Apoptosis and Surgical Trauma

Dysregulated Expression of Death and Survival Factors on Peripheral Lymphocytes

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Background: Surgery and anesthesia cause depression of cell-mediated immunity in the postoperative period, including a reduction in the numbers of circulating lymphocytes. It has been claimed that this immunosuppression is associated with an increased incidence of postoperative infections.

Hypothesis: Lymphocytopenia following surgical trauma depends on a dysregulated expression of death and survival factors associated with apoptosis that, in turn, interferes with the occurrence of postsurgical infections.

Design: Fifteen subjects undergoing elective surgery under general anesthesia entered the study. The data of the patients who had infections during the postoperative outcome were compared with the data of those who did not. The data were collected prospectively.

Main Outcome Measures: Peripheral blood samples were drawn before the operation, and 24 hours and 96 hours after the operation. Lymphocytes were isolated and examined for quantification and phenotypic analysis of apoptosis using the 7-amino-actinomycin D method, as well as for Fas and Fas ligand, interleukin 1–converting enzyme p20/caspase-1, Bcl-2, and p35 expression. The rate of apoptotic cells was correlated with the incidence of postoperative infections.

Results: Twenty-four hours after surgery, CD4+ and CD8+ cells exhibited a significantly higher frequency of apoptosis as well as of Fas and Fas ligand and interleukin 1–converting enzyme p20/caspase-1 expressions than preoperatively. This increase was paralleled by a significant down-regulation of antiapoptotic factors such as Bcl-2. However, the expression of the proapoptotic factor p35 was reduced. In addition, we found a relationship between the rate of the apoptotic CD8+ subset and the occurrence of infectious complications during the postoperative course. At 96 hours after surgery, the variables studied returned to the baseline levels.

Conclusions: In the early postoperative period, surgical trauma under general anesthesia induces an intracellular perturbation on peripheral lymphocytes, resulting in both up-regulation of death-signaling factors and down-regulation of survival-signaling factors. The increased apoptosis of CD8+ lymphocytes, but not of CD4+ cells, seemed to be associated with a greater risk of postsurgical infections.

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PATIENTS AND METHODS

PATIENTS AND BLOOD SAMPLES

We studied 15 subjects undergoing elective surgery under general anesthesia at the Department of Surgery, “La Sapienza” University, Rome, Italy, following the approval of the study protocol by the hospital institutional ethical committee and the attainment of informed written consent from the patients in all cases.

Patients were excluded if they had received treatment with immunosuppressive drugs, including corticosteroids, in the previous 3 months, and if they had a malignant disease or a history of endocrine, hematologic, or metabolic disorders. Criteria for exclusion also included a history of infection with the human immunodeficiency virus and a blood transfusion in the 12 hours preceding study enrolment.

The method of general anesthesia was standardized for all patients. After premedication with intramuscular diazepam approximately 30 minutes before the operation, anesthesia was induced with thiopentone sodium (4-5 mg/kg) and fentanyl citrate (5 µg/kg), and orotracheal intubation was facilitated with pancuronium bromide (0.08 mg/kg). Anesthesia was maintained with isotfuran at an inspiratory concentration of 0.4% to 0.6%, and bolus doses of fentanyl were administered as required. The patients’ lungs were ventilated mechanically with 40% oxygen in nitrous oxide and normobaric ventilation was established by capnometric control. A balanced salt solution was infused to compensate for fluid losses at a rate of 6 mL/kg/h during the operation. Monitoring consisted of continuous electrocardiogram and measurements of inspiratory oxygen, end-expiratory carbon dioxide concentrations, transcutaneous oxygen saturation, and indirect blood pressure.

Peripheral blood samples were drawn before surgery (t₀) and at 24 hours and 96 hours after the operation (t₁ and t₂, respectively). The samples were immediately transferred to the laboratory for evaluation of lymphocyte apoptosis and expression of proapoptotic and antiapoptotic proteins.

LYMPHOCYTE ISOLATION

Lymphocytes were separated from heparinized peripheral blood by Lymphoprep gradient centrifugation (Nycomed Pharma, Oslo, Norway) as described by the method of Boyum,7 washed twice with phosphate-buffered saline (PBS) and resuspended in Roswell Park Memorial Institute 1640 medium (Life Technologies Inc, Paisley, Scotland), supplemented with 10% heat-inactivated fetal calf serum (FCS; Life Technologies Inc), 1% penicillin with streptomycin, 10-mmol HEPES (Sigma Chemical Co, St Louis, Mo), and 1 mmol/L of glutamine (Life Technologies Inc) (complete medium). In the apoptosis assay, lymphocytes (5×10⁸/L) were cultured in complete medium for 12 hours at 37°C in a 5% carbon dioxide–humidified atmosphere.

