NaCl 0.9%, Hartmann solution (Ca^{2+} 1.8 mmol/l, K^+ 3.2 mmol/l, Na^+ 146.5 mmol/l, lactate 3.2 mmol/l), Betadine 1% (Povidone-iodine 100 g/ml diluted in NaCl 0.9%, Asta Medica BV, Diemen, The Netherlands), Dakin's solution 0.5% (NaOCl 5 mg/ml OPG Farma, Utrecht, The Netherlands), Taurolin 0.5% (5 mg/ml taurolidine, Geistlich-pharma, Wolhusen, Switzerland) and chlorhexidine 0.02% (Vifor Medical SA, Huizen, The Netherlands). Serial dilutions of lavage solutions were prepared in M-199 for optimal pH (7.2-7.4) and osmolarity (280-300 mosmol/kg).

Second passage MC were seeded into 24-well culture plates (Costar, Cambridge, UK), precoated with fibronectin, and allowed to grow to confluence before use. Medium was removed from the wells and replaced by 500 µl of lavage solutions. After 15 min of incubation at 37°C and 5% CO_2, fresh supplemented medium (500 µl) was added to replace the lavage solutions. Supernatants were collected at 24 hour, centrifuged (800 *xg*/ 5 min) and stored at -70°C.

Immunoassays. Quantitation of tPA and PAI-1 antigen was determined by means of an enzyme-linked immunosorbent assay (Biopool, Umea, Sweden).

According to the manufacturer, the PAI-1 assay detects both free (active or latent) PAI-1 and that bound to tPA. The tPA assay measures both free tPA and that bound to PAI. tPA-activity (Chromolize®) and PAI-1 activity (Spectrolyse®, Biopool, Umea, Sweden) were determined in the same supernatants. Determinations were performed in duplicate, while all experiments were performed in triplicate. Interleukin-1β (2 ng/ml; R&D systems, Abingdon, UK) is known to stimulate PAI-1 production and inhibit tPA production by MC, and served in all experiments as a positive control. Lavage solutions and reagents used were free of detectable amounts of endotoxin, as was determined with an endotoxin assay (Sigma Chemical Co, St Louis, MO).

Assessment of mesothelial cell injury. MC toxicity was assessed by examining the monolayers under phase-contrast microscopy and by measurement of lactate dehydrogenase (LDH) activity.[22] MC were seeded into 24-well culture plates and grown to confluent monolayers. Thereafter, the medium was removed, and the cells were incubated for 15 min with the various lavage solutions. After incubation, fresh medium was added. The MC monolayers were incubated with the medium for 24 hours, whereafter the medium was collected, and centrifuged (800 xg) to remove dead cells. LDH activity in this medium (50 µl aliquots) was assayed spectrophotometrically (Perkin-Elmer Lambda 2). The assay was performed according to Bergmeyer *et al.*[23] The LDH release was expressed as percentage of total (100%) cellular enzyme activity liberated from the cells by 1% (v/v) Triton-X100. All experiments were performed in triplicate.

Tissue factor activity. Studies were performed in 48-well tissue culture plates at 37°C, as described by Verhagen *et al.*[24] Six hours after stimulation the total amount of activated factor X (Xa) formed, which is directly related to TF expression on the MC membrane, has been measured. Confluent MC monolayers were washed once with a Hepes buffer (25mM Hepes, 135 mM NaCl, 5 mM KCl, 4.5 mM glucose, pH 7.4, 0.3% BSA), and then twice with the same buffer containing 5 mM $CaCl_2$. Factor VII and factor X were purified from plasma and diluted in the Hepes-$CaCl_2$ buffer to concentrations of 2.0 nM and 168 nM, respectively. The MC in each well were preincubated with 100 µl of factor VII for 5 min, after which 50 µl of factor X was added. The cells were briefly shaken and after 15 min the reaction was stopped by adding

25 µl of the sample to 50 µl of EDTA (25 mM in Hepes buffer) in a 96-well plastic assay plate. Chromogenic substrate S2765 (100 µl of 0.2 mM; Chromogenix, Nodia, The Netherlands) was added and the absorbence at 405 nm was measured for 1 min in a V-max ELISA reader (Molecular Devices, Menlo Park, CA, USA). TF expression was expressed as pM factor Xa formed per minute by 10^5 MC compared with the control (100%).

Statistical analysis. Results are expressed as mean ± standard deviation (± SD). Because standard lavage solutions were diluted in medium without serum, significant analysis was performed comparing the results with control medium without serum. The Student-*t* test was used to determine significant differences between groups.

Results

Mesothelial viability. Because exposure of 15 min to most solutions in standard concentrations (recommended by the manufacturers) were lethal for MC monolayers *in vitro*, the solutions were diluted. Serial dilutions were studied for all solutions. MC monolayers exposed to medium supplemented with 10% serum suffered less injury during 24h (7.3 ± 0.7%). A diluted lavage solution was defined as "non-toxic" and used in other experiments, when LDH activity in the supernatant 24h after the 15 min incubation was not significantly increased compared with the culture medium (M-199 without additions). The influence of several solutions on viability of MC monolayers was investigated. Table I shows the tested concentrations of diluted lavage solutions.

Influence of various lavage solutions on tPA and PAI-1 release by human mesothelial cells. tPA/PAI-1 antigens and activity were measured only in the supernatants of MC monolayers with minor cell injury, comparable with % LDH activity after exposure to culture medium (M-199). During a 24h incubation in supplemented medium, tPA and PAI-1 concentrations were 6.25 (± 0.9) ng/ml and 343.9 (± 63) ng/ml, respectivily. Exposure of MC to interleukin-1β (IL-1β) reduced the tPA release and increased PAI-1 release. No free tPA activity was detectable in conditioned medium from either stimulated or non-stimulated MC cultures (≤ 0.1 IU/ml).

Exposure of the MC monolayers to culture medium (M-199) for 15 min induced tPA and PAI-1 antigen release slightly (*table 1*) and PAI-1 activity did

Table 1. Influence of diluted solutions on viability, tPA and PAI-1 antigen release

cell damage	fibrinolytic parameters		
solution	% LDH	tPA (ng/ml)	PAI-1 (ng/ml)
supplemented medium	7.3 (0.7)	6.25 (0.9)	343.9 (63)
+ interleukin-1	6.0 (1.5)	3.55 (0.4)**	940.5 (199)*
control medium	9.3 (0.8)	7.85 (1.0)	672.5 (132)
NaCl 0.9%	13.0 (1.6)	7.75 (1.4)	699.5 (248)
Hartmann	9.7 (1.0)	7.45 (1.8)	700.0 (213)
PVP-I 1:100	6.1 (0.6)	1.95 (1.7)*	196.3 (102)*
Dakin's 1:200	9.8 (0.8)	10.0 (1.1)*	631.0 (81)
Taurolin 1:20	10.3 (0.4)	8.35 (2.5)	445.2 (128)*
chlorhex. 1:10	12.2 (1.7)	4.80 (1.5)*	696.0 (156)

Release of cytosolic LDH, tPA antigen and PAI-1 antigen in the 24h supernatant from MC monolayers exposed to medium supplemented with 10% FCS or control medium and different kinds of diluted lavage solution for 15 min. Every result is the mean (SD) of three different measurements in triplicate. LDH data are presented as the percentage LDH released during 24h divided by total LDH (100%). * Statistical significant difference ($p<0.05$) compared with MC monolayers exposed to control medium. **$p< 0.001$.

not increase significantly (*figure 1*). The physiologic salt solutions, NaCl 0.9% as well as Hartmann, also stimulated both tPA and PAI-1 antigen release of MC slightly. Only for NaCl 0.9% PAI activity increased significantly ($p< 0.01$). Diluted Dakin's was the most potent stimulator of tPA antigen. However, this was not reflected in fibrinolytic activity (PAA$\leq$ 0.1 IU/ml), and PAI-1 activity was strongly induced (28.8 $\pm$ 0.8 IU/ml, $p< 0.05$). In contrast, PVP-I (1:100) significantly reduced both tPA and PAI-1 antigen concentration ($p< 0.05$). Taurolin stimulated MC to release tPA slightly, while PAI-1 release was significantly decreased ($p< 0.05$) compared with medium without serum. Although the fibrinolytic balance was in advantage of tPA, the reduction in PAI-1 activity after exposure to Taurolin was not significant. Chlorhexidine (1:10) reduced tPA antigen production and induced PAI-1 antigen, which resulted in enhanced PAI-1 activity ($p< 0.05$).

Table 2. Influence of lavage solutions on tPA and PAI-1 antigen production

solution	tPA (-fold increase)	PAI-1 (-fold increase)	ratio PAI-1/tPA
supplemented medium	1	1	
+ interleukin-1	0.51 (0.06)	2.90 (0.66)	5.69
control medium	1.25 (0.15)	2.08 (0.49)	1.66
NaCl 0.9%	1.35 (0.21)	2.00 (0.86)	1.48
Hartmann	1.31 (0.11)	2.14 (0.65)	1.63
PVP-I 1:100	0.36 (0.20)	0.58 (0.22)	1.61
Dakin's 1:200	1.39 (0.15)	1.84 (0.51)	1.32
Taurolin 1:20	1.40 (0.32)	1.15 (0.37)	0.82
chlorhex. 1:10	0.61 (0.17)	2.00 (0.73)	3.27

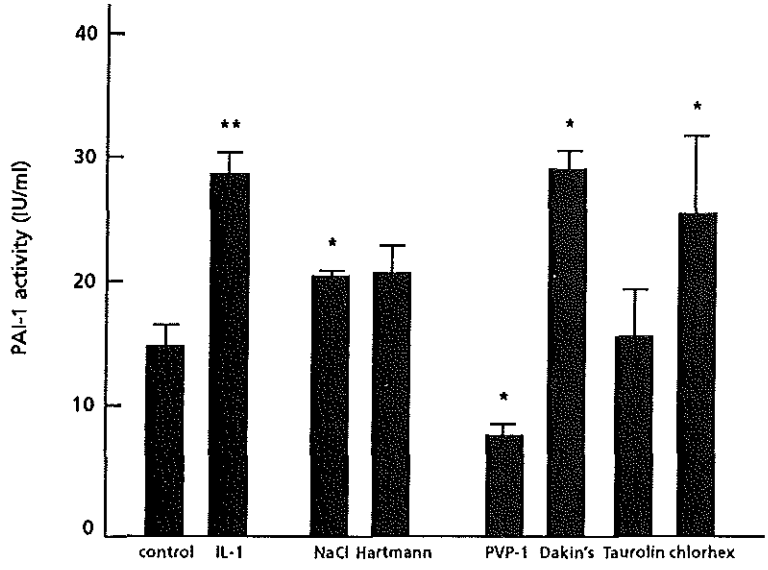
Data expressed as means of -fold increase (SD) over medium supplemented with 10% serum.

Table 2 shows the relative increases of tPA and PAI antigen concentrations for the tested solutions (supplemented medium= 1). For most solutions, PAI-1 antigen concentration in the 24h supernatant was more enhanced compared with tPA antigen concentration. To express the final effect of each solution on fibrinolytic balance, the PAI-1/tPA ratio is given in the third column. A PAI-1/tPA ratio < 1 indicates a favourable balance between concentration of the activator (tPA) and inhibitor of fibrinolysis (PAI-1). MC exposed to Taurolin resulted in the most favourable fibrinolytic ratio (0.82), while exposure to chlorhexidine (1:10) caused the most unfavourable fibrinolytic balance of all solutions tested (ratio 3.69).

A standard concentration of either tPA or PAI was incubated for 24h with the tested solutions. None of the lavage solutions interfered with the ELISAs or neutralized one of the fibrinolytic parameters. To test the siliconizing effect of PVP-I on tPA and PAI measurement we pretreated MC monolayers (IL-1β; 2 ng/ml) for 1 hour to ensure increased PAI-1 production. Exposure of the pretreated monolayers to PVP-I (1:100) for 15 min

increased the amount of PAI-1 in the 24h supernatant. This increase was less compared with no subsequent PVP-I treatment. To verify whether polyvinylpyrrolidone itself was involved in tPA and PAI-1 reduction, observed after exposure of MC to PVP-I, PVP (Sigma; MW 40,000 and intrinsic viscosity 28-32) was added to the medium for 15 min in non-toxic concentrations (0.5-0.1%). PVP did not show significant reduction in tPA nor PAI-1 release (data not shown).

Figure 1. Influence of lavage solutions on PAI-1 activity

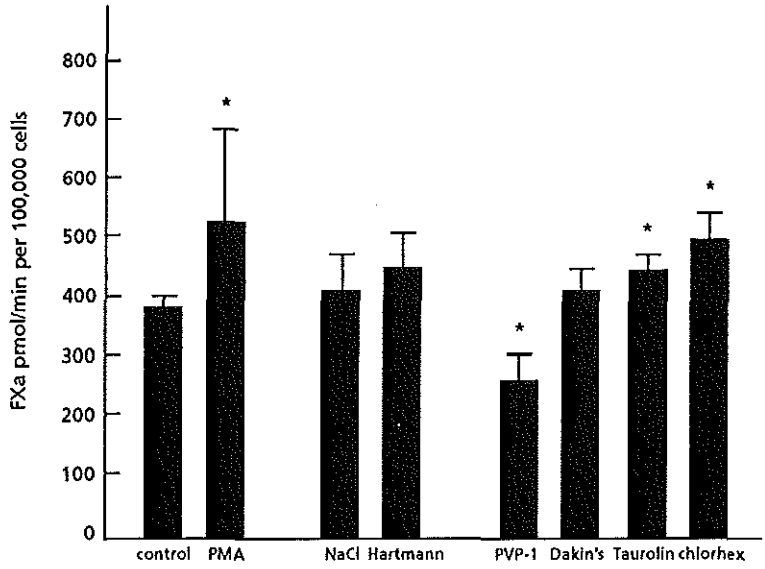


PAI-1 activity in the supernatant from MC monolayers exposed to control medium or to various lavage solutions for 15 min. Plasminogen activator activity (tPA-A) was not detectable in any tested MC culture passage (≤ 0.1 IU/ml). *Statistically significant difference ($p < 0.05$) compared with MC monolayers exposed to culture medium. **$p < 0.001$.

The influence of various lavage solutions on tissue factor expression by human mesothelial cells in culture. Addition of IL-1β (2 ng/ml) resulted in activation of the coagulant cascade by expression of TF (data not shown). However, phorbol myristrate acetate (10 ng/ml; PMA) proved to be a much more stronger inducer of TF expression on MC and was therefore used in all experiments as a positive control. In Figure 2 the effects of non-toxic concentrations of various solutions on MC factor Xa generation is given. Without cells or with one of

the clotting factors (VII or X) lacking, no Xa was formed. Most solutions induced factor Xa generation (12.5-28%). This induction was significant for chlorhexidine and Taurolin (p= 0.005 and p= 0.01, respectively). Only PVP-I reduced TF activity (15%, p= 0.001).

Figure 2. Influence of lavage solutions on procoagulant activity of MC monolayers



TF activity on cultured MC monolayers 6h after exposure to control medium and various lavage solutions. Cells exposed to medium without serum served as a control. Results are given as mean FXa generation pmol/min per 10^5 cells (SD) and based on four seperate experiments in triplicate. Student t-test was used to determine significance between groups. *Statistically significant difference (p<0.05) compared with MC monolayers exposed to culture medium.

