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Induction of Fas-Mediated Apoptosis on Circulating Lymphocytes by Surgical Stress

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Objective
The authors determined whether the decrease in lymphocytes after surgery is related to apoptosis.

Summary Background Data
Surgery induces a profound but transient depletion of circulating lymphocytes. However, the mechanism underlying this phenomenon is unclear.

Methods
Peripheral blood mononuclear cells were obtained from 18 patients before and after elective surgery and studied for morphologic and biochemical markers of apoptosis, DNA fragmentation, and Fas expression.

Results
The DNA staining of peripheral blood mononuclear cells obtained after surgery, which had been cultured for 24 hours *in vitro,* showed chromatin condensation and fragmentation of cells into collapsed spheres. Moreover, DNA isolated from these peripheral blood mononuclear cells formed a ladder of oligonucleosomal fragments. However, peripheral blood mononuclear cells obtained before surgery showed neither of these changes. The observation that none of these apoptotic cells ingested latex suggested that they were of lymphocytic origin. Fas-positive lymphocytes increased significantly 2 hours after the start of surgery and returned to preoperative levels by postoperative day 7. Anti-Fas antibody augmented apoptosis, whereas ZB4, a Fas antagonist, inhibited apoptosis in lymphocytes after surgery.

Conclusions
These results indicate that circulating lymphocytes in the early perioperative period are susceptible to Fas-mediated apoptosis, which may cause depletion of circulating lymphocytes after surgery.

There are many reports of immune system dysfunction after surgery.[1,2] Immunocompetence in a surgical patient is of vital importance as it influences an individual's susceptibility to bacterial, viral, and fungal infections.[3,4] Furthermore, in patients with cancer, immunocompetence can influence tumor dissemination and growth. Surgical stress induces a profound but transient depletion of all types of circulating lymphocytes,[1,5] which may contribute to postoperative immunosuppression. However, the mechanism underlying this decrease in circulating lymphocytes is still not clear. It may result from lymphocyte sequestration or cell death. Two forms of cell death in vertebrate tissues have been described. Necrosis, a pathologic response involving a dramatic increase in cell volume that ultimately leads to lysis, is a form of cell death induced by exposure to noxious compounds or treatment.[6] Apoptosis, conversely, is a more subtle process that commonly occurs when cell death is physiologically determined. The morphology of these two forms of cell death is clearly distinguishable.[7] Many different stimuli, both physiologic and pathologic, can induce apoptotic cell death. For example, thymocytes may be induced to undergo apoptosis by glucocorticoids[8,9] or by antigen-receptor cross linking.[10] Serum glucocorticoid concentrations are increased after surgery.[11,12] Therefore, it is quite possible that the depletion of lymphocytes after surgery because of apoptosis.

Recently, the cDNA for a transmembrane protein termed *Fas*, which mediates apoptosis, was molecularly cloned.[13] Fas is a 319 amino acid cell surface protein of molecular weight 45 kd. Fas is a member of the tumor necrosis factor receptor superfamily, which includes tumor necrosis factor receptor types I and II, the low-affinity nerve growth factor receptor, CD40, CD27, CD30, and OX40. Fas is expressed at high levels in various tissues[14,15] as well as in both T and B lymphocytes.[16,17] Expression of Fas is induced in lymphocytes by stimulation with phytohemagglutinin and pokeweed mitogen.[18] Recent studies clearly showed that Fas mediates apoptosis of activated T-cells[19] as well as clonal deletion of thymocytes.[20,21] Recently, the cDNA for the ligand to Fas also was molecularly cloned.[22,23] Fas ligand is a type II integral membrane protein homologous to tumor necrosis factor and has been detected on cytotoxic T-cells and cleared to mediate apoptotic cell death on Fas-expressing T-cells.[24] It was also reported that Fas ligand can mediate autocrine T-cell suicide[25].

