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The role of neutrophils in the formation of peritoneal adhesions

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The most common cause of intraperitoneal adhesions which may result in infertility and intestinal obstruction is previous abdominal surgery. Surgical trauma of the peritoneum in the absence of infection elicits a rapid and transient influx of polymorphonuclear leukocytes (PMN) into the peritoneal cavity. The role of neutrophils in intraperitoneal adhesion formation has not been studied. We aimed to study the effects of PMN counts and PMN functions on peritoneal adhesion formation. Forty peritoneal adhesion-induced rats were randomly divided into three groups; group I, receiving saline; group II, receiving cyclophosphamide; and group III, receiving granulocyte–macrophage colony-stimulating factor (GM-CSF). In all groups, peritoneal lavage was performed to determine PMN counts the day after adhesion induction. Blood neutrophil counts and neutrophil functions were also determined. Adhesions were evaluated blindly 14 days after the operation. Adhesion tissue samples were microscopically evaluated. Tissue hydroxyproline and collagen concentrations were measured. The neutrophil counts and phagocytosis significantly increased in group III and neutrophil counts decreased in group II ($P < 0.05$). The score of adhesion formation in group II was significantly less than that in groups I and III ($P < 0.05$). Hydroxyproline concentrations of adhesion tissue were significantly decreased in group II when compared with group III ($P < 0.05$). The present study shows that neutropenia lowers the degree of postoperative adhesion formation. It is concluded that PMN may have a role to play in modulating postoperative adhesion formation.

Key words: cyclophosphamide/granulocyte–macrophage colony-stimulating factor/neutrophils/peritoneum/postoperative complications

Introduction

Adhesions have been a major problem contributing to relatively poor results obtained in infertility surgery and have become the most frequent cause of intestinal obstruction (Stangel and Gomel, 1980; Yaacobi *et al*., 1993). Postoperative pelvic adhesions after surgery remain a major clinical concern because adnexial adhesions have the potential to impair fertility. Adhesion may enclose the ovary to form a mechanical barrier. Tubal adhesions may alter tubal anatomy, motility and function (Rock, 1991). The problem of postoperative complications due to surgical adhesions has attracted the attention of many investigators seeking agents and methods aimed at preventing adhesion formation (Yaacobi *et al*., 1993).

The mechanism of adhesion formation represents a variation in the physiological healing process. Adhesions seem to be a result of an inflammatory mechanism (Pados and Devroey, 1992). There are several indications that polymorphonuclear leukocytes (PMN) are involved in the pathophysiology of intraperitoneal adhesion formation. Peritoneal injury during surgery, in the absence of infection, causes a rapid and transient influx of PMN into the peritoneal cavity followed by the accumulation of mononuclear cells, mainly macrophages (Ellis *et al*., 1965). However, the role of neutrophils in the pathophysiological cascade leading to intraperitoneal adhesion formation has not been well studied.

The present study evaluated the role of neutrophils in postoperative adhesion formation. Cyclophosphamide and granulocyte–macrophage colony-stimulating factor (GM-CSF) were preferred because of their contrary effects on leukocytes and a number of activities such as chemotaxis, migration, cell adhesion, synthesis and release of cytokines, and superoxide release. We tested the hypothesis to ascertain whether or not the development of neutrophil function with GM-CSF would affect postoperative adhesion formation in the rat. Since adhesion formation is a kind of fibrosis, hydroxyproline concentrations as a marker of collagen metabolism have been measured in adhesive tissue.

Materials and methods

Animals

Wistar albino rats weighing 190–230 g were purchased from DETAM (Experimental and Medical Research Center) of Istanbul University and kept on a standard laboratory diet and tap water. The animals were fasted for 12 h prior to surgery.

Experiments

The animals were randomly divided into three groups, group I ($n = 12$) receiving saline (saline solution every 12 h for 2 days prior

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to the experiment, injections were continued for 5 days after induction of adhesion formation), group II (n = 14) receiving a single dose of cyclophosphamide which caused neutropenia (Dokmetas *et al*., 1991) (Sigma Chemical Co., St Louis, MO, USA) (250 mg/kg i.p. 2 days prior to the experiment), and group III (n = 14) receiving human GM-CSF (Sandoz Chem Co., Basel, Switzerland) (1 mg in phosphate-buffered saline solution every 12 h for 2 days prior to the experiment, injections were continued for 5 days after induction of adhesion formation). The neutrophil count and neutrophil phagocytosis and adherence index were determined in each group 1 day after the induction of adhesion.

