Postoperative Impairment of Cognitive Function in Rats

A Possible Role for Cytokine-mediated Inflammation in the Hippocampus


Background: Postoperative cognitive dysfunction is being increasingly reported as a complication. The authors investigated the role of cytokine-mediated inflammation within the central nervous system in the development of cognitive dysfunction in a rat model.

Methods: Adult rats were subjected to neuroleptic anesthesia (20 μg/kg fentanyl plus 500 μg/kg droperidol, intraperitoneal) for splenectomy or no surgery. On postanesthetic days 1, 3, and 7, cognitive function was assessed in a Y maze. To evaluate the immune response in the hippocampus, the authors measured glial activation, as well as transcription and expression of key proinflammatory cytokines interleukin 1β and tumor necrosis factor α. To determine propensity for apoptosis, they measured expression of Bax and Bcl-2.

Results: Cognitive function in splenectomized animals was impaired at days 1 and 3 after surgery compared with cognitive function in nonanesthetized rats. At all times, anesthetized rats that were not subjected to surgery were no different from control rats. Glial activation was observed in the hippocampus only in splenectomized rats at postsurgery days 1 and 3. Interleukin-1β messenger RNA (mRNA) was significantly increased at postsurgery days 1 and 3, with an increase in protein expression detected on day 1. There was a significant increase in tumor necrosis factor-α mRNA on day 1 after surgery, although this was not associated with an increase in protein expression. The ratio of Bcl-2:Bax was significantly decreased in the spleenectomized animals.

Conclusion: These results suggest that splenectomy performed during neuroleptic anesthesia triggers a cognitive decline that is associated with a hippocampal inflammatory response that seems to be due to proinflammatory cytokine-dependent activation of glial cells.

POSTOPERATIVE cognitive dysfunction (POCD), a distressing complication after surgery, is independently associated with poor short-term and long-term outcomes.1–4 Although it has increasingly been recognized as a complication after cardiac surgery, POCD also complicates noncardiac surgery.5 A multicenter trial noted that POCD was present in 25.8% of patients 1 week after surgery and in 9.9% of patients 3 months after surgery in patients older than 60 yr.6 POCD diminishes the quality of the patient’s life and adds cost to hospitalization and out-of-hospital care.7,8,9 Furthermore, POCD after cardiac surgery in the elderly is associated with a mortality rate (10–65%) that is comparable to that of the complication of perioperative myocardial infarction.8

Although the mechanisms for POCD remain unclear, the contribution of risk factors, including advanced age, prolonged duration of surgery, respiratory and infectious complications, and the need for a second operation must be considered.9 Possible pathogenic mechanisms may include patient-related factors (e.g., genetic susceptibility), surgical factors (e.g., embolization), and perioperative disruption of physiologic homeostasis (e.g., hypoxia-ischemia). Because the incidence of POCD does not seem to be influenced by the type of anesthesia (i.e., general vs. regional),10–12 attention has begun to focus on the role of the surgical intervention itself in the genesis of this condition.

Because increased cytokine secretion is associated with neurodegenerative disease and surgical trauma, a role for inflammatory mediators (e.g., cytokines) in the development of cognitive decline after surgery in patients with Alzheimer disease has been previously suggested.13–17 It has been postulated that a peripheral inflammatory response to a stress may induce neuroinflammation, which can result in cognitive dysfunction;18 in this setting, interruption of long-term potentiation in the hippocampus, an electrophysiologic correlate of learning and memory, has been noted.19

The aim of the current study was to determine whether a surgical procedure can trigger a proinflammatory cytokine response, in particular interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), in the hippocampus of adult rats, and to determine whether the inflammatory response is associated with cognitive dysfunction.

Materials and Methods

Animals and Surgical Procedures

Ninety-eight Sprague-Dawley male rats (weighing 300–350 g, approximately 90 days old) were housed individually in a light-, temperature-, and humidity-controlled environment.