Discussion

The use of lavage solutions is well entrenched in the present day surgical practice, which offers many situations in which peritoneal lavage could be protective. Peritonitis, colonic resection with faecal spilling, fibrous adhesion formation and prevention of tumour recurrence are all suggested indications for peritoneal lavage.[25] The evidence to date supports an effector role of MC in the acute inflammatory response and in repair of peritoneal injury. As a biologic source of TF and both plasminogen activator (tPA) and plasminogen

inhibitor (PAI-1), the peritoneal lining presumably plays a key role in the regulation of local caogulation and fibrinolysis *in vivo*. Disturbance of mesothelial function has been associated with decreased fibrinolytic activity and increased intraperitoneal adhesions.[26,27] Vipond *et al.*[28] reported that reduction of the fibrinolytic activity *in vivo* was not caused by a reduction in local tPA, which could still be assayed at normal levels, but by the release of plasminogen activators inhibitors, which competitively antagonize the action of tPA. Loss of fibrinolytic activity observed after abdominal surgery or during inflammation probably arises from a dramatic increase in PAI concentration.[14,29]

The present study shows that in an activated *in vitro* model (basal tPA and PAI-1 are both enhanced), lavage solutions can change the balance between plasminogen activator and inhibitor antigen. This resulted in enhanced activity of the fibrinolytic inhibitor (PAI-1 activity) 24h after 15 min exposure to most solutions. However, not all solutions modulated tPA and PAI antigen release of MC in a similar way; e.g. exposure to chlorhexidine caused a decrease in tPA and an increase in PAI, while Dakin's solution induced both tPA and PAI release. This finding resembles previous observations that secretion of tPA and PAI are not necessarily coupled,[30] and is of interest when pharmacological manipulation of the production of either component, but especially PAI-1, is studied in search for a solution to minimize intra-abdominal complications.

Macromolecular solutions have been popular substances in the efforts towards adhesion prevention. Polyvinylpyrrolidone, a synthetic polymer, has been claimed to be effective in preventing postoperative adhesions.[31,32] Macromolecular solutions are believed to have anti-adhesion effect by separating the viscera, and siliconizing the intestinal surface. In this study PVP-I in diluted concentrations reduced tPA and PAI-1 antigen as well as PAI-activity. Inhibition did not appear to be caused by cytotoxicity or a nonspecific effect related to interference with the assay systems. Although morphologic studies confirmed intact monolayers after exposure to diluted PVP-I, exclusion of partial loss of LDH was not possible. It is hypothesized that the observed reduction of fibrinolytic parameters is due to a partial coating effect of the MC monolayer. Recently, Meyer and co-workers suggested that macromolecular solutions increase local fibrinolytic activity by preventing removal of the local activators from the traumatized areas.[33] This is in

accordance with our observations; moreover, viscous solutions may minimize adhesion formation by reduction of overall PAI-1 activity. Anti-adhesive capacity of Taurolin has also been described.[34] In our model, Taurolin depressed the PAI-1/tPA ratio and was the only solution that did not affect PAI-1 activity significantly. This may explain less adhesion formation after Taurolin lavage compared with NaCl 0.9% or Hartmann lavage in experimental *in vivo* studies.[34,35]

Cell-mediated initiation of the coagulation cascade is another important mechanism underlying fibrin depositions.[36] TF is a membrane protein that serves as the essential cofactor for factor VII in the initiation of coagulation via factors IX and X.[37] Minor changes in TF expression have major effects on fibrin formation through amplification of the coagulation cascade. Chemical or physical damage to cell membranes can cause a rapid increase in the expression of TF procoagulant activity.[12] Therefore, the observation that lavage solutions in non-diluted concentrations caused cell membrane injury with LDH release, may be of clinical importance. We assessed TF expression on MC monolayers with minor cellular damage to determine procoagulant activity via modulation of TF synthesis 6 hours after stimulation with various lavage solutions. Only PVP-I inhibited factor Xa generation significantly ($p< 0.05$). Anticoagulant properties of PVP have been described, and this high-molecular-weight polymer prolonged the coagulation time of blood.[32] Although formaldehyde-releasing agents were also shown to have anticoagulant properties, Taurolin induced the procoagulant activity of MC monolayers ($p< 0.05$) in our model.

Peritoneal irrigation with Dakin's solution is used to prevent local recurrence from contamination by cancer cells. However, McKibbin *et al.*[38] and Oates *et al.*[1] both found that administration of Dakin's in the abdominal cavity increased tumour take above control levels in a rat model. It appeared that the irritating effect of the solution on the peritoneum enhanced the growth of exfoliated tumour cells. The mechanism of this phenomenon remained unexplained. We suggest that exposure of the peritoneum to a cytotoxic agent like Dakin's may exert local effects on the mesothelial lining, which in turn changes its regulatory function. After exposure to Dakin's solution we observed enhanced PAI-1 activity comparable with levels after stimulation with IL-1. When similar responses occur *in vivo*, local procoagulant activity and concentration of PAI-1 may remain elevated and may even

facilitate tumour recurrence.[39,40]

In conclusion, exposure of MC to lavage solutions modulates the availability of plasminogen activator and inhibitor. Moreover, the profibrinolytic entity, tPA, and the anti-fibrinolytic one, PAI-1, are not similarly regulated by the different stimuli. The activity of the fibrinolytic inhibitor (PAI-1) is also susceptible to modulation by various solutions. Furthermore, the expression of functional TF on the surface of MC can be induced after incubation with lavage solutions. These findings support the idea that lavage solutions that are used nowadays may induce local peritoneal responses and may thus affect peritoneal complications after intra-abdominal surgical intervention.

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Peroperative lavage promotes intraperitoneal adhesion in the rat

4

Abstract

This study was designed to study the effect of peritoneal lavage solutions on postsurgical adhesion formation in rats undergoing laparotomy and standardized ischemic injury to the lateral peritoneum with sutures. This reproducible model allowed semi-quantitive scoring of adhesion formation. Adhesions were induced in 33 adult female Wistar rats. The solutions RPMI medium, NaCl 0.9%, and both PVP-I 1% and chlorhexidine 0.02% in dilution were evaluated. In the control group that was operated upon (without peritoneal lavage) a mean adhesion percentage of 22.5% was scored. All solutions used for abdominal lavage in this rat model induced significantly (p= 0.0001) more adhesions (40.6-70.8%). The results found in the present *in vivo* study correlate with observations in previous *in vitro* experiments i.e. exposure of peritoneal areas to lavage solutions enhances peritoneal activation and thus promotes intra-abdominal adhesion formation.

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Introduction

Peroperative lavage following contaminated or potentially contaminated abdominal surgery has become common practice. Although peritoneal lavage may diminish the bacterial count in the abdominal cavity,[1] it may enhance peritoneal reaction and provoke an inflammatory response on the surface. Human MC in culture have been shown to have modulated inflammatory, fibrinolytic as well as procoagulant activity after exposure to lavage solutions in non-toxic dilutions.[2-4] When similar responses occur *in vivo*, peroperative irrigation of the abdominal cavity may delay postoperative restoration of physiological local defence systems, and furthermore induce adhesion of peritoneal surfaces. It has been observed both in mice and in rat that irrigation with solutions generally regarded as physiological acceptable, combined with intra-abdominal manipulations, caused more adhesions than no irrigation at all.[5,6]

Lysis of fibrous adhesions depends on the presence of an adequate amount of plasminogen activator activity.[7,8] Ischemia or inflammation of the mesothelium results in a greater reduction of fibrinolytic activity, which leads to more fibrous adhesions, than does simple trauma alone.[9] Therefore, a standardized experimental rat model with induced peritoneal ischemia was chosen to determine the effect of various solutions, commonly used in surgical practice, on peritoneal adhesion formation.

Materials and methods

Animals. A total of 33 female Wistar rats of reproductive age weighing 150-230 g (Harlan-CPB, Zeist, The Netherlands) were used. Rats were bred under specific pathogen-free conditions and kept under standard laboratory conditions. The protocol was approved by the Committee on Animal Research of the Erasmus University, Rotterdam, The Netherlands.

Surgical techniques. The model employed in this study was derived from a model recently described by van den Tol *et al.*[10] Briefly, under ether anaesthesia the abdomen was shaved and cleaned with alcohol 70%. Laparotomy was performed using a lower midline incision of 5 cm. Three 5-0 surgilene sutures were applied to the lateral peritoneum 0.7 cm from each other. Both uterine horns were exposed and sutured to the lateral peritoneum with surgilene 6-0 sutures (B Braun, Melsungen AG, Germany) proximally and distally

from the three 5-0 surgilene sutures. All knots were tightly fastened to ensure ischemia. Subsequently the abdominal cavity was exposed to 10 ml of lavage solutions (25° C) for 10 min, whereafter the fluid was suctioned out carefully. At least 5 ml of solution remained within the peritoneal cavity. In the control rats no lavage was performed after surgery. The abdomen was closed in 2 layers with dexon 5-0 and silk 2-0 sutures.

*Solutions.*The evaluated lavage solutions were: RPMI 1640 medium (Gibco, Paisly, Scotland), NaCl 0.9% (Fresenius BV, 's Hertogenbosch, The Netherlands), PVP-I 1% (povidone-iodine 100 g/ml diluted in NaCl 0.9%, Asta Medica BV, Diemen, The Netherlands), chlorhexidine 0.02% (Vifor Medical SA, Huizen, The Netherlands) and Viaspan® (an *ex vivo* storage solution, DuPont Pharma, Clarkson, Canada). Dilutions of the lavage solutions were prepared in medium (RPMI) for optimal pH (7.2-7.4) and osmolarity (280-300 mosmol/kg). All solutions were prepared under pathogen-free conditions.

Experimental design.

A. peritoneal lavage with non-aggressive solutions.

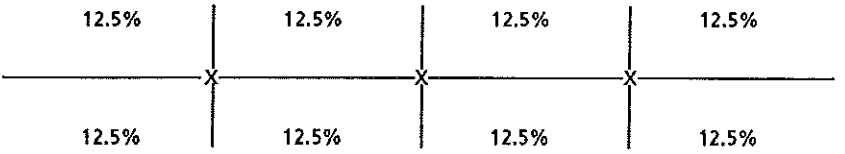
Fifteen rats divided in 3 groups underwent the described operation. Control rats (group I, n=5) did not undergo a peritoneal lavage, the other 10 underwent a lavage with non-aggressive solutions. Non-aggressive solutions in previous experiments caused only moderate activation of mesothelial functional properties *in vitro*.[2-4] Group II (n=5) underwent lavage with 10 ml NaCl 0.9% and group III (n=5) with Povidone-iodine (PVP-I diluted 1:100 in RPMI).

B. peritoneal lavage with an aggressive solution compared with non-aggressive solutions.

Eighteen rats were operated, of which six underwent a lavage with the non-aggressive solution RPMI (group IV), and another six with Viaspan® (group V). Six had lavage with the aggressive solution chlorhexidine 0.02% (diluted 1:10 in culture medium; group VI). This solution has been shown to be a major activator of mesothelial cytokine production, while serum-free culture medium, NaCl 0.9%, and PVP-I (diluted 1:100) caused less functional activation of cultured human MC.[2-4]

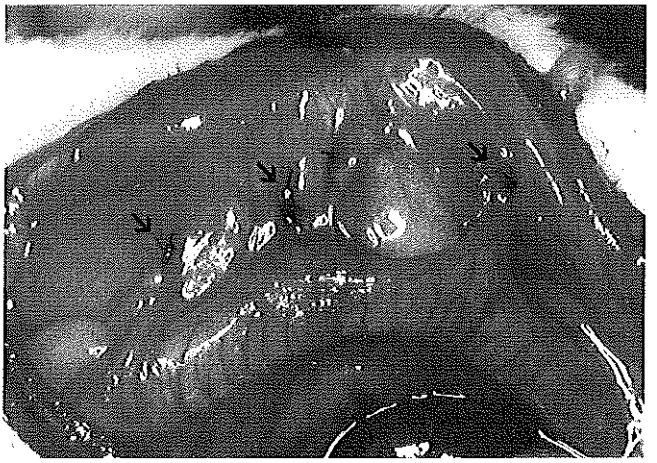
Evaluation of adhesion formation. Fourteen days postoperatively, the rats were sacrificed for assessment of postsurgical adhesion formation. Macroscopically the adhesions were scored according to their extent, at random, by three individuals. Quantification was assessed by dividing the sutured area to be scored into eight areas by means of the 3 sutures in the lateral peritoneum (*figure 1*). Each area was scored for the presence of adhesions. When adhesions were found in one area, this accounted for 12.5% adhesions (1/8). Thus, a maximum of 100% (8/8) adhesions could be scored. In one rat, two sites (uterushorns) were assessed and the mean of these two observations was used as one experimental result.

Figure 1. Scoring areas of adhesions (total of 100%) between the three sutu-



The extent of adhesion formation was quantified by dividing the "defect" area to be scored into eigth areas of 12.5% (1/8) by means of three sutures (X = suture) used to induce ischemic injury.

Figure 2.



Adhesions in a control (not irrigated) animal 14 days postoperatively. The uterushorn was sutered proximal and distal to the lateral peritoneum. Three sutures (→) induced ischemic injury and divided the "defect" into eight areas.

Statistical analysis. Mean total percentages of adhesion formation in the treated groups were statistically compared to control animals using the one-way ANOVA test followed by Bonferroni *post-hoc* tests where 3 groups were compared. Statistical significance was defined as $p< 0.05$. Data are expressed as mean adhesion percentage (SD).

Results

The percentages of mean adhesion formation after lavage with non-aggressive solutions and agressive solution are presented in Table 1 and 2. Control rats showed a mean of 22.5% adhesion formation after standard ischemic injury. There was a significant increase in the mean total number of adhesions in all five irrigated groups.

Table 1. Mean adhesion percentage (SD) in abdominal cavities lavaged with non-aggressive solutions

	group	n	mean % adhesions (SD)
I	control (no-lavage)	5	22.5 (8)
II	NaCl 0.9%	5	60.0 (11)
III	PVP-I 1% 1:100	5	63.8 (11)

Statistics: one-way ANOVA test, with a Bonferroni *post-hoc* test (I vs II, $p = 0.0001$, I vs III, $p = 0.0001$, II vs III $p = 0.613$, not significant).

Table 2. Mean adhesion percentage (SD) in abdominal cavities lavaged with non-aggressive solutions and an aggressive solution

	group	n	mean % adhesions (SD)
IV	RPMI	6	47.7 (12)
V	Viaspan®	6	40.6 (13)
VI	chlorhexidine 0.02% 1:10	6	70.8 (14)

Statistics: one-way ANOVA test, with a Bonferroni *post-hoc* test (IV vs V, $p = 0.341$; IV vs VI, $p = 0.012$; V vs VI, $p = 0.003$).

In the first experiment NaCl 0.9% and PVP-I (1:100) caused over 150% more adhesions. The differences were significant between the control group (no lavage) and the groups receiving peritoneal lavage. No significant differences were found between the two irrigated groups in this experiment. In the second experiment RPMI and Viaspan® both induced about 100% more adhesions compared with control adhesion induction without lavage (22.5%). Chlorhexidine 0.02% (1:10), an aggressive solution for MC in culture, induced significantly more adhesions than did RPMI or Viaspan® (*table 2*, p= 0.003 and p= 0.012, respectively).

Discussion

Intraperitoneal postoperative adhesion formation creates a major challenge in abdominal surgery, as it is the main cause of mechanical small bowel obstruction, "unexplained" abdominal pain, and female infertility. Furthermore, the presence of dense adhesions makes reoperation technically difficult. By far the most common cause of intraperitoneal adhesions is previous surgical intervention.[11] Intra-abdominal operation will alter inflammatory responses and disturb fibrinolytic homeostasis.[12,13]

We have assessed the effect of peritoneal lavage with non-aggressive and aggressive solutions in an established animal model in which the type of trauma mimics the injury induced in patients during abdominal operations. Adhesions were caused by ischemic injury as a result of suturing the lateral peritoneal wall. An adhesion formation of 22% occurred in control rats. This percentage was also observed in earlier experiments using the same rat model.[10] Our results show that peritoneal lavage in areas with ischemic injury is associated with increased adhesion formation in the rat. All solutions induced more adhesions in the sutured area compared with control rats without peritoneal lavage.

