Mac-1 Expression and Superoxide Generation of the Peripheral Polymorphonuclear Leukocyte following Gastrectomy and Esophagectomy

Yuji IMAMURA[1], Takashi YOKOYAMA[2], Yoshiaki MURAKAMI[1], Eiso HIYAMA[2], Yoshio TAKESUE[1], Takashi KODAMA[1] and Yuichiro MATSUURA[1]

1) The First Department of Surgery, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima,734, Japan
2) The Department of General Medicine, Hiroshima University Hospital, 1-2-3, Kasumi, Minami-ku, Hiroshima,734, Japan

ABSTRACT

The aim of this study was to evaluate the characteristics of the polymorphonuclear leukocyte (PMN) function after surgical stress. We investigated Mac-1 expression and superoxide generation by peripheral PMNs and serum granulocyte-colony stimulating factor (G-CSF) concentrations in 15 patients who underwent either gastrectomy (n=8) or esophagectomy (n=7). The serum G-CSF rapidly increased within 24 hours after operation. The maximum levels of serum G-CSF in the cases of esophagectomy were about 5–8 fold those of gastrectomy, although the increases in PMN counts within 24 hours of esophagectomy were lower than those after gastrectomy. Mac-1 expression and superoxide generation by PMNs after gastrectomy increased significantly, in parallel with increases in the PMN counts and serum G-CSF. After esophagectomy, Mac-1 expression on peripheral PMNs remained elevated through the 7th day after operation, while superoxide generation by PMNs in response to PMA declined to below preoperative levels after the 3rd day. These results suggest that, after major surgery such as esophagectomy, there is a discrepancy between Mac-1 expression and superoxide generation by the peripheral PMN despite a high level of serum G-CSF.

Key words: *PMN, Mac-1, G-CSF, Superoxide, Esophagectomy*

The polymorphonuclear leukocyte (PMN) is one of the essential components of the host defense against invading organisms and of wound healing. Circulating PMNs migrate into the extravascular space and function as scavengers of damaged tissue and of foreign material. These PMN functions require cell-cell contact and adhesion, and PMN surface molecules have been shown to be involved in the process of adhesion[23]. PMN adhesion molecules are surface glycoproteins, which mediate heterotypic or homotypic cell-cell attachment and act as ligands in the PMN-endothelial cell interaction. In particular, the Mac-1 molecule (CD11b/CD18)[1] of PMNs, which is the C3bi receptor and forms a subclass of the β_2-integrin family of adhesion molecules[10], is modulated qualitatively and quantitatively by G-CSF[16,28] both *in vitro* and *in vivo*. These changes of Mac-1 expression induced by G-CSF increase cell attachment[19], phagocytosis[7,8], superoxide anion generation[15], degranulation[17,19], and many other adhesion-dependent PMN functions[21].

After major operations such as esophagectomy for esophageal cancer, bacterial infection or an organic disorder (i.e., respiratory failure) occurs frequently[18]. Although G-CSF may be one of the humoral mediators which regulate PMN function in such clinical situations, the characteristics of Mac-1 expression on peripheral PMNs and endogenous G-CSF after surgical stress remain poorly understood. In this paper, we report that Mac-1 expression and superoxide generation by peripheral PMNs could be primed by endogenous

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Abbreviations used: CLA, chemiluminescence assay with cypridina luciferin analog (CLA-PMA, stimulated by phorbol myristate acetate; CLA-OPZ, stimulated by opsonized zymosan); EC, esophagectomy for esophageal cancer; GC, gastrectomy for gastric cancer; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HBSS, Hanks' balanced salt solution; IL-1, interleukin-1; LFA-1, leukocyte function associated antigen-1; MFI, mean fluorescent intensity; MoAb, monoclonal antibody; OPZ, opsonized zymosan; PBS, phosphate buffer saline; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; POD, postoperative day; rhG-CSF, recombinant human granulocyte colony-stimulating factor; TNF, tumor necrosis factor.