These studies prompted us to examine whether the decrease in lymphocytes after surgery is because of apoptosis or necrosis. We examined morphologic and biochemical markers of apoptosis and DNA fragmentation in peripheral blood mononuclear cells (PBMCs) before and after surgery. We also examined whether Fas expression on circulating lymphocytes is correlated with their depletion after surgery.

Table 1. PATIENT CHARACTERISTICS

Patient No.	Sex	Age (yrs)	Disease
1	F	39	Intrathoracic tumor
2	M	73	Colon Ca
3	M	73	Rectal Ca
4	M	65	Metastatic liver tumor
5	M	70	Exophageal Ca
6	M	19	Pulmonary bulla
7	F	75	Colon Ca
8	F	64	Lung Ca
9	M	66	Colon Ca
10	M	81	Esophageal Ca
11	M	78	Gastric Ca
12	F	73	Rectal Ca
13	F	71	Esophageal Ca
14	M	62	Esophageal Ca
15	M	73	Gastric Ca
16	M	70	Esophageal Ca
17	F	76	Gastric Ca
18	M	72	Hepatocellular Ca

M = male; F = female; Ca = carcinoma.

PATIENTS AND METHODS

Patients

Eighteen patients undergoing elective surgery under general anesthesia in the Department of Surgery II, Yamaguchi University School of Medicine, were studied. Fifteen patients, including 4 from the apoptosis study, were involved in the study of Fas expression in circulating lymphocytes. The clinical characteristics of the patients are summarized in Table 1. General anesthesia was maintained with 50% nitrous oxide in oxygen and isoflurane as needed. No patients had any postoperative complications. Five of the 18 patients received blood transfusions during or after surgery. Informed consent for this study was obtained from all patients.

Peripheral Blood Mononuclear Cell Preparation

Peripheral blood samples were collected aseptically from patient day 0 (before surgery), 2 hours after the start of surgery, and on days 1 (24 hours), 4, and 7 after surgery. Lithium heparin anticoagulant was used. The num-

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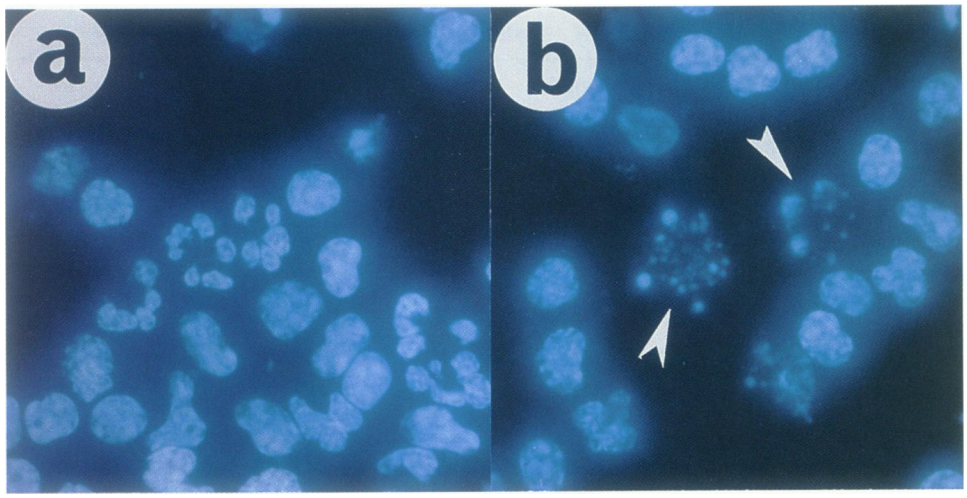

Figure 1. Apoptosis cells stained with Hoechst 33342. The peripheral blood mononuclear cell (PBMCs) isolated before surgery and 2 hours after the start of surgery from patient 1 were cultured *in vitro* for 24 hours as described in the Patients and Methods section. The DNA was stained with Hoechst 33342 for 2 hours, and cells were examined by fluorescence microscopy. (A) No apoptotic cells were observed in samples obtained before surgery (original magnification × 400). (B) Apoptotic cells with fragmented chromatin were observed in samples obtained after surgery (white arrows, original magnification × 400).