Possible explanations for induction of adhesions by lavage solutions include cellular injury, increased peritoneal permeability and peritoneal activation. We have studied cellular injury by release of an intracellular enzyme (LDH) *in vitro* and in the peritoneal fluid of patients undergoing elective colonic surgery (unpublished observations) and observed increased levels of LDH after exposure to lavage solutions. Furthermore, permeability studies with a macromolecule (inulin) after treatment of MC monolayers with lavage solutions showed disturbance of mesothelial integrity with increased

permeability. This facilitated passage for macromolecules may cause increasing fibrous exudation to the abdominal cavity and thereby induce adhesion formation. Also increased pro-inflammatory peritoneal cytokine response after exposure to various solutions may lead to enhanced inflammatory responses.[2,3] Moreover, procoagulant and fibrinolytic properties of human MC monolayers were also affected by various solutions in earlier *in vitro* experiments.[4]

Notwithstanding an overall increase in responses of human MC in culture to various kinds of clinically used solutions, not all solutions induced an equal effect. In this *in vivo* model no significant difference in the extent of postsurgical adhesion formation was observed between exposure of the peritoneal cavity to NaCl 0.9% or PVP-I 1% (1:100). This discrepancy might be explained as follows. Firstly, by observations that the cytokine profile and peritoneal fibrinolytic activity differs in different species.[14-16] Intraperitoneal responses to stimuli in man may differ and be more complicated compared with responses in the rat. Secondly, PVP-I as a highly viscous solution reduced MC activation *in vitro* probably by coating the cells. *In vivo* the coating effect over the entire peritoneal surface may be not as effective as *in vitro*.

Studies in our laboratories have emphasized the use of scavengers to minimize humanMC damage and activation during oxidative stress. The trauma induced in this rat model was mainly ischemic injury. Therefore, we also tested the organ preservation fluid Viaspan®, regarded as more biocompatible because it contains lactate and oxygen radical scavengers (gluthatione and allopurinol). Nevertheless, peroperative Viaspan® lavage also induced significantly more adhesions (40.6%) compared with no-lavage. However, the effect of antioxidants on mechanisms underlaying the initiation of fibrin appeared also to be species specific in another experimental study.[17]

In conclusion, this study is in accordance with our previous experiments with human MC *in vitro*. It has been shown that exposure to peritoneal lavage solutions may lead to more adhesion formation in the abdominal cavity. Therefore, irrigation with any solution should be viewed with caution after 'clean' surgery. Further studies appear important to establish the composition of the ideal irrigation solution.

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Changes in intraperitoneal responses of human peritoneal fluid after elective abdominal surgery with peritoneal lavage

5

Abstract

Modulation of viability and functional properties of human MC after exposure to various lavage solutions has been observed previously *in vitro*. This study was designed to evaluate the effect of peroperative lavage on postoperative peritoneal responses *in vivo*.

Drain fluid from 30 patients undergoing elective abdominal surgery, followed by lavage with NaCl 0.9% or with distilled water, or without peritoneal lavage, was collected at 6 and 24h post surgery. We determined the composition of the intraperitoneal cell population, tissue injury (LDH activity), peritoneal activation (IL-1β, -6 and -8), and peritoneal pro-coagulant (TF), and fibrinolytic status (tPA and PAI-1).

Lavage caused elevated LDH activity due to cell injury. IL-1β concentration was not significantly changed in lavaged cavities. However, IL-6 and -8 cytokine concentrations were significantly depressed in the 6h drain fluid of lavaged cavities, whereas these pro-inflammatory cytokine concentrations were increased after 24h. NaCl 0.9% induced a significant increase in TF antigen after 24h. Exposure of the peritoneal cavity to lavage solutions caused a larger increase in release of the inhibitor of fibrinolysis than in the situation without lavage.

Thus, peritoneal irrigation depressed pro-inflammatory cytokine (IL-6, -8) concentrations in the early period (0-6h) after operation, and induces a rise in IL-6 concentration after 24h. Increased procoagulant and accelerated antifibrinolytic responses in the peritoneal environment after peroperative lavage may be of clinical relevance.

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Introduction

Intra-abdominal operation modifies both inflammatory responses and fibrinolytic homeostasis.[1,2] The evidence to date supports an effector role of MC in the acute inflammatory response and in repair of peritoneal injury.[3] These cells contribute to pro-inflammatory events and actively participate in the recruitment of neutrophils into the peritoneal cavity.[4,5] As a biologic source of both tPA and PAI-1, the peritoneal lining has also been presumed to play a key role in the regulation of local fibrinolysis *in vivo*. Disturbance of mesothelial function has been associated with a decreased fibrinolytic activity and increased intraperitoneal adhesions.[6,7] Another mechanism underlaying fibrin formation is the cell-mediated initiation of the coagulation cascade.[8] TF, a membrane protein expressed by MC, is the main physiological activator of the extrinsic coagulation pathway.[9]

The use of lavage solutions is well entrenched in the present day surgical practice, which offers many situations in which peritoneal lavage could be protective. Peritonitis, colonic resection with feacal spilling, fibrous adhesion formation and prevention of tumour recurrence are all appropriate indications for peritoneal lavage.[10] However, cytokine production, fibrinolytic activity and procoagulant activity of human MC in culture were all found to be modulated after exposure to lavage solutions in non-toxic dilutions.[11,12] Peroperative irrigation of the abdominal cavity may delay, therefore, postoperative restoration of physiological local defence systems, and thus induce postoperative intra-abdominal complications. The present study was designed to test the hypothesis that peritoneal lavage can affect the surgically induced inflammatory alterations in the peritoneal cavity via cellular injury and activation. We determined the dynamics of pro-inflammatory cytokines (IL-1β, -6 and -8) and procoagulant (TF) and fibrinolytic (plasminogen activator and its inhibitor) responses in the irrigated as well as the non-irrigated peritoneal cavity 24h after elective abdominal surgery.

Methods and patients

Patients & sample collection. Peritoneal fluid was sampled via an indwelling silicone drain during 0-6h and 6-24h after operation from patients who had undergone elective colonic resection for non-disseminated malignancy. Lavage was performed at the end of the operation; one liter of lukewarm solution was poured into the cavity and was suctioned out as much as

possible after 2-3 min. The intraperitoneal drain was placed in an area where lavage had been performed, but outside the operation field. In a few patients a second drain was left behind in the operated area to compare peritoneal responses at two places. No patients showed evidence of resident macroscopic tumour. Patients received, ad random, no peritoneal lavage or peritoneal lavage with either NaCl 0.9% or distilled water. An aliquot of each drain sample was used for bacteriological studies. In the drain fluid, the total white blood cell, erythrocyte and platelet counts were measured with an automated cell counter (Sysmex F-800). Peritoneal fluid was centrifuged at 800*g* for 10 min and the supernatant was then removed, LDH (Boehringer Mannheim, according to SFBC), free hemoglobin (spectrophotometerically) and total protein (Boehringer Mannheim, Biuret method) were measured in duplicate; an aliquot was stored at -70°C until analysis.

Bacteriological study. Pellets from drain fluid samples were cultured on sheep blood agar plates under aerobic conditions and on Schaedler agar plates (Biomerieux) under anaerobic conditions, and were incubated for 4 days at 37°C. Bacterial growth on one of the plates excluded the patient from the study.

Immunoassays. IL-1β, IL-6 and IL-8 were measured with enzyme-linked immunosorbent assays (PeliKine-compact™, CLB, Amsterdam, The Netherlands). The limits of detection for IL-1β, IL-6 and IL-8 were 2 pg/ml, 2 pg/ml and 1 pg/ml, respectively. An enzyme-linked immunosorbent assay was used for the detection of human TF antigen (Imubind®, American Diagnostica Inc. Greenwich, USA). Antigenic concentrations of tPA and PAI-1 were measured by enzyme-linked immunoassay (Biopool, Umea, Sweden). The PAI-1 assay detects both free (active or latent) and tPA-bound PAI-1. The tPA assay measures both free tPA and that bound to PAI. tPAA (Chromolize®) and PAI-1 activity (Spectrolyse®, Biopool Umea, Sweden) were determined in the same samples. Determinations were performed in duplicates.

Statistical methods. Results are expressed as median values and ranges or 95% confidence intervals. Specific comparison of variables between two groups was made with the Mann-Whitney test. A $p \leq 0.05$ was considered to show significant differences.

Results

Evidence of intraperitoneal inflammation at the time of surgery was found in none of the patients. Bacteriological studies confirmed that the postoperative intraperitoneal responses were not due to bacterial contamination. All patients underwent an uncomplicated postoperative recovery. There were no significant differences in median age, length of the operation, and volume of fluid collected among the groups (*table 1*).

Table 1. Mean (range) age, length of operation, volume of drain fluid collected among group I (no-lavage), group II (NaCl 0.9%), and group III (distilled water)

	I	II	III
age (years)	74 (55-88)	72 (59-87)	69 (60-75)
length of operation (min)	98 (56-145)	102 (74-140)	117 (70-160)
volume drain fluid (ml)			
6 h	113 (15-400)	79 (10-125)	94 (20-200)
24 h	73 (5-150)	62 (5-110)	77 (5-200)

Leucocyte counts and erythrocyte counts were significantly decreased in the 24h postoperative drain fluid of patients lavaged with NaCl 0.9% (*table 2*; * $p \leq 0.05$ I *vs* II). The differential count showed the leucocyte counts to be predominantly neutrophils (90-95%). Platelet counts were not significantly different among the three groups. To exclude the possibility that lysis of blood cells was responsible for changes in LDH activity, the LDH measurements were corrected for free hemoglobin content. Hemolysis was significantly enhanced in both lavaged groups (median (range) group II 1.41 (0.47-2.11)* and group III 1.74 (0.61-2.6)* mg/ml free hemoglobin) *versus* no lavage (1.22 (0.35-1.55) mg/ml free hemoglobin; *$p \leq 0.05$) in the 0-6h drain fluid samples.

IL-1β concentrations were not significantly changed in the lavaged groups (median (range) 172 (111-307) pg/ml 0-6h and 198 (74.6-368) pg/ml 6-24h in group II; 153 (93.7-300) pg/ml 0-6h and 175 (93.7-418) pg/ml 6-24h in group III) compared with the no-lavage (group I; 137 (80.6-319) pg/ml

0-6h and 198 (74.6-368) pg/ml 6-24h). We observed a significant ($p \leq 0.05$) early decline in cytokine concentration, both for IL-6 and IL-8, in the lavaged groups (II and III) compared with the non-lavaged group (*figure 1 and 2*). IL-6 concentrations in both lavaged groups (II and III) were significantly increased in the 6-24h drain fluid compared with 0-6h drain fluid. IL-8 concentration was significantly decreased for group I and II in the 6-24h drain fluid compared with 0-6h drain fluid.

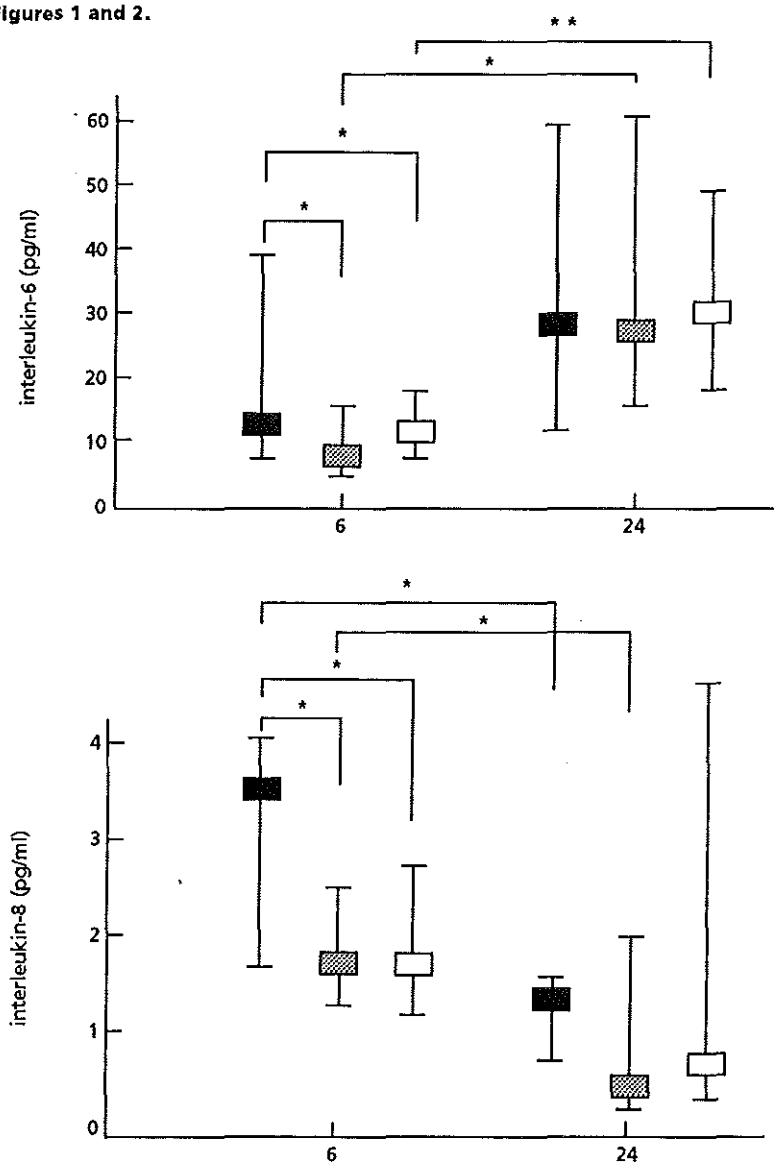
Table 2. Median (range) leucocyte-, erythrocyte-, and plateletcount, total protein, lactate dehydrogenase (LDH), and free hemoglobin (HB) in abdominal drain fluid collected among group I (no lavage), group II (NaCl 0.9%), and group III (distilled water) 6h and 24h postoperatively

	time	I	II	III
leucocytes ($x10^9/l$)	6	14.5 (0.6-37)	3.8 (0.3-24.4)	6.8(1.8-38.2)
	24	21.6 (8.6-59.6)	4.0 (0.4-17.6)*	11.0 (4.6-45.7)
erythrocytes ($x10^{11}/l$)	6	0.75 (0.1-1.9)	0.35 (0.1-0.81)	0.50 (0.1-2.8)
	24	0.45 (0.23-1.4)	0.12 (0-0.88)*	0.55 (0.14-2.7)
platelets ($x10^9/l$)	6	71 (25-145)	55 (14-105)	48 (36-101)
	24	30 (16-89)	29 (6-50)	27 (16-68)
total protein(g/dl)	6	36.9 (25-42)	36.0 (26-46)	38.0 (30-60)
	24	33.0 (24-40)	37.0 (20-44)	37.5 (28-52)
LDH (mg/dl)	6	1594 (633-2150)	1587 (588-3950)	1930 (915-2000)
	24	1117 (583-1784)	1150 (570-2285)	1446 (935-1765)
free HB (mg/ml)	6	1.22 (0.35-1.55)	1.41 (0.47-2.11)*	1.74(0.61-2.60)*
	24	0.38 (0.04-0.94)	0.29 (0.06-1.61)	0.52 (0.07-1.33)

Statistics: Mann - Witney test, *$p \leq 0.05$ *versus* no-lavage (group I)

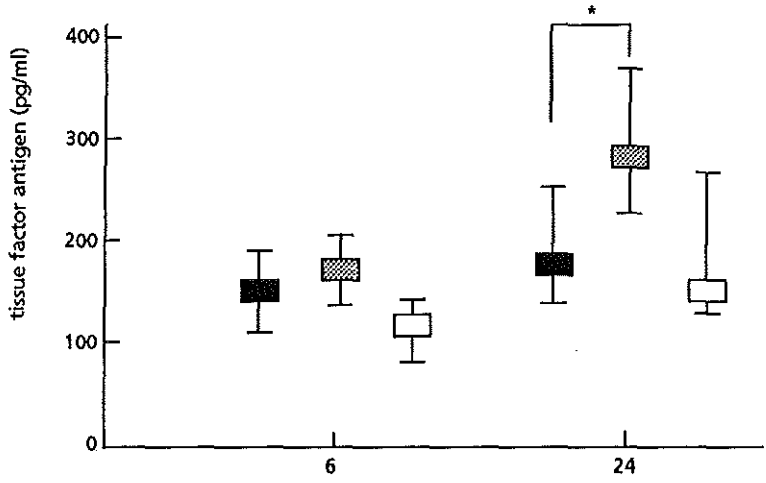
TF antigen in the 0-6h drain fluid was present in all groups in low concentration (*figure 3*). Intraperitoneal increase in TF antigen was significant in patients lavaged with NaCl 0.9%, whereas TF antigen was not increased in patients lavaged with distilled water after 24h compared with no lavage.