Received from the Department of Anaesthetics, Pain Medicine and Intensive Care, Faculty of Medicine, Imperial College London, Chelsea & Westminster Hospital, London, United Kingdom, and the Department of Anaesthesiology, Gongli Hospital, Pudong, Shanghai, China. Submitted for publication September 6, 2006. Accepted for publication November 2, 2006. Support was provided solely from institutional and/or departmental sources.

Address correspondence to Dr. Maze: Department of Anaesthetics, Pain Medicine and Intensive Care, Imperial College, London, Chelsea & Westminster Hospital, 369 Fulham Road, London SW10 9NH, United Kingdom, m.maze@imperial.ac.uk. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

Anesthesiology, 2007; 106:436–43

Copyright © 2007, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins, Inc.
POCD, SURGERY, AND CYTOKINES

437

Anesthesiology, V 106, No 3, Mar 2007

environment with free access to food and water. Experiments were conducted in accordance with the guidelines for care and use of laboratory animals from the Chinese Academy of Science, Shanghai, China, and from the Home Office, United Kingdom.

Rats were randomly assigned to three groups. Naive controls (group A; n = 14) received no intervention. In the anesthetic group (group B; n = 42), rats were anesthetized with a combination of 20 μg/kg fentanyl and 500 μg/kg droperidol (intraperitoneal). Pilot studies established that rats anesthetized with this anesthetic regimen exhibited surgical analgesia (no purposeful response to supramaximal noxious stimuli) with no evidence of cardiorespiratory dysfunction (as reflected by normal arterial blood gas measurements; data not shown). The anesthesia-plus-surgery group (group C; n = 42) underwent splenectomy during neuroleptic anesthesia (as specified for the anesthetic group). For splenectomy, a small incision was made in the left upper abdominal quadrant and the spleen was mobilized, isolated, and removed; this procedure was chosen because it represents a standardized organ or tissue removal intervention. The wound was infiltrated with 0.25% bupivacaine and then closed by suture. Postintervention animals were recovered and then returned to their cages and housed individually. Rats in groups B and C were killed at day 1, 3, or 7 after intervention (n = 14/time point).

Cognitive Testing

Aversive memory, a hippocampal-dependent place learning, was evaluated on days 1, 3, and 7 after intervention by an investigator who was blinded to intervention (surgical incision area was covered by tape in all groups) using the modified Y-maze apparatus. The apparatus consists of three opaque-white arms (30 × 5 × 20 cm) connected into a Y shape (fig. 1). The floor of the “stem” of the Y arm and one of the two branch arms was installed with electric wires through which an electric shock (40 ± 5 V for 10 s) can be applied. The branch arm lacking wires remained illuminated throughout the test (because rats are typically neophobic, they prefer to avoid an illuminated area). During the testing, rats were placed in the stem of the Y maze; when animals received an electric shock, they entered into one or the other of the two branch arms. Before the next trial, the rats were manually returned to the stem of the Y maze. The test was stopped when the animal entered into the nonshock (illuminated) arm in 9 out of 10 consecutive trials; at this trial number, the rat was considered to have developed aversive memory.

After behavioral testing, half of the animals in each group were killed by an overdose of pentobarbitone (100 mg/kg intraperitoneal) and perfused transcardially with heparinized 0.9% saline followed by 4% paraformaldehyde in 0.1 m phosphate buffer. Brains from these perfused animals were placed in 4% paraformaldehyde in 0.1 m phosphate buffer for in situ immunohistochemistry.