ber of lymphocytes was measured in each sample by use of an electronic counter. Peripheral blood was mixed with an equal volume of RPMI-1640 (GIBCO, Grand Island, NY) and centrifuged at 400 × g for 30 minutes at room temperature through Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Cells from the interface were washed with ice-cold RPMI-1640. The trypan blue dye exclusion method was used for the assessment of cell viability. Resuspended PBMCs were cultured in RPMI-1640 + 10% fetal calf serum (Whittaker Bioproducts, Inc., Walkersville, MD) for morphologic and DNA analysis.

Chromatin Staining and Assay of Phagocytic Activity

The PBMCs were cultured in RPMI-1640 + 10% fetal calf serum (1 × 106 cells/mL) for 24 hours at 37 C in CO_2 incubator in the presence of 0.013% solid latex (0.75 μm) (Fluoresbrite, Polyscience Inc., Warrington, PA). Cells that ingested latex beads were visualized by fluorescence microscopy. These PBMCs were cultured for another 2 hours in the presence of 10 μm Hoechst 33342 (Sigma Chemical Co., St. Louis, MO), and chromatin was visualized by fluorescence microscopy. The percentage of apoptotic cells was calculated as follows:

$$\frac{\text{Number of cells with fragmented chromatin}}{\text{Total cell number}} \times 100(\%)$$

More than 1000 cells were counted for each time point.

DNA Fragmentation Assay

An aliquot of 1×10^7 PBMCs was washed with phosphate buffer saline, resuspended in 500 μL ice-cold lysis buffer (10 mmol/L ethyl diaminetetra-acetic acid, 0.025% Triton X-100, 2.5 mmol/L TRIS-HCl pH 8.0), and incubated on ice for 15 minutes. Intact nuclei were pelleted by centrifugation at 15,000 rpm at 4 C for 10 minutes, and the supernatant was extracted with phenol. The DNA was precipitated with ethanol and treated with 1 μg/mL RNase for 1 hour at 37 C. Fragmented DNA was analyzed by electrophoresis through 2% agarose gels. The DNA was visualized by staining with 1 μg/mL ethidium bromide. The DNA size markers used were 4870, 2016, 1107, 926, 658, 489, 267, and 80 base pairs.

Flow Cytometry

An aliquot of 1×10^6 of freshly isolated PBMCs in 1 mL RPMI-1640 was first incubated with either 5 μg mouse anti-Fas monoclonal antibody (mAb), CH11 (MBL, Nagoya, Japan),[26] or 5 μg purified mouse IgM (ICN ImmunoBiologicals, Costa Mesa, CA). After incubation for 30 minutes at 4 C, cells were washed twice with ice-cold RPMI-1640 and then incubated for 30 minutes at 4 C with 5 μg fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Cappel, Durham, NC). More than 1000 stained cells were analyzed by EPICS profile (Coulter Co., Hialeath, FL).

Phenotypical Analysis of Apoptotic Lymphocytes

The PBMCs obtained 24 hours after surgery were cultured for 24 hours and then were stained with various phycoerythrin-conjugated monoclonal antibodies such as anti-CD3, CD4, CD8, CD11b, CD16, CD20, and CD29 (Coulter). Chromatin was stained with Hoechst 33342. Stained cells were visualized with fluorescence microscopy.