Figures 1 and 2.



IL-6 and -8 concentrations (ng/ml) in drain fluids (0-6h and 6-24h) of patients undergoing elective colectomy (■) without peritoneal lavage (n=10), or subsequent (▨)lavage with NaCl 0.9% (n=10) or (□) distilled water (n=10). Median values (95% confidence intervals) are given. *$p \leq 0.05$ tested with Mann-Whitney *versus* no-lavage (group I). **$p \leq 0.01$ *versus* groups.

Figure 3.



TF antigen concentration (pg/ml) in drain fluids (0-6h and 6-24h) of patients undergoing elective colectomy (■) without peritoneal lavage (n=10), or subsequent (▨) lavage with NaCl 0.9% (n=10) or (□) distilled water (n=10). Median values (95% confidence intervals) are given. *$p \leq 0.05$ tested with Mann-Whitney *versus* no-lavage (group I).

There was a reduction in tPA antigen concentration (ng/ml) between the early postoperative samples (0-6h) and late samples (6-24h) in all groups, but this reduction was not significant (*table 3*). Plasminogen activating activity (PAA) was present in the drain fluid 0-6h after surgery, but was reduced and undetectable in the 6-24h samples of all three groups. PAI-1 antigen concentration (ng/ml) showed an excessive rise 6-24h postoperatively. This rise started already in the early period after operation (0-6h) and was more pronounced in both lavaged groups. PAI-1 antigen as well as activity was slightly enhanced in the NaCl 0.9% lavaged group 0-6h after surgery, and significantly enhanced in the distilled water lavaged group in this period ($p \leq 0.05$ I vs III).

Discussion

Pro-inflammatory mediators are believed to regulate the host response to surgical trauma.[13] To date, this response has been principally related to

Table 3. Fibrinolytic parameters in the peritoneal fluid (0-6h and 6-24h) of patients undergoing elective colectomy without lavage (n=10), or subsequent peritoneal lavage with NaCl 0.9% (n=10) or distilled water (n=10). Median values (range) are given

6 h	no-lavage	NaCl 0.9%	distilled water
tPA antigen (ng/ml)	31.5 (15.5-60.9)	32.8 (17.9-53.7)	28.9 (18.4-50.9)
tPA activity (IU/ml)	3.2 (0.04-9.7)	5.8 (0.04-18.5)	0.2(0.03-9.5)
PAI antigen (ng/ml)	9.9 (0.03-46.7)	7.9 (3.6-80.2)	21.6 (7.0-131)*
PAI activity (IU/ml)	0.1 (0.1-1.1)	0.1 (0.1-3.3)	5.0 (0.1-24.2)*
24 h	no-lavage	NaCl 0.9%	distilled water
tPA antigen (ng/ml)	24.8 (17.2-39.9)	24.6 (2.5)	22.2 (10.6-42.5)
tPA activity (IU/ml)	0.15 (0.14-0.3)	0.16 (0.12-0.3)	0.14 (0.13-0.16)
PAI antigen (ng/ml)	428 (139-1017)	473 (138-1330)	313 (134-863)
PAI activity (IU/ml)	38.9 (20.9-55.3)	31.5 (12.2-58.1)	26.0 (9.0-57.9)

Statistics: Mann-Whitney test, * $p \leq 0.05$ tested *versus* no-lavage (group I).

factors involved in physiological tissue repair.[14,15] Other factors, rather than those eminating from the wound, may also be responsible for surgically induced immunological alterations.[1] Watson and co-workers suggested endotoxin-mediated air contamination of the peritoneal cavity as a trigger for postoperative immunomodulation. However, bacterial products are not essential for the release of cytokines, and peritoneal cells can be primed to release cytokines by other, non-endotoxin mechanisms.[16] A local cytokine response after operative trauma induces several inflammatory changes, including stimulation of MC.[17] This in its turn can induce chemotaxis of neutrophils, further production of cytokines and alterations in procoagulant and fibrinolytic activity.

This study shows the early postoperative intraperitoneal responses after surgery. Alterations in the levels of parameters, synthesized *de novo*, occur 0-6h after stimulation (operation/lavage). The 6-24h peritoneal drain fluid was collected to monitor the protracted changes in these early responses. In a few

patients drain fluid from two different sites of the abdominal cavity was collected, one drain was placed in the operated area and another on the contralateral side. In this way we excluded wound factors responsible for changes in the peritoneal responses. Different levels of mediators were found in both areas (data not shown), implying that there is not an overall reaction of the peritoneum.

Drain fluid represents the local response to stimuli. Sampling over time and storage for 6 to 18h at room temperature in plastic drain bags may influence degradation or production of e.g. cytokines and fibrinolytic enzymes by cellular components in the fluid. Therefore, the concentrations of all parameters measured may be an over- or underestimation of the peritoneal situation, and have to be considered with caution. Previously, it has also been shown that the elevated concentrations of cytokines observed in drain fluid of patients undergoing abdominal surgery correlated with peritoneal bacterial count and the length of the operation.[18] In this study, operation time, age, and indication were comparable between the three groups (mean/range). Although, bacteriologic studies confirmed the absence of gross bacterial contamination, postoperative peritoneal concentrations of IL-1β as well as IL-6 and IL-8 were elevated. IL-1β concentrations were not significantly changed in lavaged groups *versus* the control group (no lavage). Corroborating with other reports, levels of IL-1β did not differ in various patient groups operated after elective surgery.[19,20] IL-6 and IL-8 are cytokines more correlating with injury severity and appear to be regulated independently of commonly invoked mediators such as IL-1β. Interestingly, the lavaged groups, NaCl 0.9% as well as distilled water, showed a marked depression of IL-6 and IL-8 concentration in the 0-6h drain fluid ($p \leq 0.05$) compared with no lavage. Beyond its role to remove contamination, peritoneal toilet seems also effective in decreasing the intraperitoneal levels of IL-6 and IL-8 in the early period after operation. An explanation for this finding may be dilution. After abdominal lavage procedures some part of the lavage solution is inevitably left behind. Such fluid dilutes the local defence components.[21,22]

One role of IL-6 is to control the inflammatory response by inhibiting the transcription of other cytokine genes.[23] In this way IL-6 counteracts proinflammatory effects in the early postinjury period and thus prevents hyperinflammation.[19] Early depressed levels of IL-6 may therefore result in prolonged counter-regulation and late immunosuppression.

The directed secretion of IL-8 is important in the recruitment of neutrophils into the peritoneal cavity.[3] Interestingly, we observed significantly less leucocytes in the 6-24h drain fluid of NaCl 0.9% lavaged abdomen compared with no lavage. On one hand this can be due to depressed early IL-8 concentration in the NaCl 0.9% lavaged cavities. On the other hand leucocytes in the drain fluid may produce cytokines during sample time, and a lower leucocyte count may give rise to depressed cytokine concentrations compared with drain fluid of patients with no subsequent lavage. This phenomenon was not observed for lavage with distilled water and may be explained by the hypothesis that distilled water activates the peritoneum by other ways and facilitates leucocyte recruitment, for instance, via expression of adhesion molecules. In the 6-24h drain fluid we also observed significantly increased IL-6 concentrations in both lavaged groups compared with the 0-6h drain fluid, and an increased IL-8 concentration in the distilled water group. This may be due to peritoneal activation. Direct removal of cytokines by irrigation of the peritoneal cavity may induce a stronger response 24h postoperatively.

The question arises whether direct removal or prolonged activity of cytokines in the peritoneal cavity may be harmful. Cytokines such as IL-6 and IL-8 have beneficial properties in injury in low concentrations. However, enhanced local pro-inflammatory responses may lead to e.g. prolonged high amounts of toxic neutrophil products, detrimental to host tissue. This concept has been suggested in other focal infections, such as pulmonary inflammation[24] and meningitis[25]. Also both tumour stimulating, and inhibiting activities of IL-6 and IL-8 have been described.[26,27]

Serum levels of the various cytokines were not assesed, because several studies have shown absence of correlation between IL-6 and IL-8 in peritoneal fluid and serum.[28,29] Peritoneum and the vascular compartments are separately involved in the cytokine production.[30] Local peritoneal cytokine concentrations are more likely to be of primary biologic and clinical importance in regarding postoperative intra-abdominal complications.

Fibrin formation, another defence mechanism of the peritoneal cavity, is mediated via local coagulation and fibrinolytic pathways. TF is known to initiate coagulation after cellular activation. Increased levels of TF are associated with several pathologic conditions, such as inflammation, sepsis and cancer.[31,32] Cellular injury also results in an increase in TF as the cells in the

traumatized area lyse and release endogenous cell surface bound TF. Fareed reported comparatative peritoneal fluid levels for TF antigen after operation.[33] NaCl 0.9% caused a significant increase in TF antigen in the 6-24h drain fluid compared with no lavage or lavage with distilled water ($p \leq 0.05$). The reason for this may be the direct cellular damage inflicted by distilled water, with direct release of cellular bound TF.

Fibrinolytic activity in the early period after operation is important for lysis of newly formed fibrin by the coagulant cascade. In the abdominal cavity, tPA is responsible for 95% of the plasminogen conversion.[34] Therefore, intraperitoneal fibrinolytic activity is due to tPA activity. Although tPAA is reduced in the 6-24h drain fluid of all patients, we observed no significant reduction in tPA antigen compared with the 0-6h drain fluid. It is known that ablation of tPAA is secondary to a rise in PAI-1.[35,36] Only in the group lavaged with distilled water, the early (0-6h) tPAA decreased, due to an early rise in PAI-1 antigen and PAI-1 activity. However, the decrease in tPAA was not significant compared with the no-lavage group. Intraperitoneal fibrinolysis is hampered postoperatively, and peroperative lavage may accelerate this process. Because early thrombus formation is more sensitive for fibrinolytic factors than an esthablished thrombus, fibrinolytic activity in the early period after operation is probably decisive for the eventual formation of adhesions. Recently, Murthy *et al.*[37] reported fibrinogen, fibrin, and related proteins such as plasminogen activators and inhibitors may also be involved in facilitating tumor attachment at the injured site. This is mentioned because all patients underwent colectomy for malignancy. Peroperative lavage may thus also influence tumour recurrence rate by disturbing the peritoneal microenvironment.

The fact that IL-6 and IL-8, but not fibrinolytic parameters or IL-1, are sensitive to mechanical dilution may be explained by the source and biological behaviour of these products. Cellular components, in particular mesothelial cells, and their products are probably washed away during lavage. Fibrinolytic factors are not only produced by MC but the extracellular matrix is also a pool of fibrinolytic factors. This may account for the observation of depressed intraperitoneal IL-6 and IL-8 levels in the early period after lavage and the fact that tPA and PAI-1 levels are not affected by this procedure.

We have recently reported *in vitro* observations of cultured human MC exposed to various lavage solutions for 15 min. These studies have shown

important morphological and functional changes of MC. The results in the present *in vivo* study confirm these previous findings. Irrigation of the peritoneal cavity during operation affects postoperative peritoneal responses. Modulation of these responses may have the potential to reduce the morbidity and mortality of intra-abdominal surgical intervention.

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Oxidative stress in cultured peritoneal mesothelial cells

Inhibition of interleukin-8 induction by anti-oxidants

6

Abstract

Hydrogen peroxide (H_2O_2) and oxygen radicals are agents commonly produced during inflammatory processes. Exposure of human MC to H_2O_2 (100 μM) results in a cytotoxicity of 40% determined by leakage of LDH. Exposure to air (30-60 min) did not induce significant cell lysis; however, it enhanced the release of L-8 by MC monolayers time-dependently. Addition of N-acetylcyteine (NAC) and catalase to the medium partially protected the cells against H_2O_2-induced cytotoxicity. Pretreatment with combinations of intra- and extracellular scavengers also reduced the secretion of IL-8 by MC during recovery after exposure to air for 60 min. Stimulation of MC monolayers with IL-1β was significantly inhibited by pretreatment of monolayers with the oxygen radical scavenger NAC. We suggest that scavengers of reactive oxygen compounds may protect the peritoneum against oxygen-derived damage and subsequent cytokine stimulation. Addition of these agents to peroperative peritoneal lavage solutions may therefore reduce postoperative intra-abdominal complications.

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Introduction

Mesothelium is thought to be an important target site of oxidative stress under conditions such as intra-abdominal surgical intervention. Exposure to air and manipulation of this tissue may lead to ischemia, which induces peritoneal inflammatory reactions. Peritoneal injury and inflammation is associated with recruitment of neutrophils and macrophages,[1] which can produce highly potent substances. These substances include cytokines and oxidant molecules, such as H_2O_2, free radicals, and hypochlorous acid. The purpose of these reactive cell products is to destroy invading organisms and damaged tissue and thus to bring about recovery. However, oxidants and cytokines can also damage healthy tissue. Excessive or inappropriate production of these substances is associated with morbidity and mortality after peritoneal trauma and inflammatory diseases.[2,3]

Mammalian cells posses elaborate defence mechanisms to detoxify radicals. Sophisticated antioxidant defences directly and indirectly protect the host against damaging influence of cytokines and oxidants.[4] Different cell types may exhibit different mechanisms against exogenous free radicals. Antioxidant defence mechanisms in cultured MC have been studied previously by Kinnula *et al.*[5] and Breborowicz *et al.*[6,7] They observed that MC are protected mainly by the gluthatione redox system during mild oxidant exposure, and in addition by catalase during severe oxidant exposure. Effects of antioxidants in other experimental work, mainly concerning endothelial cells, suggest protection from activated neutrophils and ischemia-reperfusion-induced injury by pretreatment with various scavengers.[8-10]

This study presents three *in vitro* models of MC activation, which are supposed to mimic conditions during intra-abdominal surgery. More than any other chemotaxin IL-8 has a remarkably long duration of action and is produced by a wide variety of tissue cells.[11] We proposed that stress upon MC can act as a stimulus for the production of IL-8, with resultant *in vivo* neutrophil influx. H_2O_2 is one of the more important reactive oxygen species (ROS) produced by phagocytic cells. For this reason, $H_2O_2$was selected in the first model, as an agent for inducing exogenous oxidative stress in MC. Results obtained from *in vivo* experiments suggest that oxygen-derived free radicals are abundantly produced in ischemic tissue after reoxygenation.[12] This production far exceeds the amount of free radicals produced during normal aerobic metabolism. In the second model the influence of air-drying

conditions in cultured MC was determined by leaving the monolayers during 15-60 min without culture medium. MC responses were evaluated after the subsequent recovery period. In the third model, IL-1β was added to MC monolayers. IL-1β may cause endogenously elevated levels of H_2O_2, which appears to act as intracellular 'messenger' capable of promoting the release of chemokines such as IL-8.[13,14] The chemotactic activity of IL-8 participates in the recruitment of leucocytes. In these models, the effects on MC viability, morphology and pro-inflammatory response (IL-8) was determined and protection of MC properties in the presence of scavengers was examined.

Methods and materials

Materials. IL-1β was obtained from R&D system (Abington, UK). NAC and catalase were from Sigma (St. Louis, MO, USA). H_2O_2 30% was from Merck (Darmstadt, Germany), and NADH from Boehringer (Mannheim, Germany).

Culture and identification of omental mesothelial cells. MC were obtained from the omental tissue of patients undergoing elective abdominal surgery who had given informed consent. The MC were isolated according to techniques modified from Nicholson *et al.*[15] and Wu *et al.*[16] and cultured as described previously. MC were grown until confluence in a 37°C, fully humidified, 5% CO_2 cabinet in polystyrene culture flasks (80 cm^2; Nunc, Roskilde, Denmark) precoated with fibronectin (gift of the Central Laboratory of the Netherlands Red Cross (CLB), Amsterdam, The Netherlands). The identity of MC was demonstrated by the absence of von Willebrand factor staining[17] and the presence of intracellular cytokeratins via immunofluorescence with monoclonal antibodies (Dakopatt, Denmark).[18]

Measurement of intracellular hydrogen peroxide production by mesothelial cells. H_2O_2 production in MC was determined with the dichlorofluoresceine diacetate assay. Dichlorofluoresceine diacetate (DCFH-DA; Kodak) is a stable non-polar, non-fluorescent compound that readily diffuses into cells. Once inside cells it becomes trapped by its hydrolysis by cytosolic esterases to the polar, non-fluorescent dichlorofluoresceine. In the presence of H_2O_2 and peroxidase the latter is rapidly oxidized to a highly fluorescent compound.[19] Thus, the intensity of cellular fluorescence is proportional to levels of intracellular H_2O_2 and can be monitored by flow cytometry. Cell suspensions

(2-5 x 10^6 cells/ml) were incubated with 5 µM DCFH-DA at 37°C for 30 min in M-199 containing 1% BSA (Sigma). Preliminary experiments had shown this loading procedure to be optimal. After loading and subsequent centrifugation at 800 *g*, additions were made (time zero) and fluorescence was measured with a FACscan (Becton-Dickinson). For each analysis 5000 events were accumulated. Fluorescence was measured at 450 nm. Experiments were performed in duplicate.