Midline laparotomy was performed under ketamine–HCl (60 mg/kg) and xyalasin (4 mg/kg) anaesthesia using a clean surgical technique. The serosa was stripped from the uterine horn at its midportion for a distance of 1.5 cm and the caecum was grasped and denuded of serosa over a 2 cm length until punctuate haemorrhage occurred. The abdomen was closed at the end of the surgery in two layers with a continuous 2/0 silk (Ethicon) suture (Liebman *et al*., 1993; Heidrick *et al*., 1994).

All animals in each group underwent a peritoneal lavage 24 h after adhesion induction. Peritoneal lavage was performed using a silastic tube introduced into the peritoneal cavity through a small midline incision. 200 ml of warm sterile saline containing heparin was then infused into the peritoneum and gentle external manipulation of the abdomen was applied in order to ensure proper distribution. Neutrophil counts were determined from the aspirated fluid.

Adhesion assessment

A grading system (Linsky *et al*., 1987) was used to determine the adhesion score at the end of the second week after adhesion induction; this was practical, easy and detailed: 0, no adhesion; 1, adhesion on 25% of the traumatized area; 2, adhesion on 50% of the traumatized area; and 3, total area involved. The severity of the adhesions was evaluated according to the scale: 0.0, no resistance to separation; 0.5, moderate force required for separation; 1.0, sharp dissection needed for separation.

A total score was obtained by adding the two grading scales with a range from 0.0 to 4.0 assigned for each region evaluated. The total score represented both extent and severity.

Histological assesment

Preparation of tissues for light microscopy

The tissue from adhesions of each animal was fixed with formaldehyde for 24 h, dehydrated and embedded in paraffin wax. The thickness of the sections was 5 μm and they were stained with haematoxylin and eosin.

Preparation of tissues for electron microscopy

The abdominal cavity was opened and five tissue samples from adhesions of each group were placed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C. Fixation was continued for 24 h. Primary fixation was carried out using 1% osmium tetroxide in 0.1 M phosphate buffer. Tissue fixed in glutaraldehyde was washed in several changes of buffered sucrose and then post-fixed for 2 h in 1% osmium tetroxide in 0.1 M phosphate buffer. The specimens were subsequently dehydrated in graded solutions of ethanol, passed through epoxypropane and embedded in Epon 812. Sections 0.1 μm thick were cut on a Reichert microtome, and stained with uranyl acetate and lead citrate prior to examination through a Jeol 100-C electron microscope.

Studies on neutrophil function

White cell count, neutrophil count

Peripheral blood for blood smears and quantitation of the absolute numbers of circulating leukocytes were obtained from tail bleeding. The total circulating white blood cell count/mm^3 was determined using an automatic cell counter (Cell Dyne 300). The percentage and absolute number of circulating white blood cell subsets was determined after performing a differential count of ⩾100 cells/smear.

Neutrophil adhesiveness

Siliconized glass beads 0.1 mm in diameter were packed to a height of 3 cm over a small piece of glass wool in a siliconized glass syringe 1 cm in diameter, the whole being enclosed in a water jacket at 37°C. Two millilitres of heparinized whole blood were added to the column, and, after 10 min, allowed to flow out from the column. The flow rate was adjusted by controlling the pressure on the plunger of the syringe to 0.1 ml/min. An absolute neutrophil count (total white cell count/mm^3×% neutrophil) was performed in both influent and effluent blood and the ratio of the former to the latter termed as the adhesiveness index (AI) (Brandt, 1965).

Neutrophil phagocytosis

Heat-killed yeast cells were prepared by stirring desiccated baker's yeast in normal saline, placing the suspension in a water bath at 100°C for 30 min and filtering the suspension through layers of gauze. The suspension of yeast cells was adjusted to a particle concentration of 5×10^4/mm^3 with phosphate-buffered normal saline (pH 7.4). Vigorous shaking was important to disperse small cell clumps.