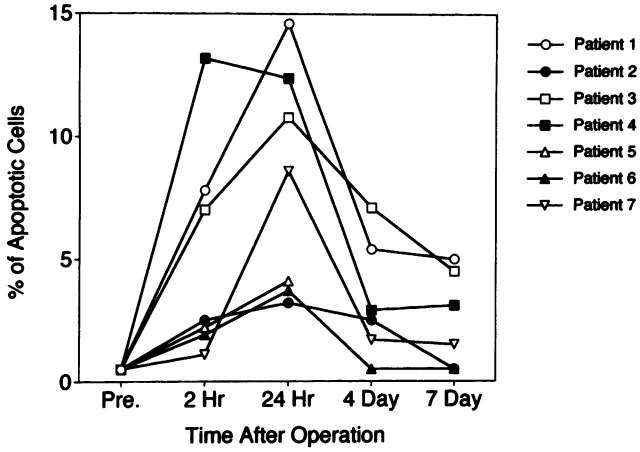

Figure 2. Changes in the percentage of apoptotic PBMCs before and after surgery. The PBMCs isolated from each patient before and after surgery were cultured as described in Figure 1, and the percentage of apoptotic cells was determined. Each point represents the average of two counts. For each point, the standard deviation was less than 10%. The PBMCs obtained after surgery showed a significant increase in the number of apoptotic cells as early as 2 hours after the start of surgery and 24 hours after surgery (before surgery *vs.* 2 hours and 24 hours after surgery; $p < 0.05$, $p < 0.01$, respectively).

Fas Agonist/Antagonist Treatment of Cultured Lymphocytes

The PBMCs obtained before day 1 and after surgery were cultured with either 0.5 μg/mL CH11 (a cytocidal anti-Fas mAb) or 5 μg/mL ZB4 (a Fas antigen agonist) (MBL, Nagoya, Japan)[20] for 1 day. Cells were examined for nuclear fragmentation as described above. Cell viability was determined by trypan blue dye exclusion method.

Statistical Analysis

Data were compared using the paired two-tailed Student's t test or, when appropriate, analysis of variance followed by Fisher's least significant difference if a significant F value was obtained using a computer (Macintosh LC, Apple Computer, Inc., Cupertino, CA) and commercial software (Statistica, Three's Company, Inc., Tokyo, Japan). A p value less than 0.05 was considered statistically significant. Data are expressed as the mean ± standard error.

RESULTS

Apoptotic Cell Detection in Peripheral Blood Mononuclear Cells After Surgery

When freshly isolated PBMCs were stained with Hoechst 33342, almost no fragmented cells were detected, regardless of whether they were obtained before or after surgery (data not shown). However, when these cells were cultured *in vitro* for 24 hours, typical apoptotic cells with bleb morphology were detected only in the samples isolated after surgery (Fig. 1).

We next quantitated the apoptotic cells as shown in Figure 2. Cultures of PBMCs obtained before surgery contained almost no apoptotic cells (less than 0.5%), whereas cultures of PBMCs obtained after surgery showed a significant increase in the number of cells committed to apoptosis as early as 2 hours after the start of surgery (before surgery *vs.* 2 hours and 24 hours after surgery; $p < 0.05$, $p < 0.01$, respectively). The percentage of apoptotic cells increased by 24 hours after surgery (except for in patient 4, who had a peak at 2 hours after surgery) and gradually decreased. As many as 14.6% of the PBMCs isolated from patient 3 at 24 hours after surgery were apoptotic.

To confirm that PBMCs isolated after surgery undergo apoptosis, we analyzed DNA from each sample by agarose gel electrophoresis. Figure 3 shows typical data from patient 4. The DNA ladder was seen in the samples obtained after surgery but not in the preoperative samples.