Mesothelial stress models. Second pasage MC were seeded into 48-well culture plates (Costar, Cambridge, UK) precoated with fibronectin and were allowed to grow to confluence before use. For inhibition studies with oxygen radical scavengers, NAC (2 mM) and catalase (25 µg/ml) were added to the serum-containing medium for 1h before adding the stimulus (IL-1β; 0.1 ng/ml) or H_2O_2 (0-100 µM)) in the continued presence of the inhibitor(s). After incubation for up to 24h, the conditioned medium was harvested and centrifuged for 5 min at 800 *g*. The samples were immediatly assayed or frozen at -70°C until use.

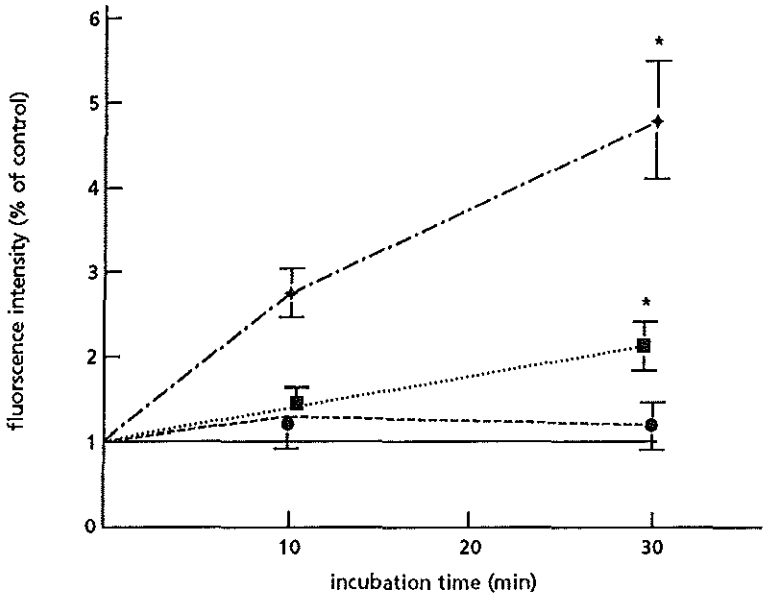
To induce an air-drying period, MC monolayers were also seeded into 48-well culture plates (Costar) and were placed with different time intervals in the 37°C, 5% CO_2 cabinet without medium. Firstly, MC monolayers were pretreated with NAC and catalase 1h before air-drying. And secondly, MC monolayers were treated after 15, 30 or 60 min, with fresh supplemented medium (NAC and catalase alone, or NAC and catalase in combination). After 24h, supernatants were collected, centrifuged at 800 *g* and assayed or frozen at -70°C until use. All experiments were performed in triplicate.

Assessment of mesothelial cell injury. Cell lysis can be measured in MC monolayers by the release of the endogenous intracellular marker LDH, as described previously.[20] The LDH release was expressed as percentage of total (100%) cellular enzyme activity liberated from the cells by 1% (v/v) Triton-X100. All experiments were performed in triplicate.

Interleukin-8 release. Supernatants were assayed for quantification of synthesized IL-8 by an enzyme-linked immunosorbent assay (Pelikine™, CLB, Amsterdam, The Netherlands). The limit of detection was 1 pg/ml. All determinations were performed in quadruplicate.

Statistical analysis. Values are expressed as mean ± SD. Statistical significance was determined with the Student-*t* test, with significance defined as $p < 0.05$.

Figure 1. H_2O_2 generation measured by the DCFH-DA assay



MC suspensions were incubated for 30 min with DCFH-DA followed by incubation with (-) control medium (M-199 with 1% BSA), (■) PMA (10 ng/ml), (●) IL-1β (2 ng/ml), or (✦) H_2O_2 (10 μM). Data are presented as the increase in fluorescence, as a percentage of control fluorescence ± SEM obtained from three experiments in duplicate. *$p < 0.05$ tested with the paired Student's-*t* test *versus* the positive control (M-199 0.5% BSA).

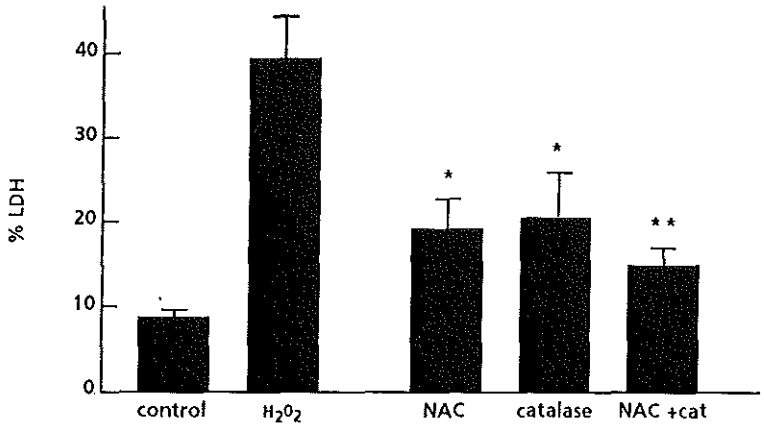
Results

Intracellular hydrogen peroxide production of mesothelial cells. MC suspensions in control medium (M-199 supplemented with 0.5% BSA) were found to have a detectable DCF fluorescence. Compared with human umbilical cord vein endothelial cells, the basal DCF fluorescence of MC was about 50% lower. Control experiments showed both cell types to have a similar pattern of changes in DCF fluorescence after stimulation with various compounds (data not shown). Incubation with PMA (10 ng/ml) induced a 2 to 3-fold increase of DCF fluorescence in MC compared with basal levels, while incubation with IL-1 (2 ng/ml) had no significant effect on the DCF fluorescence of MC

(figure 1). Exogenously added H_2O_2 (10 μM) increased the DCF fluorescence level of MC even 4 to 5 times.

Effect of oxidant scavengers on mesothelial cell injury and IL-8 release. Firstly, the ability of H_2O_2 to lyse MC monolayers was studied. The cells were incubated with concentrations of 0 to 1000 μM H_2O_2 diluted in supplemented medium. H_2O_2 dose-dependently induced MC lysis (data not shown). H_2O_2 at a concentration of 100 μM resulted in 40 ± 9.8% cytotoxicity of MC monolayers, as measured by LDH release *(figure 2)*. Pretreatment of MC monolayers with oxygen radical scavengers prevented MC lysis by 100 μM added H_2O_2. NAC as well as catalase reduced LDH release of MC to 20 ± 6.0% *(p=* 0.045) and 22 ± 8.8% *(p=* 0.016), respectively. A combination of intra- and extracellular scavengers (NAC and catalase) reduced H_2O_2 induced cell lysis even more (14 ± 3.6%, *p=* 0.006).

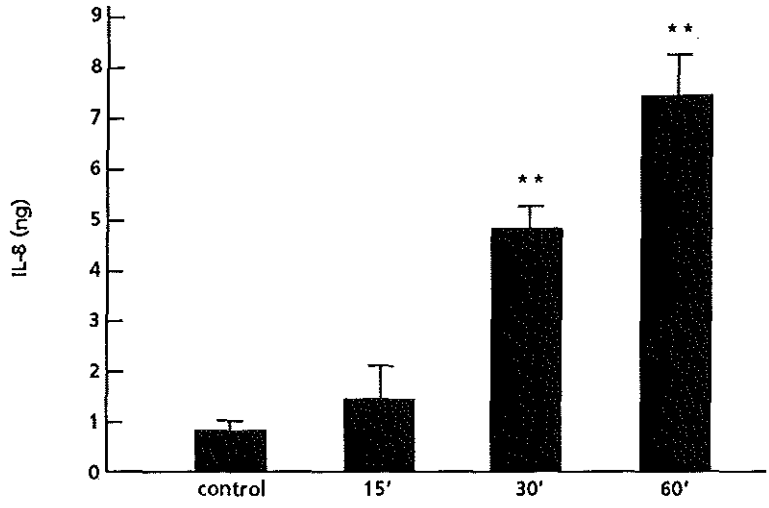
Figure 2. Human omental MC lysis by H_2O_2



Confluent monolayers of MC were incubated with 100 μM H_2O_2 in control medium (M-199 10% FCS). Cytotoxicity was measured after 24h of incubation at 37°C, 5% CO_2. Data are expressed as mean ± SD in percent LDH release obtained from five experiments in triplicate. *p< 0.05, **p< 0.001 tested with the Student's-t test *versus* the positive control (H_2O_2). NAC = N-acetylcystene, cat = catalase.

Secondly, the effect of air-drying on MC monolayers was examined. MC lysis was not significantly affected during the 15-60 min exposure to air (data not shown). However, the amount of IL-8 in the supernatant was found to

Figure 3. Human omental MC IL-8 generation after air-dry periods (15-60 min)

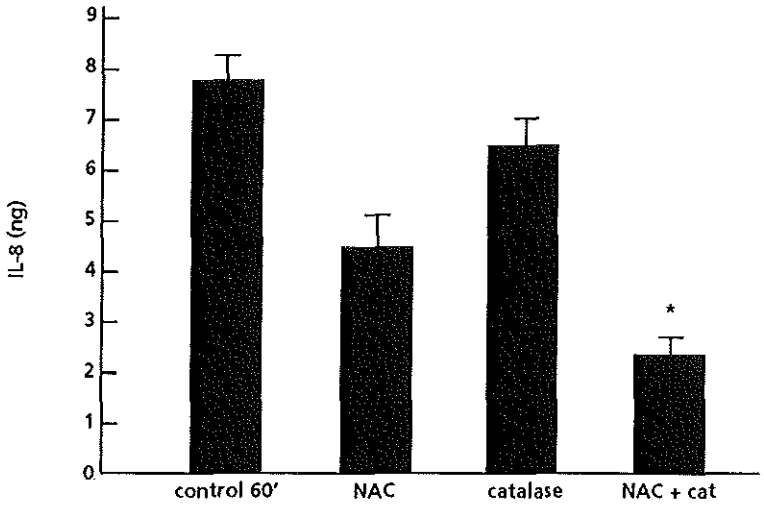


Control medium from confluent monolayers of MC was removed, and MC were exposed to air (37°C, 5% CO_2) during various time periods. Then 500 µl of medium was replaced, and after 24h of incubation, LDH and IL-8 release were measured in the supernatants. Data are expressed as mean ± SD amount of IL-8 in ng per 10^5 cells, and obtained from four separate experiments in triplicate. *$p < 0.05$, **$p < 0.001$ tested with the Student's-t test *versus* the negative control (M-199 10% FCS).

increase in a time-dependent fashion, as shown in Figure 3. IL-8 release was significantly increased in the conditioned medium 24h after 30 and 60 min (4.9 ± 0.7 and 8.0 ± 1.1 ng, respectivily) of air-drying compared with cells exposed to control medium (0.8 ± 0.5 ng). Pretreatment of the MC monolayers with NAC and catalase together before exposure to air significantly reduced the amount of IL-8 in the 24h supernatant (*figure 4*, $p < 0.05$). Treatment with the above-mentioned oxygen radical scavengers, even in combination, *after* exposure to air, during the recovery period of the MC, did not significantly reduce IL-8 release (data not shown).

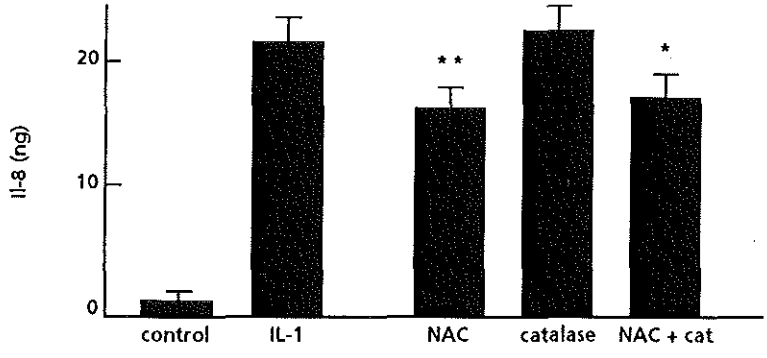
Thirdly, stimulation of MC monolayers with IL-1β (0.1 ng/ml) did not cause significant cell lysis. After 24h the amount of IL-8 in the conditioned medium was 29.3 ± 6.5 ng. Pretreated MC monolayers (NAC and catalase) showed lower amounts of IL-8 in the 24h supernatant of IL-1-stimulated MC monolayers (*figure 5*).

Figure 4. IL-8 secretion of MC after air drying and preincubation with scavangers



IL-8 secretion in the 24h supernatants of MC monolayers after 60 min of air drying, preincubated with NAC (2 mM), catalase (25 µg/ml) or a combination of both scavengers for 1h. Data are expressed as mean ± SD amount of IL-8 in ng per 10^5 cells, and obtained from four separate experiments in triplicate. *p< 0.05 tested with the Student's-t test *versus* the positive control (60 min air-drying with preincubation with M-199 10% FCS). NAC = N-acetylcystene, cat = catalase.

Figure 5. IL-8 secretion of pretreated MC following incubation with IL-1



IL-8 secretion in the 24h supernatants of MC monolayers pretreated with scavengers and subsequently stimulated with IL-1β (0.1 ng/ml). The data presented are the mean and ± SD from six separate experiments in triplicate with MC prepared from separate omental specimens. *p< 0.01, **p< 0.001 *versus* the positive control (non-pretreated MC stimulated with IL-1β). NAC = N-acetylcystene, cat = catalase.

Discussion

Peritoneal MC are uniquely located to regulate cellular events in the peritoneal cavity and are a potentially important source of various cytokines. The side effects of abdominal surgery may depend for a large part on viability and secretion of inflammatory and regulatory mediators by MC. Several studies have indicated that ROS play a central role in inflammation, adhesion formation, and/or carcinogenic responses.[21-23] Cellular injury caused by oxygen radicals is manifested by degradation of hyaluronic acid and collagen, destruction of cell membranes through the peroxidation of fatty acids within the phospholipid membrane, disruption of organelle membranes such as those surrounding lysosomes and mitochondria, and interference with important enzyme systems.[24] The possible loss of lipids from cell membranes changes receptor function and secretory capabilities of the cells.[25] The effects of ROS on cytokine stimulation, secretion and function have not been well defined. Several lines of evidence indicate that ROS may regulate production of cytokines.[26,27] ROS induce production of IL-8, which in turn induces further generation of superoxide as well as neutrophil infiltration, thereby establishing the injury. In previous studies we described increased IL-8 release after 15 min incubation of MC monolayers with micro-molar concentrations of H_2O_2.[20] We now found that air-dry conditions and treatment of MC monolayers with IL-1β also increase the release of this pro-inflammatory mediator. The three *in vitro* models described in this study are meant to mimic the type of postoperative injury induced during intra-abdominal surgery.

This raises the question whether IL-8 release, induced by different forms of oxidative stress, is sensitive to exogenously added antioxidants. Intracellular oxidants in MC may be an indication for sensitivity of these cells for the protective properties of scavengers. A low basal level of intracellular oxidants renders it a suitable signal for intracellular responses when the extracellular H_2O_2 concentration is increased during an inflammatory process.[28] Therefore, we measured basal H_2O_2 concentration in MC and changes in the cellular H_2O_2 generation in intact cells exposed to various stimuli. The results show a low basal intracellular concentration of H_2O_2, and increased levels upon activation with various stimuli such as H_2O_2 and PMA. This is in concordance with observations in rat MC and emphasizes the participation of MC in the peritoneal defence.[29] Shostak and co-workers also observed a decrease in H_2O_2 generation of rat MC after 60 min of

incubation with catalase.