The leukocyte suspension was prepared from a buffy coat, obtained by simple sedimentation (without addition of dextran) of heparinized whole blood (50 units/ml), and the concentration of mature neutrophils was adjusted to 5000/mm^3 with cell-free plasma. If the absolute neutrophil count of the buffy coat was <5000/mm^3, the concentration of yeast cells was reduced so that a constant yeast-cell to neutrophil ratio of 10:1 was maintained. The yeast cell and leukocyte suspensions was mixed together in amount of 0.4 ml, and placed in a water bath at 37°C with gentle inversion of the tube every 5 min. After 30 min, 0.08 ml of 5% dipotassium ethylenediamine tetra-acetate was added, the contents of the tube were well mixed, and films prepared rapidly on slides from the mixture, and stained with May–Grunwald–Giemsa. One hundred neutrophils were counted and classified according to their yeast-cell content; those containing more than six yeast cells were placed in one group because of the frequent difficulty of containing the exact number when more than six were packed in one neutrophil. The mean yeast-cell number per neutrophil was termed the phagocytosis index (PI) (Penny *et al*., 1966).

Biochemical analysis

Adhesive tissue samples were removed surgically and immediately frozen. These tissue samples were homogenized. Purified acid-soluble collagen (dissolved in 50 mM acetate buffer, pH 3.5) was included as a test sample for the estimation of hydroxyproline. Aliquots of standard hydroxyproline prepared from stock solution and test samples were mixed gently with sodium hydroxide in a total volume of 50 μl. Samples were hydrolysed. 450 μl of chloramine-T was added to the hydrolysate, mixed gently, and oxidation was allowed to proceed for 25 min at room temperature. 500 μl of Erlich's aldehyde reagent was added to each sample, mixed gently, and the chromophore was developed by incubating the samples at 65°C for 20 min. The absorbency of each sample was read at 550 nm using a spectrophotometer. The collagen content of adhesive tissue was calculated (Reddy and Enwemeka, 1996).

Statistical analysis

The statistical analysis of adhesion scores and blood and peritoneal neutrophil counts among the groups was carried out using non-parametric Kruskal–Wallis variant analysis and the Tukey test. Our

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Table I. White blood cell (WBC) counts (per mm^3) and some parameters of neutrophil function (mean ± SD)

	Peripheral WBC count	Peritoneal WBC count	Peripheral neutrophil count
Group I	6266.7 ± 1550.4	147.9 ± 52.7	1871.4 ± 823.8
Group II	565 ± 281.8[a]	71.4 ± 27.5[a]	105.9 ± 30.2[a]
Group III	13 450 ± 2520[b]	225 ± 91.5[b]	2545.8 ± 750.5[b]

[a]Significantly different when compared with group I.
[b]Significantly different when compared with groups I and II.

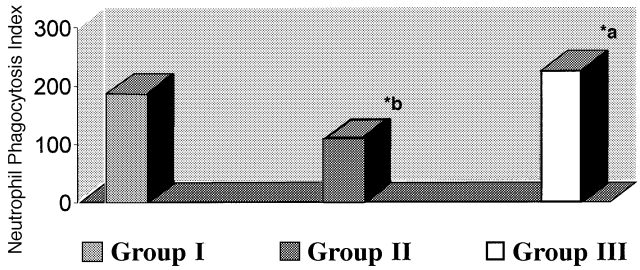


Figure 1. The neutrophil phagocytosis index of the groups. [a]Significantly different when compared with groups I and II ($P < 0.05$). [b]Significantly different when compared with group I.

data were normally distributed. $P < 0.05$ was considered to be statistically significant.

This study was approved by the ethical committee of Kocaeli University and performed following standard guidelines for the care and use of laboratory animals.

Results

In group II three animals died within 72 h of the adhesion induction. None of the rats died in the other groups. The surviving animals showed no evidence of other complications. Examination of the abdomen during autopsy revealed no residual viscous solution and no evidence of ascites.