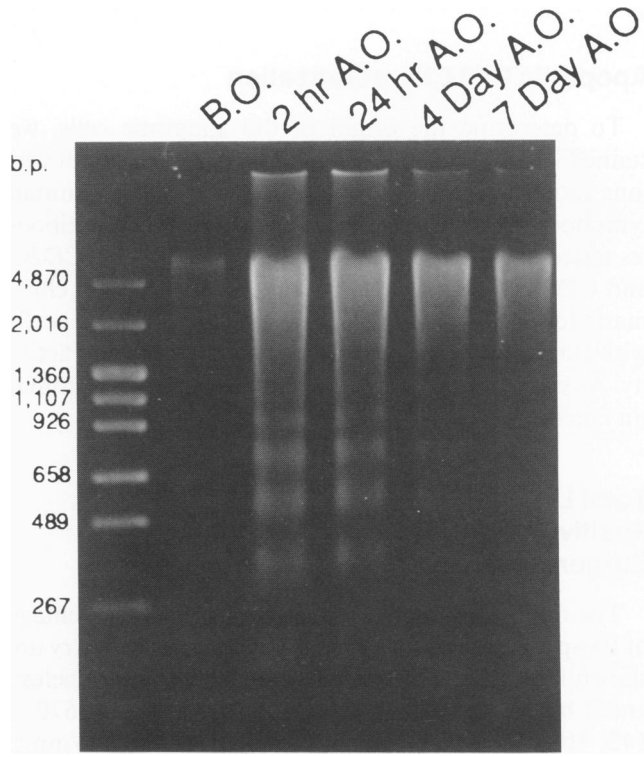

Figure 3. The DNA ladder fragmentation detected in PBMCs after surgery. Freshly isolated PBMCs were cultured for 24 hours as described in Figure 1, and DNA was extracted and analyzed by agarose gel electrophoresis. The DNA standard size markers shown are 4870, 2016, 1107, 926, 658, 489, 267, and 80 base pairs. A definite ladder pattern was detected in PBMCs obtained 2 hours and 24 hours after the start of surgery. However, occasional DNA fragmentation was observed in PBMCs before surgery. The DNA fragmentation decreased 4 and 7 days after surgery.

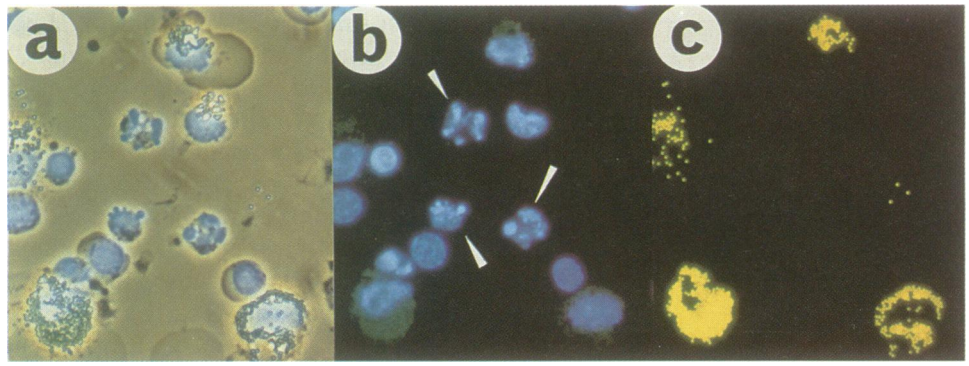

Figure 4. Lack of phagocytic activity in cells with fragmented chromatin. The PBMCs obtained from patient 2 at 24 hours after surgery were cultured with fluorescent latex microparticles. (A) Phase-contract and ultraviolet filter microscopy (excitation, 330–380 ηm, absorbance, 420 ηm); chromatin can be seen as blue intranuclear material. (B) Ultraviolet filter only; chromatin was clearly stained blue and microparticles were weakly stained. (C) Microparticles ingested by cells were visualized as yellow (excitation, 470–490 ηm, absorbance, 520 ηm). White arrowheads show cells with fragmented chromatin, which failed to phagocytose fluorescent latex.

Almost identical data were obtained with DNA from other patients (data not shown). Neither apoptotic cells nor DNA fragmentation was observed when PBMCs from healthy adults were cultured *in vitro* (data not shown).

Apoptotic Cell Characterization

To determine the origin of the apoptotic cells, we stained PBMCs obtained 24 hours after surgery with various mouse monoclonal antibodies specific for human lymphocyte CD markers. However, none of the antibodies tested (anti-CD3, CD4, CD8, CD11b, CD16, CD20, and CD29) recognized the cells with fragmented chromatin (data not shown). We next examined whether cells with fragmented chromatin possessed phagocytic activity. As shown in Figure 4, cells with fragmented chromatin failed to phagocytose fluorescent latex.