In addition, when scavengers affect various oxidant-mediated mechanisms, they may have the potential to reduce postoperative inflammation-mediated complications. NAC as an intracellular pro-glutathione drug, in combination with catalase, an extracellular scavenger of H_2O_2, may protect MC in an optimal way. NAC can only react very slowly with H_2O_2,[30] catalase is therefore essential for directly reacting with exogenous H_2O_2. Other scavengers have also been shown to be protective in many models of oxidant-mediated tissue damage.[31] However, recent data indicate that MC scavenge low exogenous H_2O_2 concentrations mainly by the gluthatione redox pathway.[5,32] Therefore, we investigated whether NAC could protect MC against the deleterious effects of oxidant-mediated conditions. Pretreatment with NAC, alone and in combination with catalase, protected MC against cell lysis. Our data also show a time-dependent relationship between duration of air-dry preconditioning and subsequent IL-8 release. The maximal duration of pre-exposure necessary to augment IL-8 generation without alterations in cellular viability was 60 min. Pretreated MC monolayers produced significantly less IL-8 in the recovery period after 60 min exposure to air. A combination of NAC and catalase protected the cells even more than pretreatment with NAC alone. Addition of scavengers in the recovery period after air-dry periods did not diminish IL-8 release. Only pretreatment prevented mesothelial cells from induction of cytokine production. Therefore, scavengers should already be introduced when the peritoneal cavity is entered, before oxidative stress occurs.

We observed significantly decreased levels of IL-8 after IL-1β stimulation of MC monolayers in the presence of NAC. The presence of catalase did not affect the IL-1β induced IL-8 synthesis. It appears that the ability of cytokines such as IL-1β to induce IL-8 expression is partly a consequence of their ability to induce specific cellular responses through activation of the transcription factor nuclear factor kappa beta (NF-kappa B).[33] Oxygen radicals may be involved in the activation of the NF-kappa B,[34,35] and intracellular gluthatione levels are assumed to play a major role in the regulation of this process.[36-38] The ability of cell membrane-permeable scavengers and the inability of nonpermeable agents to augment the reduction in IL-8 amount suggest that cytokine-mediated induction of IL-8 is mainly due to intracellular ROS.

The studies presented in this paper show that different forms of oxidative stress induced activation of IL-8 release by MC. Exposure to micromolar concentrations of H_2O_2 caused severe cell damage, and air-dry periods of more than 15 min induced significant cell activation. Pretreatment of MC monolayers with NAC and catalase prevented the increased cell damage and production of IL-8 in response to this oxidative stress. Cytokine-mediated IL-8 induction was also inhibited by treatment of MC monolayers by NAC. In conclusion, there may be a potential role for antioxidants in modifying the risk for oxidative stress during surgical interventions. We suggest that treatment of the peritoneum during surgery by supplementation of a peroperative lavage solution with these scavengers may reduce post-operative intra-abdominal complications.

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Chlamydia trachomatis infection of human mesothelial cells alters pro-inflammatory, procoagulant, and fibrinolytic responses

7

Abstract

Chlamydia trachomatis is a significant cause of complications of upper urogenital tract and intra-abdominal cavity infections, which may depend on local immunologically mediated inflammation and fibrosis. In this study we demonstrate the capability of *C. trachomatis* to infect cultured human MC monolayers. This intracellular pathogen caused significant MC monolayer damage after 72h. In decreased concentrations *C. trachomatis* induced the production of both pro-inflammatory cytokines IL-1β and IL-8. Heat-killed chlamydiae, which were ingested by MC, only induced IL-1β and not IL-8 release. Seventy-two hours after initial infection procoagulant activity of MC was induced; MC showed a 1.5-fold increase in tissue factor (TF) expression. The activity of the fibrinolytic inhibitor (PAI-1) was also enhanced in the supernatants obtained 48-72h after initial infection of MC monolayers. These findings support the hypothesis that provoked pro-inflammatory responses, induced procoagulant activity, and inhibition of fibrinolysis contribute to the development of complications after chlamydial infection.

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Introduction

Chlamydial infections of the urogenital tract are frequently asymptomatic but can result in severe complications, particularly in women. Although *C. trachomatis* preferentially infects columnar or transitional epithelium and thus most often produces mucosal infection, this micro-organism can also induce more invasive infections involving the Fallopian tube or hepatic capsule causing the Fitz-Hugh-Curtis syndrome.[1] The disease process and clinical manifestation of chlamydial infections probably represent the combined effects of tissue damage resulting from chlamydial replication as well as the inflammatory responses caused by the presence of chlamydiae and necrotic material from destroyed host cells.[2]

Progression and complications induced by ascending *C. trachomatis* infections may depend on local immunologically mediated inflammation and fibrosis.[3,4,5] However, little is known about the pathogenesis of this infection. In order to investigate the effect of chlamydial infection on peritoneal defence mechanisms, we initiated infection of human MC with *C. trachomatis*, and studied pro-inflammatory, fibrinolytic and procoagulant responses during one life cycle (24-72h period).

Materials and methods

Culture and identification of omental mesothelial cells. MC were obtained from the omental tissue of patients undergoing elective abdominal surgery who had given informed consent. The MC were isolated according to techniques modified from Nicholson *et al.*[6] and Wu *et al.*[7] and cultured as described previously. The identity of MC was demonstrated by the absence of von Willebrand factor staining[8] and the presence of intracellular cytokeratins using immunofluorescence with monoclonal antibodies (Dakopatt, Denmark).[9]

Chlamydia stock preparation. A *C. trachomatis* clinical isolate was used for all experiments. Strains were propagated on a Buffalo Green Monkey (BGM) cell line with Minimal Essential Medium with Earle's salts (EMEM, Life Technologies Ltd., Paisley, Scotland) supplemented with 10% FCS. Confirmation of strain indentity was performed with a monoclonal antibody (De Beer Medicals, Diessen, The Netherlands). After more than 80% of all cells showed changes in morphology due to infection e.g. cytopathologic

effect, as determined by light microscopy evaluation, the chlamydia suspension was frozen at -70°C after addition of SPG medium (sucrose 68.5 g/l, K_2HPO_4 2.1 g/l, KH_2PO_4 1.1 g/l, L-glutamine 7.2 g/l diluted in destilled water supplemented with 10% FCS, gentamycin 18 mg/l, vancomycin 23 mg/l, and amphotericin B 2.5 mg/l). No cyclohexamide was added to the medium, because this could interfere with MC protein synthesis.

Chlamydial inoculation of mesothelial monolayers. Mesothelial cell monolayers (second passage) grown to confluence in 48-well tissue culture plates were used for infection experiments. For all experiments MC monolayers were incubated with 500 µl of supplemented M-199 and 100 µl of (diluted) chlamydia suspension for 24h. Stock solutions were diluted in supplemented M-199. MC monolayers incubated with 500 µl of supplemented M-199 and 100 µl of SPG served in all experiment as control medium. After 24h the conditioned medium was collected, and centrifuged (800 *xg*). The supernatants were stored at -70°C for immunoassays. Fresh supplemented M-199 was added to the MC monolayers. The same procedure was followed 48h and 72h after initial infection.

For quantification of infection, cells were fixed for 30 min in absolute methanol/acetone, air dried, stained with fluorescein-conjugated murine monoclonal antibodies and examined microscopically for inclusions. The number of inclusion-forming units was counted in 10 random fields at 200x magnification. Heat-inactivated (30 min at 56°C) and ultracentrifuged (2 h at 100,000 *xg*) *C. trachomatis* suspensions were also investigated for their capability to infect MC monolayers.

Assessment of mesothelial cell injury. MC toxicity was assessed by examining the monolayers under phase-contrast microscopy and by measurement of LDH activity.[10] Mesothelial cells were seeded into 48-well culture plates and grown to confluent monolayers. Conditioned medium of MC monolayers 24, 48, and 72h after incubation with various dilutions of virus suspensions, were assayed spectrophotometrically (Perkin-Elmer Lambda 2) for LDH activity according to Bergmeyer *et al.*[11] The LDH release was expressed as percentage of total (100%) cellular enzyme activity liberated from the cells by 1% (v/v) Triton-X100. All experiments were performed in triplicate.

Immunoassays. IL-1β and IL-8 were detected with an enzyme-linked immunosorbent assay (Pelikine-compact™, CLB, Amsterdam, The Netherlands). Quantification of PAI-1 activity was determined in the same supernatants by means of an enzyme-linked immunosorbent assay (Spectrolyse®, Biopool, Umea, Sweden). Determinations were performed in duplicate, while all experiments were performed in triplicate. IL-1β (2 ng/ml; R&D systems, Abingdon, UK) is known to stimulate PAI-1 production and to inhibit tPA production by MC, and served in all experiments as a positive control.

Tissue factor activity assay. Studies were performed in 48-well tissue culture plates at 37°C. Infected MC monolayers were washed once with a Hepes buffer (25mM Hepes, 135 mM NaCl, 5 mM KCl, 4.5 mM glucose, pH 7.4, 0.3% BSA) and twice with the same buffer containing 5 mM $CaCl_2$. Factor VII and factor X were purified from plasma from healthy donors and diluted in the Hepes-$CaCl_2$ buffer to concentrations of 2.0 nM and 168 nM, respectively. The MC in each well were preincubated with 100 µl of factor VII for 5 min, after which 50 µl of factor X was added. The cells were briefly shaken and after 15 min the reaction was stopped by adding 25 µl of the sample to 50 µl of EDTA (25 mM in Hepes buffer) in a 96-well plastic assay plate. To measure the total amount of activated factor X (factor Xa) formed, 100 µl of 0.2 mM chromogenic substrate S2765 (Chromogenix, Nodia, The Netherlands) was added and the absorbance at 405 nm was measured for 1 min in a V-max ELISA reader (Molecular Devices, Menlo Park, CA, USA). TF expression was expressed as pM factor Xa formed per minute by 10^5 MC compared with control (100%).

Statistical analysis. Results are expressed as mean and standard deviation (SD). The data were submitted to statistical analysis with the paired two-tailed Student-*t* test where groups were compared with a control.

Results

Susceptibility and cell damage of mesothelial monolayers. MC monolayers were highly susceptible to infection with *C. trachomatis.* After infection all monolayers were intact as determined by phase-contrast microscopy and LDH activity (*table 1*). Significant cell injury occurred 72h after initial infection for non-diluted (1:1) and 1:5 diluted chlamydiae suspensions. Therefore, a 1:10

dilution of the chlamydia stock solution was used in all subsequent experiments. Actual infection percentage of MC monolayers after 48h incubation with 1:10 diluted stock solution of *C. trachomatis* was comparable with that of the standard BGM cell line and showed approximately 17% (30 inclusion bodies per 500 MC at 200x magnification).

Table 1. Cell damage (% LDH release) after infection with *Chlamydia trachomatis*

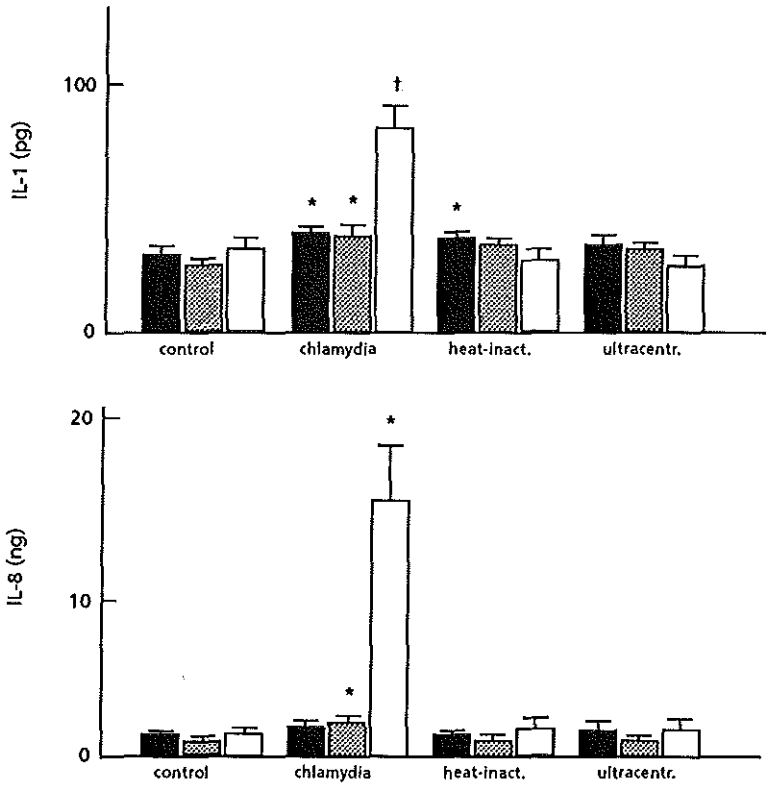
	0-24h	24-48h	48-72h
control	8.4 (2.2)	7.3 (1.1)	7.0 (0.2)
IL-1	6.9 (1.5)	8.1 (1.6)	10.1 (1.1)
C. trachomatis stock			
1:1	10.2 (2.2)	8.4 (1.4)	25.2 (5.7)‡
1:5	7.5 (0.5)	6.4 (1.1)	13.1 (3.6)*
1:10	7.2 (1.1)	7.4 (1.5)	9.7 (2.0)
1:20	6.5 (1.0)	8.8 (1.6)	8.5 (2.4)
heat-inactivated	6.2 (1.6)	6.8 (0.6)	6.4 (0.1)
ultracentrifuged	5.2 (0.4)	6.3 (0.5)	6.5 (0.8)

Statistics: * $p < 0.05$, ‡ $p < 0.001$, compared to control medium (M-199 0.2% SPG).

Interleukin-1β and interleukin-8 release by human mesothelial cells. Unstimulated cultured MC monolayers released small amounts of IL-1β (28.2 ±7.4 pg) and IL-8 (1.38 ±0.2 ng) during incubation with control medium (M-199 with 0.2% SPG). When MC were cultured with viable chlamydiae, increased IL-1β and IL-8 responses were observed (*figure 1*). Incubation with a 1:10 diluted stock solution resulted in an induction of IL-1β concentration in the supernatants reaching maximal levels by 72h after initiation of cultures (78.9 ±13.5 pg). Stimulation with IL-1β (2 ng/ml) induced the release of 37.6 ±13 ng of IL-8 (data not shown). The release of IL-8 in response to infection with 1:10 diluted stock of *C. trachomatis* rose significantly above background levels after 24-48h (2.0 ±0.4 ng), and rapidly increased after 48-72h incubation (11.9 ±3.9 ng). Heat-killed chlamydiae were ingested by MC but no intracellular growth was observed (*figure 2*). Figure 1 also indicates that heat-killed

C. trachomatis significantly enhanced the amount of IL-1β 0-24h after initial infection. IL-8 generation was not induced by heat-killed chlamydiae during the 72h period.

Figure 1. IL-1β and -8 release in the supernatant of MC infected with *C. trachomatis*

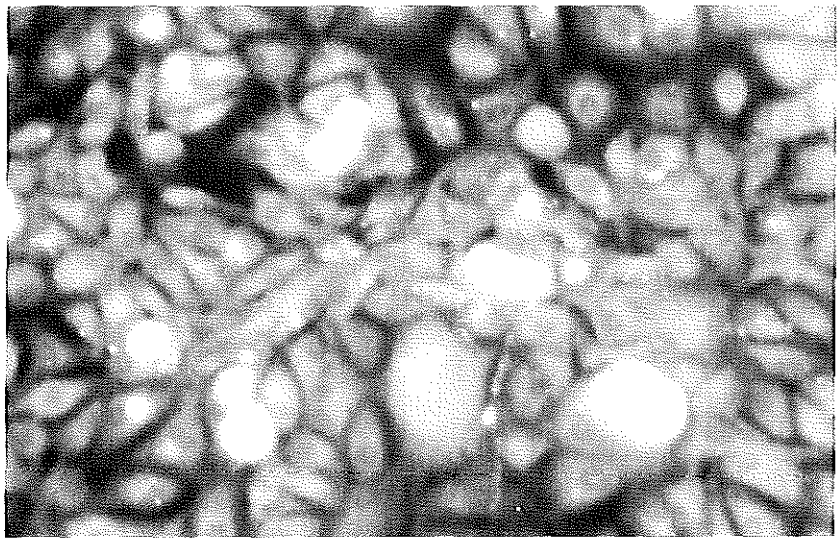


Levels are expressed as mean IL-1β amount in pg (± SD) and IL-8 amount in ng (± SD) per 10^5 MC, based on four separate experiments in duplicate. (■) 0-24h, (▨) 24-48h, and (□) 48-72h. *$p<0.05$, †$p<0.01$, statistical significant differences compared with MC monolayers exposed to control medium (supplemented M-199 containing 0.2% (v/v) SPG medium). heat-inact= heat-inactivated chlamydia suspension, ultracentr= ultracentrifuged chlamydia suspension.