Blood cell counts, neutrophil functional parameters

The peripheral white blood cell counts were determined in each group and were found to be decreased in group II and increased in group III as expected. The same results were also recorded for the neutrophils ($P < 0.05$). Thus, neutropenic and neutrophilic effects of cyclophosphamide and GM-CSF respectively, were observed (Table I). Additionally the total numbers of white blood cells recovered from the peritoneum significantly decreased in group II and increased in group III. These neutropenic and neutrophilic effects of the agents were also reflected in the peritoneal fluid and the number of the cells in group III was significantly higher than in both group I and group II ($P < 0.05$) (Table I).

The neutrophil phagocytosis index which was recorded as 223.9 ± 31.9 was found to have increased significantly in group III as compared with group I and II ($P < 0.05$) (Figure 1). However the neutrophil adherence index was significantly indifferent between the groups (Figure 2).

Table II. Adhesion scores, hydroxyproline and collagen concentrations of the groups (mean ± SD)

	Peritoneal adhesion scores	Hydroxyproline concentrations (g/100 g tissue)	Collagen concentrations (g/100 g tissue)
Group I	3.25 ± 0.39	0.17 ± 0.06	2.12 ± 0.94
Group II	1.50 ± 1.2[a]	0.16 ± 0.05	1.99 ± 0.62[c]
Group III	3.93 ± 0.18	0.23 ± 0.04[b]	2.46 ± 0.52[c]

[a]Significantly different when compared with groups I and III.
[b]Significantly different when compared with groups I and II.
[c]Non-significant difference.

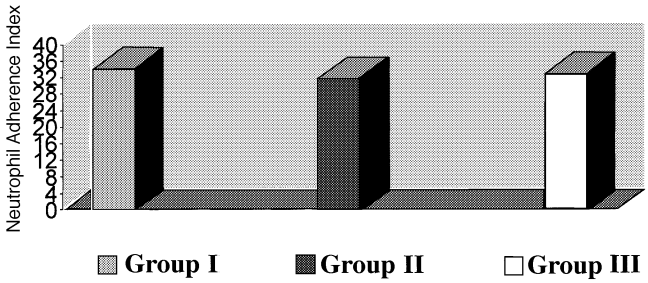


Figure 2. The neutrophil adherence index of the groups. The differences were not statistically significant.

Adhesion score

The mean combined score of area involvement and the character of adhesions was 3.25 in group I, 1.50 in group II and 3.93 in group III. Adhesion formation in group II was significantly less than that of group I and III (Table II).

Hydroxyproline concentrations

Although collagen concentrations did not differ significantly between the groups, hydroxyproline concentrations in surgically removed adhesive tissue were significantly increased in the neutrophilia-induced group III compared with the neutropenia-induced group II ($P < 0.05$) (Table II).

Histological results

Light microscopic findings

Group I: Collagen formation was pronounced and fibroblasts were prominent. Small blood vessels were also present within the adhesive tissue. Occasional foreign body granulomas and PMN were seen (Figure 3a).

Group II: Fewer bundles of collagen were present; fine reticulin fibres were seen (Figure 3b).

Group III: Several macrophages and PMN were seen within the adhesive tissue. Inflammatory granulation tissue with fibroblasts and new blood vessels were observed.

Electron microscopical findings

Micrographs were usually taken from the middle of the adhesive area. The results obtained using the electron microscope largely supported those of the light microscopical study but also revealed some interesting features not shown by light microscopy.

Group I: Collagen formation was well advanced and fibroblasts

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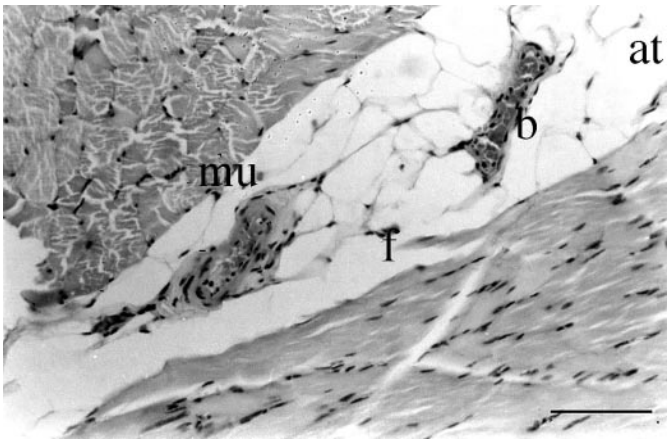


Figure 3. Light microscopy of adhesive tissue of group I: a sample of adhesive tissue in control group (haematoxylin and eosin). mu = muscle; at = adipose tissue; f = fibroblast; β = blood vessels. Bar 1 = 100 μm.