Total Lymphocyte Number and Fas-Positive Lymphocyte Percentage After Surgery

The number of total lymphocytes and the percentage of Fas-positive lymphocytes before and after surgery are shown in Figure 5. The number of lymphocytes before and 2 hours, 1, 4, and 7 days after surgery was 1670 ± 143, 1087 ± 105, 1128 ± 189, and 1530 ± 190/mm^3, respectively. These numbers were significantly decreased on days 1 and 4 after surgery ($p < 0.02$, $p < 0.05$, respectively) and returned to preoperative levels 7 days after surgery.

The percentage of Fas-positive lymphocytes before and 2 hours, 1, 4, and 7 days after surgery was 40.3 ± 2.5, 46.3 ± 3.6, 43.0 ± 3.4, 44.4 ± 4.1, and 38.5 ± 4.6%, respectively. These percentages were significantly increased 2 hours after the start of surgery ($p < 0.02$) and decreased to preoperative levels 7 days after operation.

Anti-Fas Antibody Effect and Fas Antigen Antagonist on Apoptosis in Lymphocytes

To determine the relationship between apoptosis in circulating lymphocytes after surgery and Fas expression, we cultured PBMCs obtained from patients 17 and 18 at 1 day after surgery in the presence of either anti-Fas antibody (CH11) or Fas antigen antagonist (ZB4). Figure

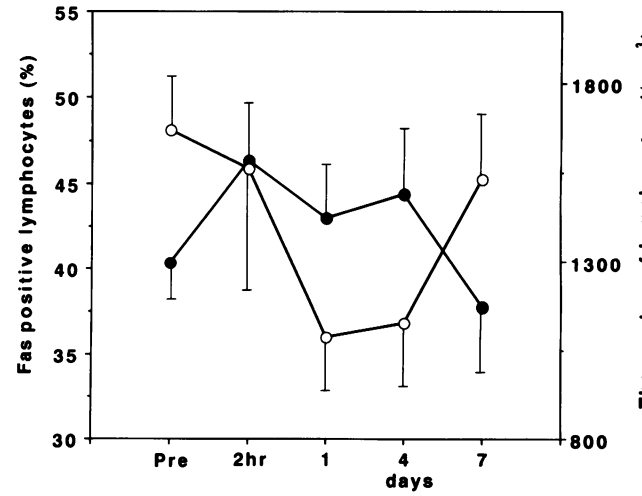

Figure 5. Changes in the number of total lymphocytes and percentage of Fas-positive lymphocytes after surgery. The number of lymphocytes was significantly decreased on days 1 and 4 after surgery ($p < 0.02$, $p < 0.05$, respectively). The Fas-positive lymphocytes were significantly increased 2 hours after the start of surgery ($p < 0.02$) and decreased to preoperative levels 7 days after operation.

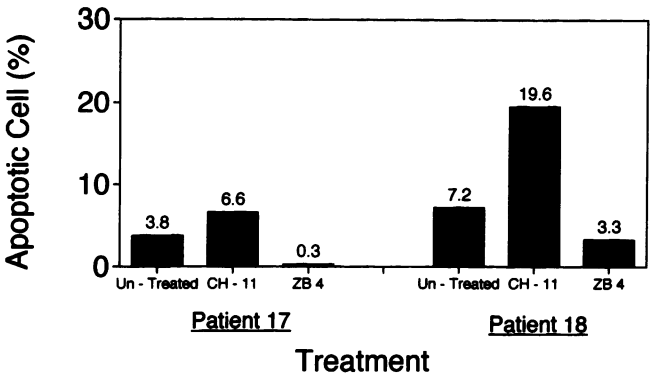

Figure 6. Augmentation of apoptosis in PBMCs by CHII, an anti-Fas mAb, and inhibition of apoptosis of PBMCs by ZB4, a Fas antagonist, in patients 17 and 18. The PBMCs obtained 1 day after surgery were cultured for 24 hours with or without anti-Fas mAb or ZB4 as described in the Materials and Methods section. The DNA was stained with Hoechst 33342 for 2 hours, and cells were examined by fluorescence microscopy. The percentage of apoptotic cells was determined as described in Figure 3. The anti-Fas mAb accelerated apoptosis and ZB4 inhibited apoptosis.