G-CSF after gastrectomy. However, after esopha-
gectomy, there was a discrepancy between Mac-1
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SUBJECTS AND METHODS

Reagents and antibodies

The monoclonal antibody (MoAb) against the al-
pha chain of Mac-1, the anti-Leu15 Ab and the
negative control MoAb (mouse IgG2a) were ob-
tained from Becton Dickinson, Mountain View,
CA. Phorbol myristate acetate (PMA) and Hanks'
balanced salt solution (HBSS) came from Sigma
Chemical Co, St.Louis, MO. The immunolysing
solution and fixative came from Coulter Electron-
ics, Hialeah, FL. Recombinant human G-CSF
(rhG-CSF), produced in CHO cells, was provided
by the Kirin Brewery (Tokyo, Japan). Zymosan
and cypridina luciferin analog (CLA) came from
Tokyo Kasei, Tokyo, Japan.

Patients

The subjects were patients with gastric cancer
(n=7) or esophageal cancer (n=8) who were ad-
mitted to the Hiroshima University Hospital.
They had no major complications and had not re-
ceived either steroid therapy or radiation therapy.
All patients underwent curative operations, ei-
ther gastrectomy for gastric cancer or esophagec-
tomy with thoracotomy and reconstruction with
stomach or colon for esophageal cancer. All opera-
tions were performed under fentanyl and nitrous
oxide-oxygen-sevofulurene anesthesia in combina-
tion with epidural anesthesia. There were no sig-
nificant differences in clinical background
between the two patient groups, except for opera-
tion time and volume of intraoperative bleeding
(Table 1). All esophagectomy patients received
mechanical ventilation during the first 3 days or
more after operation. All patients gave informed
consent for this study before their operations.

Isolation of peripheral blood PMNs

PMNs were prepared as previously described by
Eggleton et al[4]. Briefly, PMNs were isolated from
heparinized venous blood by differential centrifu-
gation in isotonic ammonium chloride at 4°C.
Cells were then resuspended at 1×10^6 cells/ml in
HBSS with 10 mM HEPES buffer, pH 7.20, and
were kept at 4°C for no longer than 2 hours be-
fore use. Cell viability, as determined by trypan
blue exclusion, was always greater than 95%.
PMN purity was more than 90% as determined
by Giemsa staining.

Determination of superoxide generation by PMNs

Superoxide generation by PMNs was measured
by CLA-dependent chemiluminescence[14]. The
reaction mixtures contained 1×10^5 PMNs, 1 μM
CLA, 4.5 mg/ml opsonized zymosan (OPZ) or 100
ng/ml PMA in 2.0 ml of continuously stirred
HBSS. Maximum luminescence was measured
with a lumiphotometer (Labo. Science Co., Osaka,
Japan, TD-4000).

Immunofluorescence staining

Mac-1 expression by PMNs was quantified by
flow-cytometry as previously described[9]. Briefly,
5 ml of freshly drawn venous blood was added to
50 U heparin sodium and immediately placed on
ice. Heparinized whole blood (100 μl) was added
to 20 μl of MoAb (anti-Leu 15, anti-alpha chain
of Mac-1) directly conjugated to phycoerythrin
(PE) and incubated on ice for 30 minutes. The
cells were then washed twice with 2.5 ml of ice-
cold PBS, with 2.5% pooled AB serum and 0.1%
NaN$_3$ at pH 7.2. The cells were fixed and the
erythrocytes were lysed with immunolysing solu-
tion and fixative, and the cells were then washed
with ice-cold PBS twice and resuspended in PBS.
Cell-surface fluorescence was analyzed by flow
cytofluorography with FACScan (Becton Dickin-
son, Mountain View, CA) flow-cytometry. PMNs