Immunohistochemistry

Wax sections (5 μm) of the hippocampus were immunostained on slides. Briefly, after preincubation with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase, the sections were rinsed in phosphate-buffered saline. Glial fibrillary acidic protein (GFAP), S100β, Bax, Bcl-2, and caspase 3 were visualized immunohistochemically using rabbit anti-GFAP antibody (1:80; Sigma, St. Louis, MO), rabbit anti-S100β antibody (1:100; Dako, Carpenteria, CA), Bax, Bcl-2, and caspase 3 (1:100; Delta Biolab, Gilroy, CA), respectively. After incubation for 60 min with the primary antibodies, the sections were washed three times in phosphate-buffered saline at room temperature. The biotinylated secondary antibodies (Sigma) and the avidin–biotin–peroxidase complex (Vectastain Elite ABC-Kit; Vector Lab, Burlingame, CA) were applied (both diluted 1:200), and the sections were incubated for 30 min at room temperature. After the sections were washed with phosphate-buffered saline, the peroxidase reaction was developed by incubating the section in the 0.02% 3,3’-diaminobenzidine tetrahydrochloride (Sigma) solution containing 0.003% Western blot studies. The remaining animals in each group were killed by an overdose of pentobarbitone (100 mg/kg intraperitoneal) and perfused transcardially with heparinized 0.9% saline followed by 4% paraformaldehyde in 0.1 m phosphate buffer. Brains from these perfused animals were placed in 4% paraformaldehyde in 0.1 m phosphate buffer for in situ immunohistochemistry.
hydrogen peroxide. The sections were then counterstained with Mayer hematoxylin. Finally, the sections were dehydrated through a gradient of ethanol solutions (70–100%). Control sections were processed identically and in parallel; however, they were incubated with phosphate-buffered saline instead of the primary antibodies. No labeling was detected in these controls. For quantification, the number of immunopositive cells per a total of 1,000 in the hippocampal sections was counted at 400× magnification.

RNA Extraction and Reverse-transcription Polymerase Chain Reaction

Total RNA was isolated from homogenization of the hippocampal tissue samples in Trizol reagent. Chloroform (200 µl) was added to each tissue homogenate. Samples were centrifuged (12,200g, 15 min, 4°C), and the aqueous phase was transferred to a clean tube. An equivalent volume of sterile DNase and RNase-free H2O. and the pellet was allowed to air dry before reconstitution in 200 U Superscript II RNase H-reverse transcriptase (Life Technologies, Gaithersburg, MD). Reverse transcription was performed at 42°C for 60 min and 15 min, 4°C. Reverse transcription was performed at 42°C for 60 min and 15 min, 4°C). The amount of mRNA was expressed as a ratio of gene-specific mRNA and β-actin, which amplified a 578-bp product. The primers for TNF-α and IL-1β protein were normalized by the ratio of TNF-α and IL-1β to β-actin, respectively.

Western Blot Analysis

Hippocampi were homogenized in lysis buffer (5 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors; pH 7.5). The homogenates were centrifuged for 15 min at 12,000g at 4°C. The quantity of protein in the supernatants was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Protein extracts (60 µg per sample) were denatured in Laemmni sample loading buffer (10% SDS, 0.1 M Tris pH 8.0, 50 mM DTT, 3 mM EDTA, 0.001% bromphenol blue) at 100°C for 5 min, separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). After blocking with 5% skim milk in TBS-T (0.1% Tween 20, 20 mM Tris-HCl, 137 mM NaCl, pH 7.6) for 2 h, the membrane was incubated with rabbit anti-TNF-α (1:800) or anti-IL-1β (1:1,000; Sigma, St. Louis, MO) in TBS-T overnight at 4°C. Subsequently, membranes were incubated for 2 h with goat anti-rabbit HRP-conjugated secondary antibody (Amersham Pharmacia Biotech) at room temperature. The membranes were treated with the ECL system (Amersham) and then were developed on film. The bands were analyzed by densitometric analysis (Kodak Digital Science and 1D Image Analysis Software; Rochester, NY). The membrane blots were stripped and reprobed with polyclonal anti-β-actin (20-33; Sigma). Relative expression levels of TNF-α and IL-1β protein were normalized by the ratio of TNF-α and IL-1β to β-actin, respectively.

Results

Impairment of Aversive Learning and Memory after Surgery

To elucidate the effect of surgery on learning and memory, we conducted the Y-maze test, which is used widely to evaluate spatial learning and memory in rodents. In naive animals, it took 28 ± 7 trials for them to remember the impending risk of being shocked. The anesthetic regimen used in this study did not affect their learning ability, with the number of trials (28 ± 7, 29 ± 6, and 29 ± 7 at days 1, 3, and 7 after experiments, respectively) being no different than that seen in the control group. However, on days 1 and 3 after surgery, the number of trials of the test was significantly increased to 67 ± 26 and 60 ± 18 (P < 0.01 vs. control)
in the surgical animals. Although the number of trials was still higher in the splenectomized animals (41 ± 11) on day 7 after surgery, this did not differ statistically from that of naive control (P > 0.05 vs. control; fig. 1). These results suggest that surgery induces a temporary impairment in spatial learning and memory.