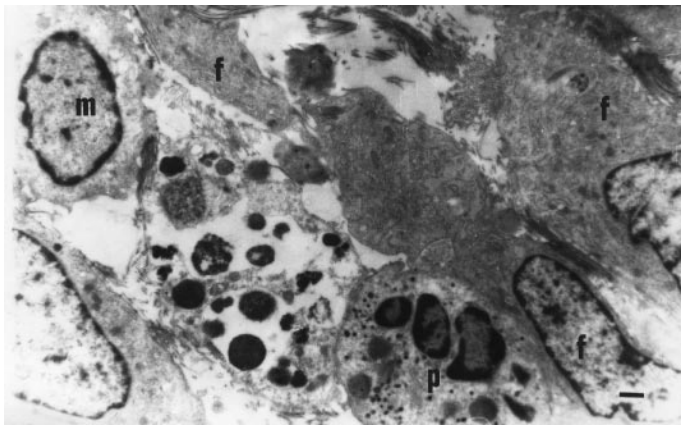


Figure 4. Electron micrograph showing fibroblasts (f), a monocyte (m) and a neutrophil (p) in adhesion tissue of control group. Bar = 1 μm.

were rarely beginning to align themselves parallel with one another (Figure 4).

Group II: The cells within adhesions were mainly typical fibroblasts and monocytes showing changes in organelles. PMN and macrophages were rarely seen in most of the areas examined. In some of the cells chromatin material was condensed, and pyknosis, dilated cisternae of granular endoplasmic reticulum, and karyorrhexis lysis were observed in several cells (Figure 5).

Group III: Irregular bundles of collagen were abundant and many cells with phagocytic vacuoles and lysosomal vesicles were observed. Macrophages and PMN were the most numerous cells. Angiogenesis was obvious in most areas (Figure 6a and b).

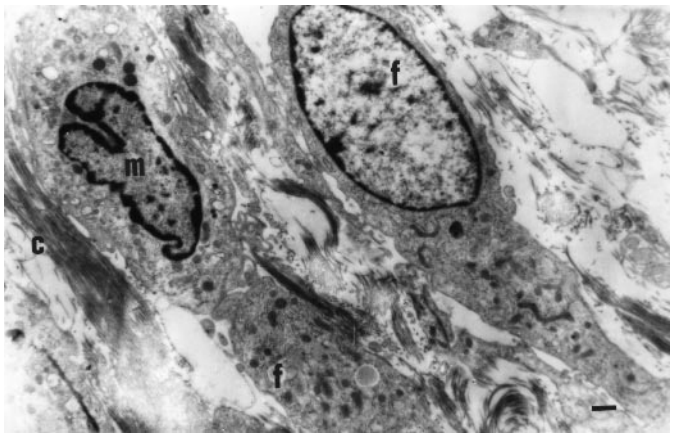
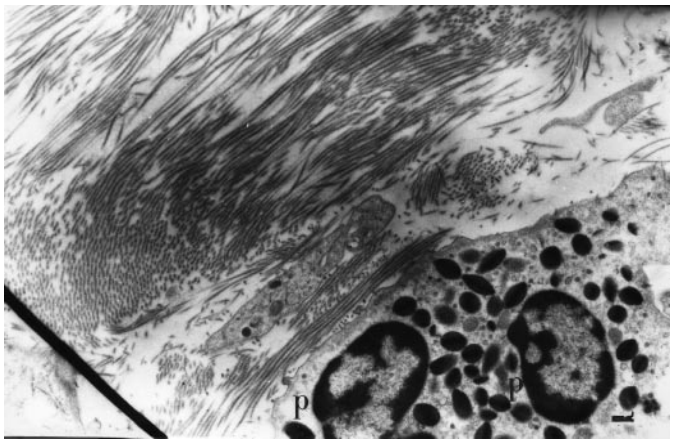


Figure 5. Fibroblasts (f) were more numerous in adhesion tissue of neutropenia-induced animals. A macrophage (m) and collagen fibres (c) can also be seen. Bar = 1 μm.