Table 1. Patient Characteristics

	Gastric cancer (n=7)	Esophageal cancer (n=8)	
Age	5±12	62.0±6	N.S.#
PMN counts (/μl)	4057±1338	3813±1301	N.S.
Lymphocyte count (/μl)	1429±570	1185±651	N.S.
Serum albmin (g/dl)	3.84±0.5	3.69±0.2	N.S.
Operation time (min)	170±23	316±108	*
Bleeding volume (ml)	437±185	906±485	**
blood transfused (ml)	57±151	317±485	N.S

*, p<0.01; **, p<0.05; #N.S., not significant, gastrectomy versus esophagectomy

were gated for forward and right angle light scatter, which were measured simultaneously. PMN fluorescence-intensity was reported as the mean fluorescence (log 10, 10^4 cells), which was the average fluorescence intensity of all cells chosen for measurement. The mean fluorescence intensity (relative MFI)[24] was standardized to determine the specific anti-Mac-1 fluorescence of the PMNs by subtracting the mean fluorescence of a parallel sample of cells that had been reacted with mouse control IgG$_2$a-PE.

Serum granulocyte-colony stimulating factor (G-CSF)

Serum was separated from venous blood and stored at $-20°C$. G-CSF concentration was estimated by ELISA (R&D Co., Minneapolis)

Priming of PMNs by recombinant human G-CSF

(1) Mac-1 expression on PMNs.

Heparinized venous blood of healthy adults (n=2) was incubated with rhG-CSF (0, 0.5, 1.0, 2.5, 5.0, 10 ng/ml) at 37°C and cooled down on ice. Then the PMNs in the whole blood were stained by monoclonal antibody (anti-Leu 15-PE conjugated) and their Mac-1 expression were measured by FACScan.

(2) Superoxide generation by PMNs.

Isolated PMNs (1×10^5 cells/ml) of healthy adults (n=2) were incubated with rhG-CSF (1.0, 5.0, 10 ng/ml) for 15 minutes at 37°C. Then the PMNs were mixed with 1 μM/ml cypridina luciferin analog (CLA) and 4.5 mg/ml opsonized zymosan (OPZ). Luminescence by activated PMNs was measured with a lumiphotometer.

Statistical analysis

Data are expressed as mean ± standard error. To evaluate the alterations of peripheral PMN function after surgery, we compared each PMN assay result with the preoperative data. The results were analyzed by Wilcoxon's unpaired test. Significance was accepted for $p < 0.05$.

RESULTS

Postoperative changes in peripheral PMN counts

Circulating PMN counts increased significantly by the end of the operation (Fig. 1). After gastrectomy, the PMN counts peaked dramatically at 4 hours after operation and declined to normal after 3 postoperative days (POD). In contrast, the PMN counts after esophagectomy increased gradually and remained high until 7 POD.

Changes in Mac-1 expression by PMNs after gastrectomy

Mac-1 expression level by PMNs increased at

the end of the operation and peaked at 4 hours after operation (Fig. 2). There was a significant correlation between PMN counts and Mac-1 expression ($p < 0.01$).
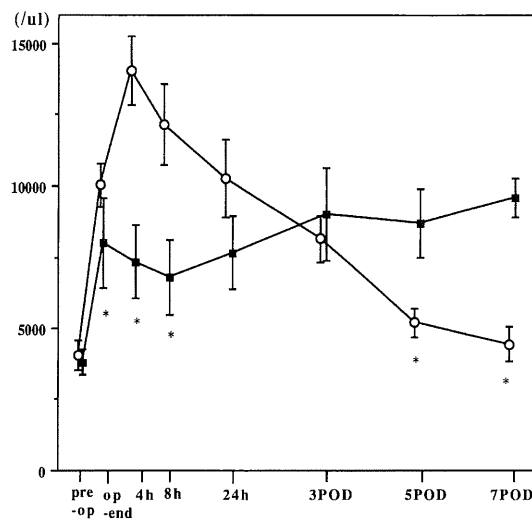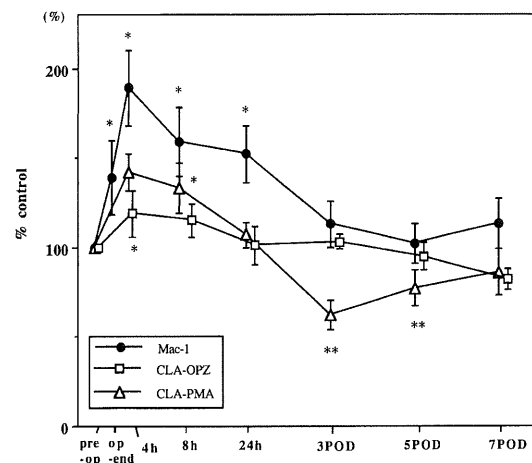