Ten age-matched and sex-matched healthy individuals were selected from among the hospital and laboratory personnel to serve as controls.

EXPRESSION OF SURFACE AND INTRACELLULAR PROAPOPTOTIC AND ANTIAPOPTOTIC PROTEINS

The absolute counts of cells bearing either the CD4+ or CD8+ and the Fas and FasL phenotype were determined by flow cytometry. Lymphocytes were stained with the following monoclonal antibodies: phycoerythrin–labeled anti-hCD4+ or anti-hCD8+ (Becton Dickinson Immunocytometry Systems; Becton Dickinson and Co, San Jose, Calif), anti-hCD95/Fas/APO1 (Upstate Biotechnology Inc, New York, NY), and fluorescein isothiocyanate–labeled anti-mouse IgM (Sigma Chemical Co), anti-hCD95L/FasL (Pharmigen, San Jose, Calif) and anti-mouse IgG FITC conjugate (Sigma Chemical Co). For staining of surface proteins, 5×10⁸/L lymphocytes were washed in PBS containing 1% bovine serum albumin (BSA; Sigma Chemical Co) and 0.1% sodium azide (NaN₃).

The mean ± SD duration of postoperative hospital stays was 8.72 ± 4.13 days. During this period, 1 patient died on the sixth day after operation because of the disruption of the aortic aneurysm that was surgically repaired. Six patients had infectious complications in the postoperative course, 2 of whom had bronchopneumonia on the fourth and fifth postoperative day, respectively; 1 patient who had thrombophlebitis on the sixth day; and 3 subjects who had wound infections on the sixth, fourth, and sixth day, respectively.

These complications were treated successfully with antibiotic therapy, but they delayed patient discharge from the hospital. The longest postoperative stay was 16 days.

APOPTOTIC CD4+ AND CD8+ LYMPHOCYTES

As expected, by measuring apoptosis with 7-AAD, the great majority of CD4+ and CD8+ cells were not stained by the fluorescent intercalating agent in control individuals (mean ± SD percentage, 0.9% ± 0.4% and 1.1% ± 0.3% of apoptotic cells for CD4+ and CD8+ subsets, respectively) nor in surgical patients at the time of baseline measure-
(PBS-BSA-NaNO₃) followed by incubation for 20 minutes at 4°C with the monoclonal antibodies previously described. For determination of background staining, cells were incubated with 20 µL each of the mouse IgG FITC and mouse IgG1 phycoerythrine (Becton Dickinson and Co). After one wash with 2 mL of PBS-BSA-NaNO₃, the supernatant was removed, and the cell pellet was resuspended in 1 mL of PBS-BSA-NaNO₃.

To evaluate the intracellular expression of ICEp20, p35, and Bcl-2 proteins, freshly isolated peripheral lymphocytes (5 × 10⁶/L) were washed in PBS-BSA-NaNO₃. Cells were then incubated with the conjugated monoclonal antibodies specific for the CD4⁺ and CD8⁺ surface markers described above. The stained cells were further washed in PBS-BSA-NaNO₃ fixed in 1 mL of 0.25% paraformaldehyde, while vortexing at 300g for 5 minutes and then incubated in the dark for at least 15 minutes at room temperature. After washing with PBS-BSA-NaNO₃, the samples were added to 1 mL of cold (4°C) 70% methanol, and incubated in the dark for 60 minutes at 4°C to make the cell and nuclear membrane permeable. After this washing, the samples were incubated in the dark with an anti-ICEp20 or an anti-p35 monoclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) and anti-rabbit FITC-labeled IgG (Sigma Chemical Co) or with a FITC-labeled anti-Bcl-2 monoclonal antibody (DAKO, Glostrup, Denmark) for 30 minutes at 4°C. Then, after 2 washings with PBS-BSA-NaNO₃ containing 2% fetal calf serum, the samples were analyzed using flow cytometry.