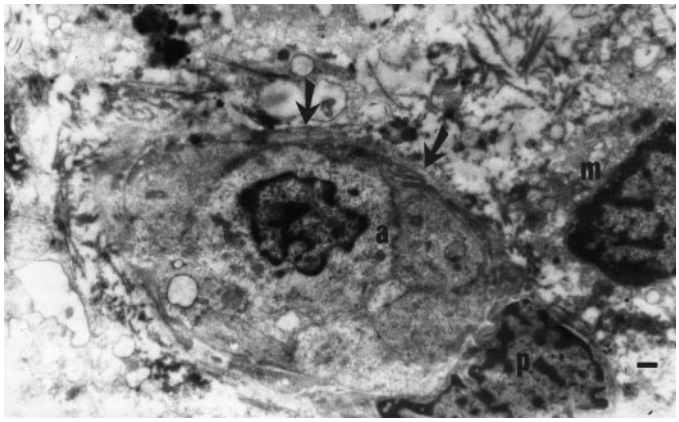


Figure 6. (**a**) Electron microscopy of adhesion tissue of group III: one neutrophil (p) was observed along with collagen fibres. (**b**) Electron micrograph of newly formed blood vessels in granulomatous adhesion tissue of animals administered with granulocyte–macrophage coony-stimulating factor: proliferated endothelial cell (a); pericyte (p). The pericyte, which guides neovascularization, and the endothelial cell share the common basal laminate (arows). A macrophage (m) is seen on the right side. Bar = 1 μm.

Discussion

Intraperitoneal adhesion formation after major abdominal and gynaecological surgery may result in long-term sequelae, such as bowel obstruction, infertility and pain. To address this problem, investigators have attempted to reduce intraperitoneal adhesion formation using different approaches (Liebman *et al*., 1993; Pijlman *et al*., 1994).

Although many multifactorial processes are involved in the adhesion formation, one of these has been shown to be the

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inflammatory process (Pados and Devroey, 1992). Surgical injury of the intact peritoneum produces a serosangineous exudate within 3 h (Pados and Devroey, 1992). Following an immediate inflammatory reaction, vascular permeability is increased and a fibrin-rich exudate is released (Ellis *et al*., 1965; Liebman *et al*., 1993). If the fibrin is not completely lysed through the plasminogen–plasmin cascade, adhesions may form through collagen deposition on a framework of proteoglycan and fibronectin. This exudate includes various inflammatory cells such as neutrophils, and mononuclear cells (Rodgers and DiZerega, 1992). However, under normal conditions the peritoneal cavity contains mainly macrophages and lymphocytes in a small amount of fluid (Pados and Devroey, 1992). During the early period of injury (up to 3 days), the most pronounced elements in the healing of peritoneal lesions are PMN and fibrin (Raftery, 1973; Rodgers and DiZerega, 1992). The PMN cells, recruited in large numbers into the peritoneal cavity after the injury, play a role in the formation of peritoneal adhesions.