Fig. 1. Postoperative changes in peripheral PMN counts.
Gastrectomy, (○); esophagectomy, (■);
*: $p < 0.01$, gastrectomy versus esophagectomy.
POD: postoperative day.

Fig. 2. Time course of Mac-1 expression and of superoxide anion generation by peripheral PMNs in cases of gastrectomy (n = 7).
Mac-1 expression (●) was indicated by rMFI, and superoxide generation by PMNs was measured by chemiluminescence in response to PMA (100 ng/mL, CLA-PMA), (△) or to opsonized zymosan (5 mg/mL, CLA-OPZ), (□). Each preoperative value of Mac-1, CLA-PMA and CLA-OPZ was 29.9 ± 2.9 (channel), 278 ± 34.6 and 133 ± 4.5 (light unit). All data are shown as % control (preoperative) data.
*: $p < 0.01$; **: $p < 0.05$ versus preoperative data.

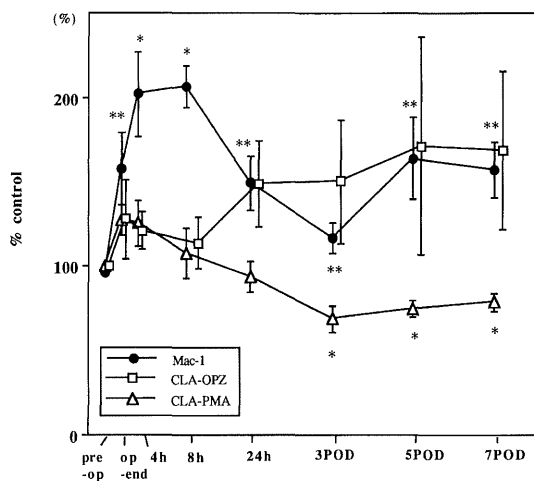## Changes in Mac-1 expression by PMNs after esophagectomy

After esophagectomy, increased Mac-1 expression by PMNs remained until 7 POD (Fig. 2). The increase in Mac-1 expression after esophagectomy was larger than that after gastrectomy, but not significantly so. No correlation was found between Mac-1 expression and PMN counts.

Superoxide generation and Mac-1 expression by PMNs after gastrectomy

CLA-PMA, reflecting the activity of superoxide generation mediated by $NADPH^+$–oxidase on PMNs, increased within 24 hours after gastrectomy and then decreased to below the preoperative level during PODs 3–5 (Fig. 2). On the other hand, superoxide generation by PMNs in response to opsonized zymosan (CLA-OPZ) showed no remarkable change.