ASSESSMENT OF CELLS UNDERGOING APOPTOSIS

Quantification and phenotypic analysis of apoptotic cells from the short-term cultured lymphocytes were performed by staining the apoptotic cells with 7-aminoactinomycin D (7-AAD; Sigma Chemical Co) or with a FITC-conjugated FITC-labeled IgG (Sigma Chemical Co). The spectral properties of 7-AAD allow the staining of apoptotic cells and to avoid nonspecific staining in their staining solution by a fluorescent-activated cell scan flow cytometer (Becton Dickinson and Co). The spectral properties of 7-AAD allow the staining of apoptotic cells by fluorescence emission in the red channel fluorescent 3 (650 nm < wavelength < 850 nm), and the easy and simultaneous labeling of cell surface proteins (fluorescein 1 and fluorescein 2). Identification of apoptotic CD4⁺ and CD8⁺ cells was assessed also by considering scatter characteristics, based on the evidence that apoptotic cells can easily be distinguished from viable cells via measurement of forward scatter and side scatter light parameters, which are proportional to cell diameter and internal granularity, respectively.⁹ Indeed, while living cells displayed relatively high forward scatter and low side scatter properties, cells undergoing programmed cell death shifted to a lower forward scatter and to a higher side scatter compartment, consistent with the cellular changes occurring during apoptosis (reduction of cell size, cytoplasmic volume, and chromatin condensation).⁹,¹⁰

STATISTICAL ANALYSIS

Statistical analysis was performed using the Statgraphics Plus program (Bisstream Inc, Cambridge, Mass). Changes over time of the variables studied were examined using 1-way analysis of variance for repeated measurements. The t test was used to compare the data of the patients who developed infections with the data of those who did not. P < .05 was defined as significant.

The Table presents the percentage of apoptotic CD4⁺ and CD8⁺ T cells detected at t₁ in the patients studied. Subjects who went on to suffer postsurgical infections had a

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significant increase in the frequency of apoptotic CD8+ lymphocytes compared with subjects who did not develop infectious complications (P<.05). Conversely, no significant difference was found in the rate of apoptotic CD4+ cells between the 2 groups.

Furthermore, we were able to establish a cut-off level in the percentage of apoptotic CD8+ cells that was associated with the occurrence of infections; in fact, infections occurred in all patients who had a frequency of apoptotic CD8+ cells that was greater than or equal to 12%, whereas none of the subjects with a rate of apoptotic CD8+ cells that was less than this value went on to have these complications.

Expression of both Fas and FasL was significantly elevated in CD4+ and CD8+ cell subsets at t1 with respect to baseline values, paralleling the increased lymphocyte apoptosis. In fact, at t1, the mean ± SD percentages of CD4+ and CD8+ cells bearing the Fas protein was 43.7% ± 2.4% and 45.7% ± 3.2%, respectively, while at the time of baseline measurement, mean ± SD percentages of Fas-positive CD4+ and CD8+ cells were 26.4% ± 3.2% and 26.5% ± 3.3%, respectively (P<.05 for the 2 parameters).

Likewise, the mean ± SD percentage of CD4+ and CD8+ cells expressing FasL was also increased at t1, compared with baseline values (39.1% ± 3.9% vs 20.7% ± 1.8% for CD4+, P<.05; and 44% ± 3.9% vs 19.9% ± 2% for CD8+, P<.05). However on t2 measurements, the expression of Fas and FasL by CD4+ and CD8+ lymphocytes returned to preoperative values and no significant change was observed.

When we further investigated the intracellular expression of ICEp20/caspase-1, we found a significant elevation in the frequency of ICEp20-positive CD4+ and ICEp20-positive CD8+ cells at t1 (50.1 ± 5.9 vs 27.8 ± 3.6 for CD4+, P<.05; and 47.2 ± 7.4 vs 23.6 ± 4.6 for CD8+, P<.05) but both subsets returned to the baseline values at t2. By contrast, at this same time point, the frequency of p35-positive lymphocytes was significantly reduced compared with the preoperative levels (49.8 ± 7.1 vs 69.1 ± 7.9, P<.05, for p35-positive CD4+ cells and 44.3 ± 4.4 vs 61.7 ± 3.9, P<.05, for p35-positive CD8+ cells) but promptly returned to the baseline frequency on t2 measurement.

Finally, at t1, we observed a significant reduction in the percentage of CD4+ and CD8+ cells bearing the anti-apoptotic Bcl-2 phenotype compared with preoperative time (61.7 ± 5.2 vs 81.3 ± 3.8, P<.05, for Bcl-2–positive CD4+ cells and 57.1 ± 5.2 vs 75.9 ± 4.9, P<.05, for Bcl-2–positive CD8+ cells). In turn, on t2 measurement, the frequency of CD4+ and CD8+ lymphocytes expressing Bcl-2 was comparable with the baseline frequency.

The mean percentages of CD4+ and CD8+ cells expressing Fas, FasL, ICEp20/caspase-1, Bcl-2, and p35 at each point in the study are given in Figure 2 and Figure 3, which show a strongly similar trend in the kinetics of expression of those proteins by both CD4+ and CD8+ lymphocyte subsets.