It has also been shown that enhancement of macrophages markedly decreases postoperative adhesion formation (Rodgers and DiZerega, 1992). Additionally, it has been reported that inhibition of PMN adherence results in increased peritoneal adhesion formation (Arfors *et al*., 1987; ArRajab *et al*., 1996). The opposite mechanisms were also suggested. It was shown that neutrophils migrate into the peritoneum 1 day after adhesion-producing intraperitoneal injuries. PMN first adhere to the vascular endothelial surface and highly reactive metabolites derived from both the endothelium and PMN as a result of the adherence, produce endothelial injury and, consequently, microvascular permeability, oedema and thrombosis occur (Harlan, 1987a,b; Harlan *et al*., 1987; Price *et al*., 1987; Barton *et al*., 1989; Argenbright *et al*., 1991; ArRajab *et al*., 1996). Neutrophil–neutrophil aggregation further compromises the microvascular circulation by occluding capillaries and postcapillary venules, extending the zone of ischaemia and subsequent necrosis (ArRajab *et al*., 1996). Additionally, most animal studies have shown that corticosteroids and non-steroidal anti-inflammatory drugs, which prevent leukocyte migration besides their other effects, have reduced adhesion formation (Replogle *et al*., 1966; Siegler *et al*., 1980). In the present study cyclophosphamide-induced neutropenia resulted in decreased peritoneal adhesion formation. The decreased number of neutrophils was accompanied by a decreased phagocytosis index but an unchanged adherence index. Although GM-CSF-induced neutrophilia was accompanied by an increased phagocytosis index, the adherence of PMN did not change in this group. Adhesion formation was increased in this group but the difference between this and the control group was statistically insignificant. So we conclude that changes in phagocytosis index could be more important in the formation of adhesion than changes in the adherence index.

As shown previously, adhesion formation can form through collagen deposition (Thompson *et al*., 1989). Collagen principally forms the matrix of the developed adhesion. Neutrophils were claimed to have the potential to degrade the extracellular matrix (Sopata *et al*., 1989). Serine protease and metalloproteinases released from the neutrophils have been shown to play a role in neutrophil-mediated degradation of basement membrane collagen (Vissers and Winterbourn, 1988). In some studies, the release of free oxygen radicals and neutral protease from neutrophils has been claimed to cause collagen degradation (Hogstrom and Haglund, 1986; Jonsson and Hogstrom, 1991). Although collagen synthesis is known to reach significant amounts on the fifth day, controversial results have been reported (Baykal *et al*., 1997). Collagen solubilization by neutrophils was measured by hydroxyproline release (Miligan and Raftery, 1974; Palmgren *et al*., 1992; Baykal *et al*., 1997). In this study we also used a similar method to determine the amount of connective tissue present on adhesive tissue as a chemical marker. Interstitial connective tissue fragments were known to be chemotactic for neutrophils. Native and synthetic collagen peptides were shown to stimulate free oxygen radical and elastase release as synthetic peptides containing hydroxyproline from neutrophils (Laskin *et al*., 1994). Although the collagen concentrations did not show a significant difference between the groups in this study, the degradation product, hydroxyproline, was significantly increased in the neutrophilia-induced group. But the adhesion formation was not found to have decreased in this group. This may be explained by extensively increased numbers of neutrophils in the peritoneum, since we believe that neutrophils cause adhesion formation but, at the same time, they start the degradation of collagen through the substances released by themselves and through other undetermined factors. Thus in the GM-CSF-induced neutrophilia group this balance can be changed in favour of adhesion formation. Increased phagocytosis, which was present in accordance with the increased hydroxyproline concentrations, may not be sufficient to prevent the adhesion formation. In the neutropenia induced group, hydroxyproline concentrations were not different since phagocytosis was very low. At the same time adhesion formation was significantly low which might be explained by a decreased number of neutrophils. Light and electron microscopy findings also supported this suggestion. It has been reported that inhibition of neutrophil adherence and consequently neutrophil activation and migration into the peritoneum reduce neutrophil-dependent collagen degradation and result in increased adhesion formation (ArRajab *et al*., 1996). On the contrary, our results have shown that in the neutrophilia-induced group adhesion formation has been increased. Moreover, as stated in the literature (Bigatti *et al*., 1995), vascularization played a role in the organization of adhesions during the early stages of this study.

Finally, it is concluded that inhibition and activation of neutrophil counts and function affect the cascade of postoperative adhesion formation. These results might suggest that neutrophils have a role in modulating postoperative adhesion formation. Although we showed the beneficial effects of cyclophosphamide on adhesion formation, by significant reduction of neutrophil counts and neutrophil phagocytosis index and insignificant inhibition of neutrophil adhesivity index, the bioacceptance and usefulness of these factors in the formation of postoperative abdominal and pelvic adhesion require further investigation prior to possible clinical use.

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