**GFAP and S100β Expression Triggered by Splenectomy**

Glial fibrillary acidic protein immunoreactivity was increased in the splenectomized rats when compared with the naive and anesthetized rats that did not undergo splenectomy. The GFAP-positive cells increased from 99 ± 24 in controls to 228 ± 39 (P < 0.01) and 169 ± 18 (P < 0.05) at days 1 and 3 after surgery, respectively (fig. 2). A similar pattern change was seen with S100β; S100β-positive cells were significantly increased from 106 ± 10 in controls to 203 ± 18 (P < 0.01) and 193 ± 60 (P < 0.05) at days 1 and 3 after surgery, respectively (fig. 3).

**IL-1β mRNA and Protein Expression Triggered by Surgery**

The anesthetics did not increase IL-1β mRNA expression at any time point when compared with the naive controls (P > 0.05). However, on days 1 and 3 after splenectomy, the level of IL-1β mRNA was increased by

---

**Fig. 2.** Glial fibrillary acidic protein (GFAP) expression in the CA1 area of hippocampus in rats. Representative photomicrographs of the CA1 area of hippocampus illustrating expression of GFAP from a control (no-intervention) rat (A), or from rats receiving anesthesia alone tested at 1 (B1), 3 (B3), or 7 (B7) days after anesthesia or animals tested 1 (C1), 3 (C3), or 7 (C7) days after anesthesia and surgery. (D) Histogram plotting the number of GFAP-positive cells (mean ± SD; n = 6). * P < 0.05 versus control. ** P < 0.01 versus control.
67% \( (P < 0.01) \) and 46% \( (P < 0.05) \), respectively, when compared with the naive controls. Protein expression of IL-1\( \beta \) was significantly increased by 40% on the first postsurgical day \( (P < 0.05) \) but not at other time points (fig. 4).

TNF-\( \alpha \) mRNA and Protein Expression Triggered by Surgery

The general anesthetic did not induce an increase of TNF-\( \alpha \) mRNA expression at any time point when compared with the naive controls \( (P > 0.05) \). However, on postsurgical day 1, the level of TNF-\( \alpha \) mRNA was increased by 38% \( (P < 0.05) \), but no difference in transcription was noted at days 3 and 7. There was no change in the expression of TNF-\( \alpha \) protein at any time after surgery (fig. 5).

Expression of Bax, Bcl-2, and Caspase 3

There was almost no Bcl-2 and Bax staining in hippocampal sections from controls and animals treated with anesthesia without surgery; a strong cytoplasmic signal for both Bax and Bcl-2 was detected in neurons in the CA1 area of the hippocampus in postsplenectomy rats (fig. 6). On days 1, 3, and 7, the numbers of Bax-positive cells were 378 ± 72, 272 ± 48, and 73 ± 1, respectively. The numbers of Bcl2-positive cells on postoperative days 1, 3, and 7 were 214 ± 25, 216 ± 31, and 62 ± 9, respectively. When considered as a ratio of Bcl2:Bax, the values on postoperative days 1, 3, and 7 were 0.57 ± 0.12, 0.79 ± 0.13, and 0.85 ± 0.09, respectively. Caspase 3 was not detected in any section examined.
Discussion

We investigated the association of an inflammatory response in the hippocampus with the development of POCD and found that after surgery (splenectomy) under neuroleptic general anesthesia (fentanyl–droperidol), rats

Fig. 4. Expression of interleukin-1β (IL-1β) messenger RNA (mRNA) and protein. Reverse-transcription polymerase chain reaction (RT-PCR) (a) and immunoblotting (b) images for IL-1β are represented for mRNA and protein, respectively, extracted from the hippocampus of a control (no-intervention) rat (A), or from rats receiving anesthesia alone tested at 1 (B1), 3 (B3), or 7 (B7) days after anesthesia or animals tested 1 (C1), 3 (C3), or 7 (C7) days after anesthesia and surgery. (c) Densitometry data (mean ± SD; n = 7) from the RT-PCR and immunoblotting images compared with that of the housekeeping gene β-actin are plotted as a histogram for each cohort. *P < 0.05, **P < 0.01 versus control. WB = Western blotting.