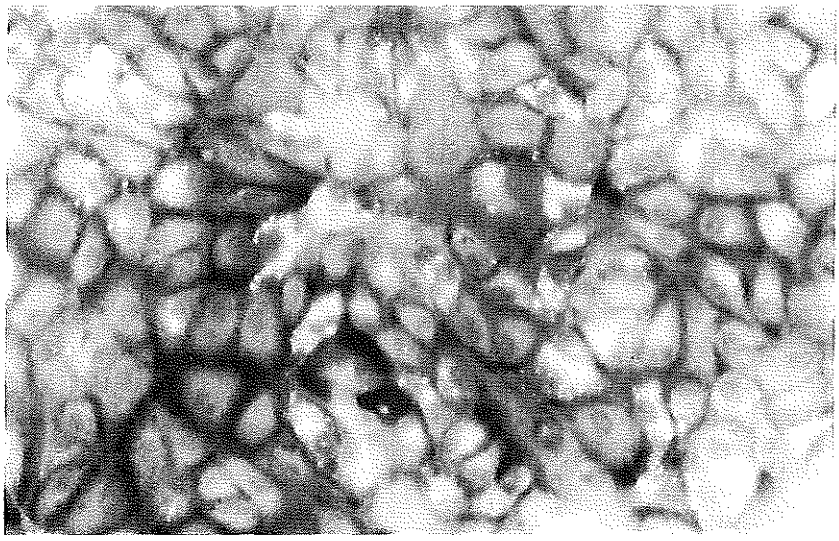
Procoagulant activity by human mesothelial cells. Procoagulant activity was studied on MC monolayers with a chromogenic substrate technique that meaures factor Xa activity. Factor Xa generation is directly related to TF

Figure 2.

a.



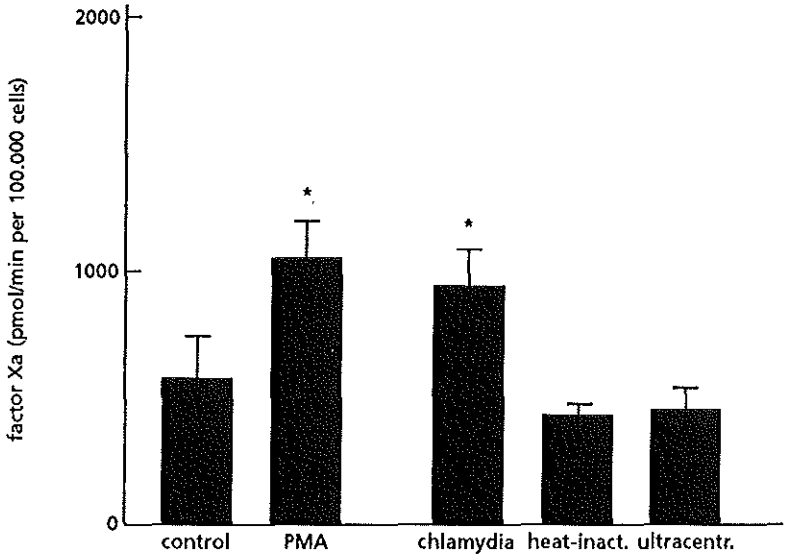
b.



Phase-contrast microscopy of chlamydia uptake in MC during 48h incubation with *a)* viable *C. trachomatis* (1:10 diluted stock suspension) and *b)* heat-killed chlamydiae suspension (400x magnification).

expression on the MC membrane. Without cells or with one of the clotting factors (VII or X) lacking, no Xa was formed. The results are shown in Figure 3. As a positive control we used PMA (10 ng/ml), because this agent proved to be a stronger inducer of TF expression on MC than IL-1β. Fourty-eight hours after initial chlamydial infection TF expression was significantly induced. Compared to the negative control a 1.5-fold increase in TF expression was observed. Incubation with heat-inactivated or ultracentrifuged chlamydia did not result in significantly enhanced TF expression.

Figure 3. TF activity of MC 72h after initial infection with *C. trachomatis*

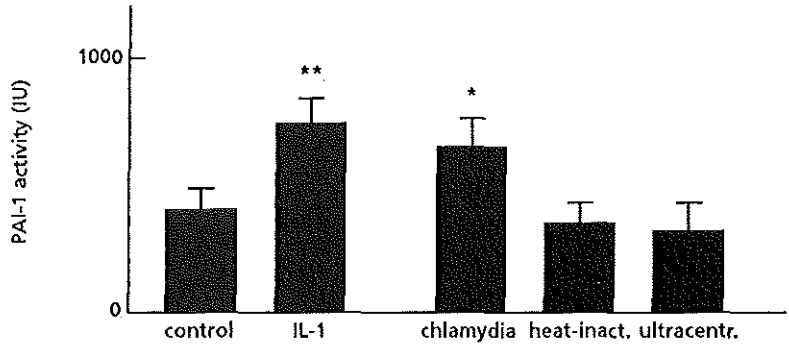


Results are given as mean factor Xa generation in pmol/min per 10^5 cells (± SEM) and are based on four separate experiments in triplicate. *$p<0.05$, statistically significant differences compared with MC monolayers exposed to control medium (supplemented M-199 containing 0.2% (v/v) SPG medium). PMA= phorbol myristrate acetate, heat-inact= heat-inactivated chlamydia suspension, ultracentr= ultracentrifuged chlamydia suspension.

Antifibrinolytic (PAI-1) activity of human mesothelial cells. The supernatants from unstimulated, non-infected and infected MC monolayers were assessed for fibrinolytic activity by ELISA. In response to IL-1β, PAI-1 activity of MC monolayers is altered directly after stimulation. This response continued 48

and 72h after initial stimulation. Kinetics of chlamydia-induced PAI-1 activity were different compared to those of the IL-1β-induced response: PAI-1 activity was only significantly enhanced in supernatants obtained 72h after initial chlamydia infection (*figure 4*).

Figure 4. PAI-1 activity of MC 72h after initial infection with *C. trachomatis*



Results are given as mean PAI-1 activity (IU per 10^5 cells and SD) and based on four separate experiments in triplicate. *$p<0.05$, **$p<0.01$ statistical significant differences compared with MC monolayers exposed to control medium (supplemented M-199 containing 0.2% (v/v) SPG medium). heat-inact= heat-inactivated chlamydia suspension, ultracentr= ultracentrifuged chlamydia suspension.

Discussion

Local immune system activation by *C. trachomatis*, and not the infection per se, may be responsible for the tissue damage and fibrosis seen in chlamydial associated infections. Immune mediated mechanisms may alter the pathogenesis or the course of the disease. Complications such as peritonitis, and the typical violin string adhesions seen may occur after chlamydial infection of the peritoneal serosal lining. *C. trachomatis* has a very limited host range *in vivo* and appears to be exclusively a parasite of squamocolumnar epithelial cells. In this study MC, obtained from human omental species, were shown to be very sensitive host cells *in vitro*. The inflammatory changes of these host cells induced by *C. trachomatis* was the subject of this study. During inflammatory events MC are activated either directly by bacterial products or by cytokines secreted by peritoneal cells, such as IL-1.[12] Intraperitoneal

administration of IL-1, which is not chemotactic *in vitro*, provokes a rapid influx of PMN.[13] The mesothelium is involved in the process of transmesothelial migration of neutrophils, via synthesis of IL-8.[14,15] The inflammatory response after primary inoculation with chlamydia spp. *in vivo* is characterized by infiltration with neutrophils and cytokine secretion in the acute phase.[16-18] The results of this study show induction of IL-8 release by MC at the onset of infection with *C. trachomatis*. We consider that the chemoattractant IL-8, produced at the serosal surface, is responsible for the neutrophil influx that occurs during invasive chlamydia infection. Epithelial cells may be an important source of IL-8 during the initial urogenital infection.[19] Recently, Rasmussen and co-workers showed increased production of pro-inflammatory cytokines by epithelial cells in response to chlamydial infection.[20] It can be hypothesized that chlamydial inclusion results in endogenous production of IL-1β by mesothelial cells, which might activate neighbouring cells, and thus spread the inflammatory response.

In contrast, Rothermel *et al.*[21] and Magee *et al.*[22] reported that *C. trachomatis* induced IL-1 production *in vitro* and *in vivo*. However, there are contradictory results as well.[23] Therefore, we also measured the concentration of IL-1β in the supernatants of MC monolayers in response to infection with *C. trachomatis*. We observed significant induction of IL-1β in the supernatants of infected MC. This parameter is therefore likely to influence the immune-mediated responses. Heat-killed chlamydiae induced IL-1β release after 0-24h only slightly, and this may be explained by activation in response to the outer membrane of *C. trachomatis* or to ingestion of the heat-killed chlamydiae as we observed after staining with monoclonal antibodies. In contrast, Rothermel *et al.*[21] did not observe uptake of heat-killed chlamydiae by monocytes and found inactivated chlamydiae to induce IL-1 production by monocytes as effective as did viable chlamydiae. In our experiments heat-killed chlamydiae failed to initiate prolonged production of IL-1, a prerequisite to start the inflammatory cascade. However, Kinnaert *et al.*[24] observed both heat-killed E. *coli* and Staphylococci spp. to activate MC to induce IL-6 as well as IL-8 production, although with different efficacy. This difference in induction of IL-8 may be explained by the different pathogens used in these studies. Although chlamydiae possess cell-wall antigens analogous in structure to the cell walls of gram-negative bacteria, the fact that they are ingested by MC and not effective in continuing IL-1 induction may explain the

different IL-8 response after incubation with heat-killed chlamydiae compared to E. *coli*. Ultracentrifuged suspensions fail to alter inflammatory as well as procoagulant or fibrinolytic responses. In this way we excluded MC activation by the medium in which the chlamydiae had been suspended.

Local cytokine production during infection may play an important role in modulating host defences to *C. trachomatis*. The formation of fibrin is mediated via local coagulation and fibrinolytic processes. Under normal circumstances, there is a balance between coagulation and fibrinolysis in the abdominal cavity. If coagulation is activated, TF expression on the cell membrane initiates the extrinsic pathway (directly activating factor VII, which subsequently activates factor X), leading to thrombin generation and fibrin formation from fibrinogen.[25] The fibrin clot formed is susceptible to lysis by tPA. The fibrinolytic process is dampened when tPA is blocked by PAI-1. This reduction in peritoneal fibrinolytic activity, as occurs after injury or inflammation, is associated with adhesions,[26] and may facilitate e.g. post-infectious tubal infertility. We demonstrated induction of TF expression by MC monolayers in the early stage of infection with *C. trachomatis*. High levels of TF were detectable after 48-72h incubation. Damage to cell membranes can cause a rapid increase in the expression of TF procoagulant activity[27]; however, this does not explain the increased TF expression observed after incubation with 1:10 diluted chlamydia suspension. Upregulation of TF expression can be due to both activated inflammatory responses, or to interaction of the intracellular parasite with MC cytoplasmic systems. Infection of MC monolayers with *C. trachomatis* resulted also in enhanced activity of the inhibitor of fibrinolysis, PAI-1.

In conclusion, the present study demonstrates that, after initial chlamydial infection, local pro-inflammatory responses and procoagulant activity are enhanced, whereas fibrinolytic activity is inhibited. Heat-killed chlamydiae failed to induce a prolonged IL-1 response. These data further implicate the pivotal role of local immune system activation in the pathogenesis of intra-abdominal complications after chlamydial infection.

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General discussion

The immunological consequences of intra-abdominal operations have attracted attention as they may influence the outcome of surgical intervention. Manipulation of factors that contribute to the postoperative inflammatory response may have the potential to minimize intra-abdominal complications. In this light this thesis focuses on the question whether peritoneal lavage, a peroperative procedure generally regarded as protective and unharmful, may have adjuvant effects on peritoneal responses. This section will discuss the results obtained from the experimental and clinical studies performed with regards to the three objectives formulated in the preface.

The influence of peroperative lavage solutions on peritoneal defence mechanisms in vitro.

The peritoneal cavity may be seen as an organ lined with metabolically very active cells, i.e. mesothelial cells. One method to observe the reactivity of this particular important cell layer to stimuli is by *in vitro* experiments. During *in vitro* exposure of mesothelial cells to lavage solutions that are nowadays used we have shown most lavage solutions to affect cell morphology *in vitro*. Clinically used lavage solutions were shown to be cytotoxic (Chapter 1), and to increase the permeability of the mesothelial monolayer (Chapter 1). This is in concordance with *in vivo* observations of Tolhurst and co-workers who demonstrated that after peritoneal lavage with Hartmann's solution serosal healing was delayed. They also observed severe morphologic changes of the mesothelial layer in histological studies. In addition, we found that the functional properties of mesothelial cells, pivotal in the peritoneal defence, can also be disregulated by exposure to various solutions. Lavage solutions appear to affect mesothelial cell function in the following way:

- by enhancing the pro-inflammatory cytokine (IL-8) release (Chapter 1)
- by altering the expression of the adhesion molecule ICAM-1 (Chapter 2)
- by changing the balance between plasminogen activator and inhibitor release and by inducing the activity of the inhibitor of fibrinolysis (Chapter 3)
- by affecting the procoagulant activity (Chapter 3)

Although the mesothelial reaction to lavage solutions can be considered as an overshoot of a normal physiological response to stimuli, the observation that the effects on mesothelial functional properties different per solution (*table 1*)

may be of great interest in the search for an ideal solution. This ideal solution should at least be non-toxic and should be supplemented with a potential agent that favourably influences the peritoneal inflammatory response. However, in any consideration of the composition of such a solution, the potential damaging effects of an excessive inflammatory response must be outweighed against the beneficial effects.

Table 1. Overall reaction of various solutions on mesothelial cellular functions

	salt solutions	PVP-I	Dakin's	Taurolin	chlorhex
IL-8	+	↓	++	++	++
ICAM-1	-	++	+	+	++
PAI-1	+	↓	++	-	++
TF	-	↓	-	+	+

+ = stimulating, - = no effect, ↓ = inhibiting.
ICAM-1= intercellular adhesion molecule-1, IL-8= interleukin-8, TF= tissue factor, PAI-1= plaminogen activator inhibitor-1, PVP-I= povidone iodine, chlorhex= chlorhexidine.

A characteristic intra-abdominal complication induced by ascending chlamydial infection is the typical violin string adhesion formation which may result from local immunologically mediated inflammation and fibrosis. When we compared the changes in functional properties of mesothelial cells after incubation with various lavage solutions with the responses of mesothelial cells to infection with *C. trachomatis* (Chapter 7), we observed much resemblance in reactivity. Pro-inflammatory, procoagulant and fibrinolytic responses were altered to a proportional extent. Because activation of the inflammatory cascade may contribute to complications seen after abdominal surgery, we consider that this overshoot induced by exposure to nowadays used lavage solutions should be avoided.