Mac-1 expression and superoxide generation by PMN after esophagectomy

The increase in the number of peripheral PMNs after esophagectomy was sustained until 7 POD (Fig. 1), but the magnitude of the increase within 24 hours over preoperative levels was smaller than that after gastrectomy. No correlation between Mac-1 expression and peripheral PMN count was found in these periods. The activity of
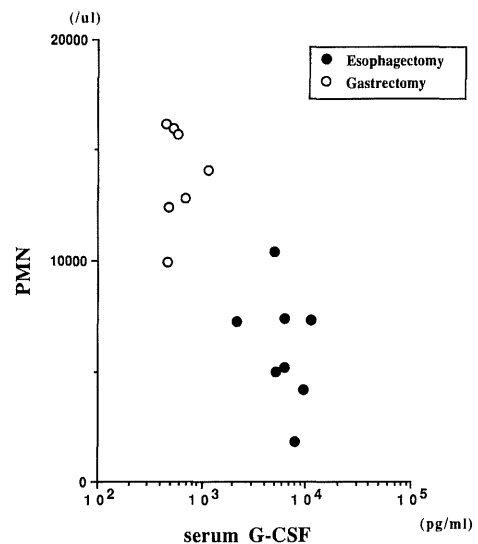
CLA-PMA by PMNs decreased significantly during PODs 3–7. Though CLA-OPZ was increased after 1 POD, it was not significantly different (Fig. 3).

Postoperative levels of serum G-CSF

After gastrectomy, serum G-CSF increased to 1311 ± 106 pg/ml at 1 POD and returned to preoperative levels at 7 POD. On the other hand, serum G-CSF in cases after esophagectomy increased dramatically at 4 hours after operation and its maximum levels were 5–8 fold those after gastrectomy (Table 2). In contrast to the high level of serum G-CSF within 24 hours after esophagectomy, the peripheral PMN counts were lower than those after gastrectomy (Fig. 4).

G-CSF enhances Mac-1 expression and superoxide generation by PMNs

The incubation of PMNs with recombinant human G-CSF resulted in an increase of Mac-1 expression by PMNs in both a dose- and time-dependent manner (Fig. 5). Mac-1 expression on incubated PMNs with rhG-CSF in 5 ng/ml concentration was increased to 350% of that of preincubated PMNs. In contrast, Mac-1 expression on peripheral PMNs was 163 ± 28% of preoperative PMNs at 4 hours after esophagectomy when serum G-CSF peaked to 6016 ± 950 pg/ml (Table 2). On the other hand, the treatment of rhG-CSF enhanced superoxide generation by PMNs in response to OPZ (Fig. 6). However, enhancement of the responsiveness of PMN to PMA was not found (data was not shown).

Fig. 3. Time course of Mac-1 expression and of superoxide anion generation by peripheral PMNs in cases of esophagectomy (n=8).
Mac-1 expression (●) was indicated by rMFI, and superoxide generation by PMNs was measured by chemiluminescence in response to PMA (100 ng/mL, CLA-PMA), (△) or to opsonized zymosan (5 mg/mL, CLA-OPZ), (□). Each preoperative value of Mac-1, CLA-PMA and CLA-OPZ was 28.0 ± 4.7 (channel), 326 ± 20.8 and 110 ± 14.6 (light unit). All data are shown as % control (preoperative).
 *: p<0.01; **: p<0.05 versus preoperative data.

Fig. 4. Relation of peripheral PMN count to serum G-CSF level at 4 hours after operation.
Gastrectomy, (○); esophagectomy, (●).

Table 2. Changes in Serum G-CSF

	Gastrectomy (n=7)	Esophagectomy (n=8)
pre	116.4± 48.3 (pg/ml)	94.8± 56.6
4 hours	664.9± 74.7	6016.3±950.0*
8 hours	616.7±106.6	4011.3±687.8*
24 hours	1311.0±641.0	1637.5±159.0
3 days	425.6±105.7	605.5±185.0
5 days	361.4± 85.4	644.1±315.1
7 days	127.3± 41.0	966.3±320.6**