Apoptosis occurs through the activation of a cell-intrinsic suicide program. A finely tuned balance between the action of apoptosis-signaling systems such as Fas and FasL, tumor necrosis factor α, and the caspase system, in addition to the action of antiapoptotic systems such as Bcl-2, is crucial to the maintenance of normal immune responses.11,12

A depletion of circulating lymphocytes is usually observed during the postoperative period and different plausible explanations have been proposed; however, so far the ultimate mechanisms have not been definitively clari-
fied. In particular, the discussion is centered on the possibility that surgery-induced lymphocytopenia may result from either lymphocyte redistribution, inappropriate cell death, or both. In this study, we demonstrated that the rate of lymphocyte apoptosis is significantly increased in patients undergoing surgical stress. The frequency of apoptotic CD4+ and CD8+ T cells was significantly elevated at t1 compared with at the preoperative measurement. However, the commitment of lymphocytes to undergo apoptosis seemed to be transient, as at t2 measurement, the frequency of apoptotic cells was comparable in each population subset with the low levels observed in the same patients at the baseline measurement performed at t0, and in healthy control individuals.

This temporal profile of accelerated lymphocyte death was closely paralleled by an augmented expression of proapoptotic signaling systems (Fas and FasL, and ICEp20/caspase-1); in turn, factors that act as antiapoptotic signaling systems (Bcl-2) were downregulated. The kinetics of the altered expression of these proteins was strictly comparable with the temporal profile observed in the frequency of apoptotic lymphocytes. In fact, CD4+ and CD8+ cells expressing Fas and FasL and ICEp20/caspase-1 were strongly increased at t1, but returned to low baseline levels at t2. Likewise, the rate of cells bearing Bcl-2 was greatly reduced at t1 and returned to baseline levels at t2 measurement. Furthermore, this perturbation in the signaling systems involved in the regulation of apoptosis with the resulting accelerated rate of lymphocyte apoptosis was associated with a decrease in the absolute counts of CD4+ lymphocytes on measurements performed at t1 and that returned to the normal range at t2.

Our finding of increased ICE/caspase-1 expression by lymphocytes at t1 requires discussion. Based on substrate specificity, caspases are grouped into 3 categories. Several studies have indeed shown that group I caspases (-1, -4, -5), which include ICE/caspase-1, are primarily involved in inflammation rather than in apoptosis, whereas group II and group III caspases are most important for the execution and signaling events of apoptosis. Interleukin 1–converting enzyme p20 (ICEp20), p35, and Bcl-2–positive CD4+ cells before surgery (t0), at 24 hours after surgery (t1), and at 96 hours after surgery (t2). Values are expressed as means ± SD. Asterisks indicate P < .05 vs baseline values.

expression of ICE/caspase-1 has a role even in the increased process of lymphocyte apoptosis that we measured after surgery. Further investigations are needed for a better understanding of this issue.
The significance of the reduced expression of p35 is also unclear and difficult to explain, though it is known that p35 is a regulatory subunit of cyclin-dependent kinase-5 (cdk-5), a key regulator of cell-cycle progression.\(^1\)\(^9\)\(^,\)\(^20\) There is evidence that cdk-5 expression is specific to apoptotic cells and that high levels of expression and kinase activity are found in dying cells.\(^21\)\(^,\)\(^22\) Cyclin-dependent kinases are considered to be candidate apoptotic effectors, given their activation during cell proliferation.\(^23\) Since the highest levels of cdk-5 activity are expressed in the brain with low or undetectable levels in all other tissues,\(^23\)\(^-\)\(^26\) it is likely that the p35 and cdk-5 pathway has only a marginal impact on lymphocyte apoptosis. This hypothesis could be in agreement with the reduced lymphocyte expression of such a proapoptotic factor as p35 despite the accelerated rate of lymphocyte apoptosis that we measured during t\(_1\) in our patient sample. However, when our results are altogether considered, it appears conceivable that the surgical trauma itself triggers a complex alteration in the intracellular pathways and substrates that regulate survival and death of lymphocytes, with an ultimate net effect of a greatly accelerated rate of lymphocyte apoptotic death.

We cannot rule out the hypothesis that a redistribution of lymphocytes between peripheral blood and either lymphoid organs or sites of inflammation such as the surgical wound also contributes to the lymphocytopenia commonly recognized in the early postoperative period. However, our results strongly support the view that an unregulated activation of the apoptotic process is the main mechanism responsible for the decrease in the absolute counts of peripheral blood lymphocytes in subjects undergoing surgery, and this probably explains the immune dysfunction seen in those subjects and their susceptibility to viral and bacterial infections.