Fig. 5. Expression of tumor necrosis factor-α (TNF-α) messenger RNA (mRNA) and protein. Reverse-transcription polymerase chain reaction (RT-PCR) (a) and immunoblotting (b) images for TNF-α are represented for mRNA and protein, respectively, extracted from the hippocampus of a control (no-intervention) rat (A), or from rats receiving anesthesia alone tested at 1 (B1), 3 (B3), or 7 (B7) days after anesthesia or animals tested 1 (C1), 3 (C3), or 7 (C7) days after anesthesia and surgery. (c) Densitometry data (mean ± SD; n = 7) from the RT-PCR and immunoblotting images compared with that of the housekeeping gene β-actin are plotted as a histogram for each cohort. *P < 0.05 versus control. WB = Western blotting.

Fig. 6. Expression of Bax and Bcl-2 in the CA1 area of hippocampus in rats. Representative photomicrographs of the CA1 area of hippocampus illustrating Bax and Bcl-2 expression from a control (no-intervention) rat (A), or from rats receiving anesthesia alone tested at 1 (B1), 3 (B3), or 7 (B7) days after anesthesia or animals tested 1 (C1), 3 (C3), or 7 (C7) days after anesthesia and surgery. (D) Histogram plotting the number of Bax– and Bcl-2–positive cells in the cohorts undergoing anesthesia and surgery (mean ± SD; n = 6). **P < 0.01 versus C7.
exhibited impaired memory that was temporally associated with glial activation and proinflammatory cytokine expression in the hippocampus; neither cognitive dysfunction nor neuroinflammatory changes were noted in rats that were subjected only to neuroleptic general anesthesia.

Postoperative cognitive dysfunction affects postoperative surgical patients irrespective of the anesthetic technique used.\(^{21–23}\) POCD is particularly prevalent in cardiac surgical patients but also occurs in noncardiac surgical patients in whom putative mechanisms such as embolism, hypotension, and hypoxemia do not obtain.\(^{2}\) Although especially frequent in elderly patients,\(^{24}\) the incidence depends to a large degree on the sensitivity of the tests that are used to detect the cognitive deficit. In fact, the unequivocal establishment of POCD as a distinct condition has been questioned because its occurrence in the postoperative period has not been prospectively compared with an appropriate control group, e.g., patients with a surgical condition who are not subjected to surgery and anesthesia.\(^{25}\) Intercurrent illness (both medical and surgical) may be associated with “sickness behavior,” one component of which is cognitive dysfunction.\(^{26}\) A series of studies have now demonstrated the pivotal role that neuroinflammation exerts to impair the hippocampal-dependent memory that is part of sickness behavior.\(^{6,20,27}\)

Previously, it had been speculated that proinflammatory cytokines could play a role in the development of cognitive decline that may acutely follow surgery.\(^{28}\) The peripheral IL-6 response to surgical injury exemplifies the vast literature on the proinflammatory cytokine response to surgery. Within 2 h of skin incision, IL-6 levels increase, peak between 4 and 12 h, and remain elevated for up to 3 days postoperatively.\(^{29–31}\) Extent of surgical trauma is an important factor; there was a greater degree of IL-6 elevation after abdominal aortic and colorectal procedures (mean peak concentrations, 400–550 U/ml, ranging as high as 1,220 U/ml) than after hip replacement (208 U/ml) despite similar surgical procedure times.\(^{30}\) Further, four separate studies illustrate that the increment in IL-6 is less after laparoscopic versus open cholecystectomy, despite similar surgical times.\(^{32–35}\) The IL-6 response is not modified by anesthetic regimens\(^{36,37}\) but is decreased by use of antiinflammatory analgesic agents.\(^{38}\)