G-CSF concentrations were determined by ELISA.
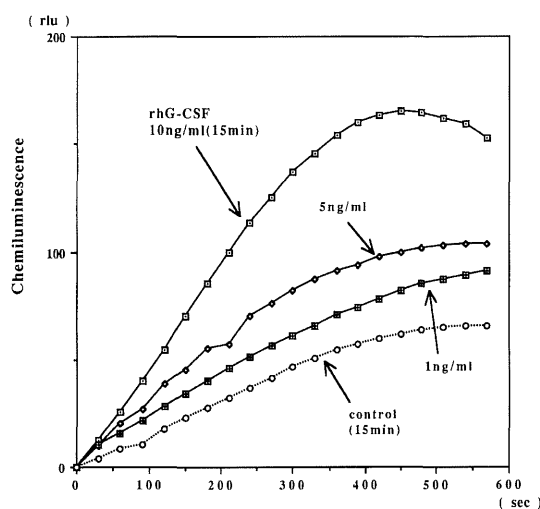*, p<0.01; **, p<0.05; gastrectomy versus esophagectomy.
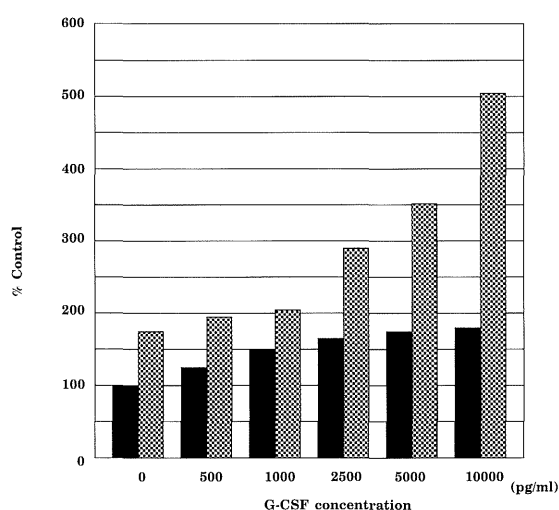
Fig. 5. Increase of Mac-1 expression on PMNs by exogenous G-CSF.
PMNs of healthy adults (n=2) in whole blood were incubated with recombinant human G-CSF for 5 min (■) or 15 min (▨) at 37°C, and then analyzed by FACScan. All data are presented as % control (control was 13.1 channel in mean fluorescence intensity of the PMNs at 0 minutes before rhG-CSF 0 pg/ml.)

Fig. 6. Priming of PMN to generate superoxide anion by G-CSF.
Isolated PMNs (1×10^5 cells/ml) of healthy adults (n=2) were incubated with recombinant human G-CSF for 15 minutes at 37°C. Luminescence by activated PMNs was measured with a lumiphotometer.

DISCUSSION

Mac-1 molecule on PMN is an important ligand in the PMN-endothelial interaction required for PMN migration to the extravascular space at sites with inflammation[12,13]. This interaction depends upon several adhesion molecules from the β_2-integrin family (Mac-1, LFA-1)[23] and selectin family (MEL-14)[12,13,21] on PMN. During the first and transient step in this interaction, PMN adheres to the endothelial cells by a ligand of MEL-14, and then the second step requires tight adhesions by LFA-1 and Mac-1. LFA-1[24] on activated PMN changes qualitatively, but not quantitatively, while Mac-1 expression on PMN is

modulated in quantity by several cyotokines including G-CSF[16,19] and GM-CSF[9]. In our study, we showed that endogenous G-CSF was considered to increase Mac-1 expression on peripheral PMNs after surgical stress. Investigation of Mac-1 expression on PMN in clinical patients is useful for the monitoring of alterations in the PMN adhesion molecules. For this purpose, we recommend use of our rapid method keeping PMNs at 4°C, as Griffin[9] and Ferron[6] reported, because we found that the PMN Mac-1 expression can be modified easily by isolation procedures including density gradient centrifugation (data not shown).