Our study is also in agreement with this hypothesis from a clinical standpoint. We provided data that add weight to the opinion that the accelerated apoptosis of immune cells in the early postoperative period could have important implications for the outcome of patients. In particular, subjects with a more elevated rise in apoptosis of CD8\(^+\) lymphocytes seemed to have a greatly increased risk of infections, as a higher rate of apoptotic CD8\(^+\) cells at 24 hours after surgery was associated with the occurrence of infections during the postoperative follow-up. Specifically, we observed a cutoff level in the percentage of apoptotic lymphocytes in the CD8\(^+\) subset that significantly predicted the occurrence of infections after surgery. In fact, only those subjects with a frequency of apoptotic CD8\(^+\) cells equal to or greater than 12% had infections, whereas none among the subjects with a smaller frequency of apoptotic CD8\(^+\) cells went on to have infectious complications. We were unable to establish a similar relationship between the frequency of apoptotic CD4\(^+\) lymphocytes and the subsequent occurrence of postsurgical infections. The reasons for this discrepancy are unclear and require further investigation, even though a probable explanation could be that our study did not have sufficient statistical power because of the small sample size of patients that was enrolled.

The question remains open with regard to whether soluble factors or cytokines released during operative trauma or associated with surgery could be the ultimate cause of the perturbation in lymphocyte apoptosis that we observed.

It is well known that surgical procedure promotes a neuroendocrine/inflammatory response that is mediated by stress hormones including cortisol and catecholamines, as well as by inflammatory cytokines such as tumor necrosis factor \(\alpha\) and interleukin 6.\(^27\)\(^,\)\(^28\) We did not investigate the kinetics of plasma concentrations of those cytokines and stress hormones, but there is clear evidence that these substrates are involved in regulating apoptosis and that in particular, tumor necrosis factor \(\alpha\) is main signaling messenger for cell apoptosis.\(^29\)\(^-\)\(^32\) Each one or all of these substances together could be implicated in the induction of postsurgical lymphocyte apoptosis and in the dysregulated balance between the expression of proapoptotic and antiapoptotic factors resulting in T-cell death.

An additional hypothesis to be tested is whether anesthesia could participate either directly or indirectly in the induction of postoperative apoptotic death occurring in T cells. This view is supported by the recent demonstration that anesthetic agents such as fentanyl or isoflurane may modulate cytokine release and interleukin 6 response to surgery.\(^33\)\(^,\)\(^34\)

In conclusion, our data strongly support the observation that surgical procedure in general anesthesia promotes an intracellular perturbation resulting in T-cell apoptosis which accounts for postoperative lymphocytopenia. Of relevance, this phenomenon is associated not only with up-regulation of the Fas and FasL system, as previously reported, but also with an altered expression of other proapoptotic and antiapoptotic factors. Furthermore, our study suggests that an accelerated rate of lymphocyte apoptosis, particularly among the CD8\(^+\) subset, in the early postoperative period could correlate with infectious complications following surgery.

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REFERENCES

Use of Calcium Channel Blockers and Risk of Hospitalized Gastrointestinal Tract Bleeding

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Background: We conducted a case-control study of the association between calcium channel blocker use and gastrointestinal (GI) tract bleeding in hypertensive members of a health maintenance organization.

Methods: Case patients (n=174) were treated hypertensive health maintenance organization members hospitalized for GI tract bleeding in hypertensive members of a health maintenance organization.

Results: Compared with β-blocker users, calcium channel blocker users had an age-, sex- and calendar year–adjusted relative risk (RR) of GI tract bleeding of 2.60 (95% confidence interval [CI], 1.71–3.96). The RR associated with calcium channel blocker use was 2.05 (95% CI, 1.33–3.17) after further adjustment for number of recent visits, diastolic blood pressure, chronic congestive heart failure, and duration of hypertension. No significant dose-response relationship was observed. Compared with β-blocker users, angiotensin-converting enzyme inhibitor users had an RR of 1.22 (95% CI, 0.75–1.97). Calcium channel blocker use tended to be more strongly associated with risk of lower GI tract bleeding (RR, 2.56; 95% CI, 1.08–6.05) than with risk of upper GI tract bleeding (RR, 1.54; 95% CI, 0.91–2.59) or peptic ulcer–related bleeding (RR, 1.17; 95% CI, 0.62–2.21), although these results were compatible with chance.

Conclusions: Calcium channel blocker use might be associated with an elevated risk of GI tract bleeding. These findings require confirmation in randomized studies.

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