Cytokines originating from the periphery can exert effects within the central nervous system (CNS) through both direct and indirect means. Both IL-1\(^+\)\(^{39}\) and TNF-\(\alpha\)\(^{40}\) have been shown to gain entry into the CNS through the relatively permeable blood–brain barrier in the periventricular regions. Further, IL-1\(\beta\) can also directly bind to its cognate receptors on the endothelial cells within the brain microvasculature where these can elaborate a central inflammatory response.\(^{13}\) Indirectly, cytokines can induce changes within the CNS through vagal afferent nerves.\(^{41}\) In an elegant series of studies, either lipopolysaccharide\(^{42}\) or IL-1\(\beta\)\(^{43}\) when administered peripherally, induced brainstem and hippocampal IL-1\(\beta\) production and CNS expression of IL-1, IL-6, and TNF-\(\alpha\), all of which could be abolished by vagotomy.

Within the CNS, the glia can be activated by above-mentioned events that are initiated in the periphery.\(^{44}\) Glial activation and the consequent release of proinflammatory cytokines within the hippocampus interfere with cognitive function as evidenced by abnormal memory and learning in the intact organism and/or inability to develop long-term potentiation in hippocampal slice preparation.\(^{45}\) Interestingly, cytokines seem to have a biphasic effect on long-term potentiation, the synaptic plasticity marker that is the neurobiologic correlate of memory; low concentrations seem to enhance long-term potentiation, whereas higher concentrations impair memory. Furthermore, the memory-enhancing and memory-impairing effects of the proinflammatory cytokine TNF-\(\alpha\) seem to be mediated by different receptor subtypes.\(^{46}\)

Within the intrinsic apoptotic pathway, interactions between proapoptotic members of Bcl family seem to play a critical role in determining the ultimate fate of injured cells.\(^{47}\) Neurons in the hippocampus of splenectomized rats increased expression of both Bcl-2 and Bax. However, the ratio of Bcl-2:Bax was decreased after surgery, suggesting that neurons in the hippocampus are inclined toward apoptosis,\(^{48}\) however, caspase-3 activation, a marker of apoptotic cell death, was not detected in splenectomized rats at the time points examined. We speculate that the cognitive dysfunction that we observed is not necessarily associated with the presence of actual cell death in hippocampus; rather, there may be abnormal interaction between neurons and glial cells in the hippocampus.\(^{49,50}\) Such hippocampal dysfunction in the absence of morphologic injury has been recently noted after induction of a proinflammatory cytokine response.\(^{51}\)

There are several caveats that must be considered. Spleectomy itself can alter the inflammatory response of an organism; however, this results in a decrease in inflammation rather than a proinflammatory state.\(^{52}\) The abnormality in cognitive function did not persist up to 7 days postoperatively; therefore, it is possible that our observed phenomenon is more reflective of the cognitive impairment associated with delirium, a postoperative state that does not necessarily progress to POCD.\(^{53}\) Possibly, an additional provocation (e.g., infection, advanced age, or underlying subclinical neuropathology) is required to convert this phenomenon into a more permanent cognitive impairment. At this juncture, it is not possible to establish that the cognitive impairment is causally related to the inflammatory response and whether the proinflammatory cytokine expression and/or transcription initiated, or followed, glial activation.

In summary, we have demonstrated that surgery triggers a transient neurocognitive decline in a rat model that is temporally associated with glial activation and increase in proinflammatory cytokines in the hippocampus. Although further data are needed to establish the robustness of these observations (including other anesthetic regimens and surgical procedure), we interpret
these data to mean that glial activation and consequent release of proinflammatory cytokines in the CNS (noted with a variety of “stressors”) may also occur after the “stress” of a surgical intervention. Strategies designed to mitigate the central neuroinflammatory component of surgery may represent a means of preventing postoperative cognitive dysfunction.

References