6 shows that CH11 augmented apoptotic cell death of lymphocytes after surgery in both patients. Conversely, ZB4 inhibited apoptotic cell death almost completely in patient 17 and decreased the number of apoptotic cells by 54.2% in patient 18.

DISCUSSION

In 1972, Kerr et al. proposed the term *apoptosis* to describe a common series of morphologic changes that accompany a characteristic form of death.[7] The hallmarks of apoptosis are delayed loss of membrane integrity and rapid onset of oligonucleosomal length DNA fragmentation.[27] Apoptosis, which is a form of physiologic cell death, is observed in immature T-cells on T-cell receptor stimulation,[10] in irradiated lymphocytes,[28] cytotoxic T-lymphocyte target cells,[29] metamorphosing tadpole tails,[30] and regression tumors.[31] Our study clearly showed that PBMCs obtained after surgery, but not before, underwent apoptosis by morphologic (chromatin condensation) and biochemical (DNA fragmentation) markers. Cells committed to apoptosis appeared as early as 2 hours after the start of surgery and peaked at 24 hours after the operation. By day 7 after surgery, the proportion of apoptotic cells had decreased significantly. We initially examined the peripheral blood cells on smears stained by Wright stain. However, no apoptotic cells were observed (data not shown). Because apoptotic cells are rapidly cleared by phagocytosis *in vivo*,[32] circulating apoptotic cells were probably eliminated by phagocytosis or were physically destroyed in the peripheral blood stream. However, culture of PBMCs *in vitro* allowed detection of apoptotic cells.

An electron microscopic study of apoptosis revealed that apoptotic cells rapidly lose their microvilli.[33] Thus, it was predictable that none of the apoptotic cells detected in this study reacted with mouse monoclonal antibodies raised specifically against human lymphoid cell surface marker. However, the observation that none of these apoptotic cells ingested latex suggested that the cells might be of lymphocytic origin. These results indicated that circulating lymphocytes may be susceptible to apoptosis after surgery.

Yonehara et al. have described a mouse monoclonal antibody-defined cell surface antigen that is cytocidal to a variety of human cells, including those of hematopoietic origin.[26] The cell surface antigen recognized by this monoclonal antibody was designed Fas, and a study using cloned Fas cDNA showed that Fas mediates cellular apoptosis.[13] Thus, we asked whether apoptosis in circulating lymphocytes induced by surgical stress may be related to expression of Fas. We examined Fas expression on circulating lymphocytes after surgery as well as induction of apoptosis by an anti-Fas mAb and inhibition of apoptosis by a Fas antagonist. The Fas-positive lymphocytes increased significantly 2 hours after the start of surgery and returned to preoperative levels by postoperative day 7. Namely, surgical stress made an influence on Fas expression in circulating lymphocytes. It has been reported that normal PBMCs are resistant to the cytocidal effects of anti-Fas mAb (CH11), even though they can be stimulated to express Fas by phytohemagglutinin and interleukin-2.[18] In fact, in this study few PBMCs obtained before surgery became apoptotic with any treatment. However, PBMCs obtained after surgery were killed by an anti-Fas mAb (CH11). Apoptosis in lymphocytes obtained after surgery was almost completely (patient 7) or significantly inhibited by an antagonist against anti-Fas antibody (ZB4). These results strongly suggest that Fas may mediate apoptosis in circulating lymphocytes after surgery.

We have shown that apoptosis in circulating lymphocytes, which may be mediated by Fas, is responsible for the depletion of lymphocytes after surgery. However, it is still unclear which factors augment Fas expression in circulating lymphocytes after surgery. Many factors such as anesthetics, cortisol, catecholamines, and cytokines may be involved. Further studies are required to address this question.

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