Studies on another cell type, the neutrophil, which is important in the non-specific defence mechanism of the peritoneal cavity, resulted in similar findings. The neutrophil plays a central role in the pathogenesis of tissue injury and has two prerequisites to become injurious; adhesion to tissue cells and the secretion of histotoxic agents. We have shown that there is evidence of an alteration of the neutrophil activation response after exposure to lavage solutions in terms of rate of hydrogen peroxide production (Chapter 1). At the same time, the expression of the ICAM-1 on the mesothelial membrane,

important in neutrophil-mesothelial adherance, was also enhanced after exposure to some solutions (Chapter 2). It has been shown that activated and therefore adhesive neutrophils induce more damage to the tissue they adhere to than non-adhesive neutrophils. Therefore, our observations support the theory that after peroperative exposure to lavage solutions, postoperative modulation of neutrophil function and increased adhesiveness may occur, which may predispose for intra-abdominal complications. However, it is generally considered that the injurious neutrophil effects in acute inflammation are normally outweighed by their positive role in tissue defence mechanisms. It may be hypothesized that an alteration in neutrophil activation and expression of adhesion molecules induced by peritoneal lavage may therefore not aggravate the response of the host to surgical injury. However, irrigation will not be restricted to the operation field, but will cover much more of the peritoneal surface and reach even those parts which were not initially activated by surgery or infection.

The effect of peroperative lavage on postoperative responses in vivo.

It is questionable whether the effects of peroperative lavage determine the final postoperative response of the peritoneum during conditions in which lavage will be performed. To verify our *in vitro* observations, studies *in vivo* were conducted to investigate the postoperative effects of various lavage solutions when exposed to the peritoneum during laparotomy. In both *in vivo* studies the contribution of lavage to an already changed environment was tested. Moreover, in contrast with the *in vitro* situation, not only the effect of exposure to various solutions but also the effect of the operating procedure has to be taken into account.

Firstly, we studied the incidence of adhesions after laparotomy and lavage in a rat adhesion model (Chapter 4). The main conclusion drawn from this study is that exposure to lavage solutions during intra-abdominal surgical intervention increases postoperative adhesion formation. Considering the pathogenesis of adhesion formation, we may deduct that lavage solutions interfere with beneficial postoperative peritoneal responses. By lack of suitable assays to study the dynamics of mediators in a rat model, we only indirectly proved that as a result of peroperative irrigation, the postoperative

peritoneal environment changed and in this way induced adhesion formation. Another conclusion derived from this study is that there seems to be a correlation between the *in vitro* results of mesothelial reactivity to solutions and the percentage adhesions found after peritoneal ischemic injury and lavage *in vivo*. From our *in vitro* experiments we could distinguish between very aggressive, aggressive and less aggressive solutions. This classification could also be made on the basis of the *in vivo* data. However, lavage with PVP-I failed to reduce adhesion formation *in vivo* in spite of the favourable results observed *in vitro*. The assumption that *in vitro* PVP-I, as a highly viscous solution, reduced the mesothelial activity by its coating effect may be clinically not significant and may account for the discrepancy with the *in vivo* result. Another reason may be the differences in species, in particular the difference in peritoneal fibrinolytic activity between human and rat peritoneal tissue.

Secondly, the dynamics of cytokine production, procoagulant and fibrinolytic responses in elective surgery with peroperative lavage were studied (Chapter 5). During the past few years plasma levels of inflammatory mediators in intra-abdominal infection have been extensively studied, but may only indicate the spill over from the peritoneal cavity. Local peritoneal concentrations are more likely to be of primary biologic and clinical importance. It appears that these local concentrations act in a paracrine fashion, and that the intensity of the peritoneal response (humoral as well as cellular) correlates with adverse postinjury events. Local measurement of important mediators involved in wound healing, adhesion formation and tumour recurrence may reveal the severity of an initially local process.

Operation itself obviously induces a distinct effect on the mediators we assessed. A detailed evaluation of changes in cytokine, coagulant and fibrinolytic responses postoperatively has been described. Interestingly, peritoneal lavage is effective in decreasing the peritoneal levels of cytokines in the early period (0-6h) after operation, although exposure of the peritoneum to the solution may initially activate the mesothelial monolayer. The use of these solutions as prophylactics during routinely performed intra-abdominal surgery deserves further attention: whether initially impaired peritoneal cytokine levels after surgery are beneficial or not remains to be investigated. On the one hand, enhanced cytokine levels in the 6-24h drain fluid postoperatively may indicate that feedback occurs of the early decreased levels. On the other hand, enhanced levels could also result from activation of the peritoneum in

response to peroperative lavage, which may exceed the initial dilution 6-24h after operation. The relationship between changes in reactivity of the peritoneum and exposure to lavage solutions may be demonstrated when the fibrinolytic mediators are taken into account. Indentical changes in levels of the fibrinolytic activator (tPA) and the fibrinolytic inhibitor (PAI-1) were observed, as in *in vitro* experiments.

Measurements via drains in the abdominal cavity at the non-operated and operated site showed different levels of mediators in both areas. In the operated area the effects of lavage may not be as dramatic as in parts of the peritoneal cavity that are not related to the wound. Other factors may overcome the effects induced by lavage. Therefore, it is concluded that the influence of lavage may especially affect those parts of the abdominal cavity with minimal damage and activation of the peritoneum. In accordance, our rat experiments showed that in the area with induced ischemic injury to the peritoneum, subsequent lavage increased adhesion formation.

Modulation of biochemical parameters of mesothelial cells by oxygen radical scavengers.

The inflammatory response to injury, infection or even the presence of cancer cells in the peritoneal cavity is at least partially mediated through the effects of reactive oxygen compounds. Among these is hydrogen peroxide, which acts on peritoneal cells either directly, or indirectly through augmentation of for instance cytokine synthesis. Protection against activation by reactive oxygen compounds may have the potential to decrease the inflammatory response of the peritoneum and thus the complication rate after intra-abdominal surgery. To simulate oxygen stress as may occur during surgery, mesothelial cells were exposed to extracellularly added hydrogen peroxide, air-drying conditions as well as cytokine stimulation (IL-1β). In these *in vitro* models we found that mesothelial cells are very sensitive to oxygen-mediated activation and damage. Recent data descibe mesothelial cells to protect themselves against oxygen stress mainly by the gluthatione pathway. We observed that addition of reactive oxygen scavengers to the culture medium, in particular N-acetylcysteine and catalase, prevented mesothelial cell activation and damage induced by oxidative stress (Chapter 4). The combination of

intra- (N-acetylcysteine) and extracellular (catalase) acting scavengers may complement the natural defence against reactive oxygen compounds and we therefore suggest that addition of scavengers to an irrigation solution may be beneficial to prevent an overshoot of the inflammatory response after intra-abdominal intervention.

In our studies, pre-incubation with scavengers showed to be more effective than exposure to scavengers after stimulation. Therefore, in case of proposed irrigation with scavengers the time of action may be important in preventing non-specific tissue activation and injury. This implicates that lavage with scavengers at the onset and during operation may be more effective than at the very end of the procedure in reducing the side effects of oxidative activation and injury in the postoperative period.

Final conclusion

Peroperative lavage is more than mechanical cleansing of the abdomen. These studies have shown that lavage may contribute to the postoperative peritoneal inflammatory response and affect intra-abdominal coagulant and fibrinolytic activity. The biological significance of these observations requires further study, in particular in a peritonitis and tumour model. The clinical implication is, however, that to prevent non-specific inflammatory responses we should either not irrigate the peritoneal cavity or at least use a non-toxic solution, because this preserves in particular the mesothelial cell layer and its regulatory function. Addition of specific compounds to this solution such as serum, proteins or scavengers may prevent peritoneal cell activation and restore natural peritoneal defences. It seems prudent to take every precaution to minimize injury and activation of the peritoneum by restricted use of any solution, until *in vivo* studies provide support for irrigation with a newly composed solution. Nowadays available lavage solutions induce local peritoneal responses and may thus lead to intraperitoneal complications after abdominal surgery.

Summary

The **General Introduction** outlines the history of peroperative peritoneal lavage. It overviews the pathophysiology of local peritoneal defence mechanisms and the responses of cellular and humoral components. Factors detrimental to the host response and options for controlling or modification of these processes are discussed. A definition of the ideal lavage solution is given.

Chapter 1 examines the effects of seven peroperative lavage solutions on mesothelial and neutrophil viability and function. Incubation with most solutions caused severe cell damage, disturbance of the integrity and activation of cytokine (interleukin-8) release of mesothelial monolayers. Neutrophil function was also affected, as shown by changes in superoxide anion release after exposure to some solutions.

Chapter 2 describes the influence of lavage solutions on the expression of mesothelial cell-associated molecules that play a major role in the pathophysiology of abdominal inflammatory processes. After short exposure to various solutions, the expression of intercellular adhesion molecule (ICAM)-1 but not that of vascular adhesion molecule (VCAM)-1 was significantly increased. In this way, peroperative lavage may contribute to neutrophil-mediated damaging conditions and tumour cell adhesion.

In **Chapter 3** we studied the procoagulant and fibrinolytic responses of mesothelial cells after exposure to various lavage solutions. Besides modulation of the release and activity of the inhibitor of the fibrinolytic cascade (PAI-1), we observed also increased expression of functional tissue factor on the surface of mesothelial cells after exposure to some solutions. Therefore, peroperative lavage may delay postoperative restoration of the physiological balance between peritoneal coagulation and fibrinolysis.

Chapter 4 gives the results of an experimental study in rats. In an adhesion model with induced peritoneal ischemic injury and subsequent lavage the postoperative response of the peritoneal cavity was determined by scoring the extent of adhesion formation. Laparotomy followed by peritoneal lavage induced significantly more adhesions than without lavage. These results correlate with the hypothesis that peritoneal lavage induces postoperative peritoneal activation and thus adhesion formation.

Chapter 5 contains a study in patients in which the peritoneal reactivity after elective abdominal surgery with or without subsequent peritoneal lavage was evaluated. In collected peritoneal drain fluid various mediators were determined. Irrigation depressed pro-inflammatory cytokine concentrations in the early period after operation, and induces a rise in interleukin-6 and 8 after 24h. Increased procoagulant and accelerated anti-fibrinolytic responses in the peritoneal fluid after peroperative lavage were observed. These data indicate that as a result of peroperative lavage peritoneal responses are altered. The clinical relevance in terms of these alterations being directly responsible for intra-abdominal complications remains to be elucidated.

In **Chapter 6** the effects of reactive oxygen compound scavengers on mesothelial cells were investigated. Oxidative stress is commonly generated during intra-abdominal surgical intervention and may play a key role in the pathogenesis of postoperative complications. This chapter presents three different *in vitro* models of mesothelial cell activation as they may occur during operation. Addition of reactive oxygen scavengers such as N-acetylcysteine and catalase to the culture medium protected mesothelial monolayers against damage and activation.

Chapter 7 focuses on the responses of mesothelial cells infected with *Chlamydia trachomatis*. Intra-abdominal complications induced by ascending chlamydiae may depend on local immunologically mediated inflammation and fibrosis. We studied the pro-inflammatory, procoagulant, and fibrinolytic responses of mesothelial cells after initial infection and found much similarity with the responses of mesothelial cells to lavage solutions *in vitro*.

Samenvatting

De **Algemene Introductie** bevat een overzicht van de literatuur op het gebied van peritoneaal spoelen. De pathofysiologie van de locale verdediging en de cellulaire en humorale afweerreacties worden besproken. Factoren die de lokale afweerreactie zouden kunnen verstoren dan wel opties om de afweerreacties te versterken worden bediscussieerd. Eigenschappen van de ideale spoelvloeistof worden beschreven.

In **Hoofdstuk 1** worden de effecten van zeven spoelvloeistoffen op de vitaliteit en functionaliteit van mesotheelcellen en neutrofielen beschreven. Blootstelling aan de meeste vloeistoffen veroorzaakte ernstige schade, verstoring van de integriteit en activatie van mesotheelcellagen. Ook de neutrofielenactiviteit in de vorm van zuurstofradicaalproduktie gaf duidelijke veranderingen te zien na incubatie van deze cellen in sommige spoelvloeistoffen.

Hoofdstuk 2 geeft een beschrijving van de invloed van spoelvloeistoffen op de expressie van cel-geassocieerde moleculen. Deze adhesiemoleculen spelen een rol in de pathofysiologie van abdominale inflammatoire processen. Na korte incubatie van mesotheel met verschillende spoelvloeistoffen blijkt met name de expressie van ICAM-1, maar niet de expressie van VCAM-1, te veranderen. Op deze manier zouden spoelvloeistoffen kunnen bijdrage aan neutrofiel-gemedieerde beschadigende processen en aan tumorceladhesie.

In **Hoofdstuk 3** bestudeerden we de stollings- en fibrinolytische eigenschappen van mesotheel na blootstelling aan verschillende spoelvloeistoffen. Met name de release en activiteit van de remmer van de fibrinolyse (PAI-1) veranderdt na incubatie met sommige vloeistoffen. Daarnaast blijkt ook de stollingsactiviteit in de vorm van tissue factor expressie op de mesotheelcelmembraan aangedaan. Hieruit mag worden geconcludeerd dat het peroperatief blootstellen van mesotheel aan spoelvloeistoffen het postoperatieve herstel van het peritoneale evenwicht tussen stolling en fibrinolyse zou kunnen verstoren.

Hoofdstuk 4 bevat de resultaten van een experimentele studie in ratten. In een rattenadhesiemodel, waarin ischemische schade wordt aangebracht aan de laterale peritoneumwand door middel van hechtingen, werd de buikholte aan het einde van de operatie niet gespoeld of gespoeld met een bepaalde

vloeistof. Na twee weken werd het percentage adhesies in het ischemische gebied gescoord. Laparotomie gevolgd door peritoneale lavage veroorzaakt significant meer adhesievorming dan in het geval dat de spoeling achterwege blijft. Dit gegeven komt overeen met de gedachte dat peroperatief spoelen postoperatieve peritoneale activatie kan veroorzaken en hiermee adhesievorming kan bevorderen.

In **Hoofdstuk 5** wordt een studie gepresenteerd waarin de peritoneale reactiviteit na een electieve darmoperatie met en zonder spoelen wordt geëvalueerd. In het verzamelde drain-vocht van 30 patienten werden diverse ontstekings-, stollings- en fibrinolytische mediatoren bepaald. In gespoelde buiken blijkt de inflammatoire cytokine-concentratie in de vroege periode na operatie significant verlaagd te zijn, maar na 24 uur zagen we juist weer een stijging van zowel interleukine-6 als -8. Tevens wordt een toename van de stollingsmediator (tissue factor) en verandering in de anti-fibrinolytische reactie (PAI-1) in het drainvocht van gespoelde buiken ten opzichte van niet gespoelde buiken gevonden. Deze studie toont aan dat als gevolg van peroperatief spoelen de peritoneale reactiviteit wordt beinvloed. Of deze veranderingen in reactiviteit direct bijdragen aan intra-abdominale complicatievorming moet nog worden onderzocht.

In **Hoofdstuk 6** worden de effecten van zuurstofradicalen op mesotheel onderzocht. Gedurende een intra-abdominale ingreep speelt oxidatieve stress een belangrijke rol in het onstaan van complicaties. In drie verschillende *in vitro* modellen werd mesotheelactivatie bestudeerd zoals tijdens een operatie zou kunnen plaatsvinden. Toevoeging van N-acetylcysteine en katalase aan het kweekmedium blijkt bescherming te bieden tegen mesotheelbeschadiging en activatie.

Hoofdstuk 7 beschrijft de *in vitro* reactie van mesotheel na infectie met *Chlamydia trachomatis*. Intra-abdominale complicaties na een opstijgende chlamydia-infectie zijn mogelijk het gevolg van immunologisch gereguleerde inflammatoire en fibrotische reacties. Wij bestudeerden inflammatoire, stollings en fibrinolytische reacties na infectie en vonden veel gelijkenis met de reactie van mesotheel na blootstelling aan sommige spoelvloeistoffen.

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In fancy they pursue
The dream-child moving through a land
of wonders wild and new
In friendly chat with bird or beast-
And half believe it true.

And ever, as the story drained
The wells of fancy dry,
And faintly strove that weary one
To put the subject by,
"The rest next time-" " It is next time!"
The happy voices cry.

Thus grew the tale of Wonderland:
Thus slowly, one by one,
Its quaint events were hammered out-
And now the tale is done,
And how we steer, a merry crew,
Beneath the setting sun.

(uit: Alice's adventures in Wonderland)

Colofon

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