The superoxide generation by PMN in response

to opsonized zymosan (OPZ) is different from that in response to PMA. OPZ activates $NADPH^+$–oxidase in PMN by Mac-1-triggered oscillation of cytosolic free Ca^{2+}[17]. On the other hand, PMA stimulates $NADPH^+$– oxidase by direct activation of protein kinase C without any ligands. In our study, we showed that G-CSF can enhance superoxide generation of PMNs stimulated by OPZ, while G-CSF failed to enhance superoxide generation of PMNs stimulated by PMA *in vitro*, as reported by Ohsaka[16]. In cases after esophagectomy, although superoxide generation by PMNs in response to PMA (CLA-PMA) decreased gradually after 3 POD, responsiveness to opsonized zymosan (CLA-OPZ) was increased by high levels of serum G-CSF. Therefore, the activity of peripheral PMNs after surgical stress reflects to the upregulation of Mac-1 molecule induced by G-CSF and other cytokines such as IL-1, TNF[2] and/or GM-CSF[2,9,25] *in vivo*. Cannon[3] reported the detection of TNF and IL-1, which could stimulate Mac-1 expression on the PMN, in the sera of patients with severe burns or sepsis. In our previous study of surgical patients, we could not detect either cytokine in the sera[11], but GM-CSF could be detected between 10–30 pg/ml in the thoracic fluids or sera in some cases after major surgical stress such as esophagectomy (data not shown).

In the cases of esophagectomy, the number of peripheral PMNs within 24 hours after the operation was less than that after gastrectomy in spite of high levels of serum G-CSF. Mac-1 expression on PMNs increased, but the magnitude of these increases were lower than those of mature PMNs enhanced by treatment of G-CSF *in vitro*. We suppose that PMN sequestration mediated by the PMN-endothelial interaction diminished the primed PMN with high Mac-1 expression in the peripheral blood, which resulted in these phenomena after esophagectomy. Vedder[26] and Wash[29] demonstrated that PMN adherence to endothelial cells by a ligand of Mac-1 may elicit massively PMN accumulation in peripheral organs in critical conditions such as post-ischemic reperfusion injury and adult respiratory distress syndrome. The esophagectomy procedure with laparotomy and thoracotomy elicits greater tissue damage as compared with gastrectomy in terms of operation time, intraoperative bleeding volume, and frequency of postoperative lung damage. We also showed that G-CSF production *in vivo* in the cases after esophagectomy is higher than that after gastrectomy, and that G-CSF *in vitro* can induce the upregulation of Mac-1 expression on PMN within a few minutes. In addition to PMN sequestration, our results suggested an alteration in PMN subpopulations after surgical stress. It has been shown that administration of the G-CSF[25] and GM-CSF[22] cytokines *in vivo* activates mature PMNs in circulation and induces influx of premature cells from the bone marrow to the peripheral blood, followed by alteration of peripheral PMN subpopulations. Seligman[20] demonstrated a functional heterogeneity of peripheral PMNs of healthy adults when they were stimulated with PMA *in vitro*. Fein[5] has reported that the susceptibility of peripheral PMNs to PMA in septic patients was different from that in healthy adults. In our study, the activity of peripheral PMNs in generating superoxide anion in response to PMA decreased after 3 POD. From these behaviors of PMN after esophagectomy, we hypothesize that primed PMNs with enhanced expression of Mac-1 may be sequestrated to lung or other tissue by PMN-endothelial interactions at early postoperative periods, which is reflected by a decline in the ability of the remaining circulating PMNs to generate superoxide in response to PMA.

Our results showed that surgical stress enhanced the expression of PMN adhesion molecule Mac-1 and superoxide anion generation by PMNs. Moreover, surgical stress induced a dramatic increase in serum G-CSF, which may be one of the major humoral influences on PMN functions in early postoperative periods in surgical patients. Certainly G-CSF can cause increases in Mac-1 expression and superoxide generation by PMNs. In this paper, we demonstrated that, in the case of major surgery such as esophagectomy, superoxide generation by peripheral circulating PMNs decreases phenomenally, although the level of serum G-CSF and Mac-1 expression by peripheral PMNs increases remarkably. PMN-endothelial cell interaction mediated by a ligand of Mac-1 molecule on the PMN acts as one of the important roles in peripheral PMN behavior after surgical stress.

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