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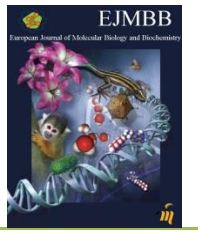

POST-SURGERY OR ANESTHESIA, MONONUCLEAR CELLS EXPRESS AND RELEASE HIGH MOBILITY GROUP BOX 1 (HMGB1) PROTEIN

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ABSTRACT

During major elective surgery, High mobility group box 1 (HMGB1) proteinv production kinetics were investigated, along with circulating mononuclear cells. Interleukin-6 (IL-6) and HMGB1 were also examined. We enrolled 94 patients scheduled for major abdominal procedures. In addition to midazolam, thiopentone sodium and fentanyl were given to all patients as well as Vecuronium. In total, 6 samples were collected from patients before, after, and 24 hours following surgery, t0: before surgery, t1: immediately following surgery, and t2: 24 hours after surgery. Anti-CD14-coated microbeads were used to purify monocytes, followed by magnetic sorting. HMGB1 cellular localization was determined by flow cytometry and serum HMGB1 release by Western blotting. ELISA was used to measure serum levels of IL-6. HMGB1 concentrations in monocytes and serum were assessed using one-way repeated-measures analysis. At t1, HMGB1 expression was significantly higher in monocytes than at t0; at t2, serum HMGB1 levels increased significantly. As a result of such an increase, cellular HMGB1 was significantly downregulated, suggesting that HMGB1 might be partly produced by monocytes; c) monocytes treated with HMGB1 released IL-6 in vitro; d) at t2, levels of circulating IL-6 were elevated. Surgical/anesthesia trauma can cause monocytes of surgical patients to express HMGB1, suggesting these cells contribute to the production of HMGB1.

INTRODUCTION

Stress response to surgery/anesthesia trauma has been poorly understood. In spite of this, the field's research on this topic is growing. There is a massive neuro-endocrine-hormone flux that contributes to surgical/anesthesia trauma-induced stress, activating intracellular signaling pathways, releasing cytokines and maintaining body homeostasis [1,2]. Several factors influence the inflammatory response, such as tissue damage, preexisting diseases, surgery type, surgeon experience, and the type of anesthesia used during surgery [3, 4].

Specifically, anesthetic agents impair perioperative inflammation through direct and indirect effects on immune balance [5]. Studies have demonstrated that volatile and nonvolatile anesthetics inhibit lymphocyte proliferation in vitro and in vivo. In addition, drugs used to induce and maintain general anesthesia decreased mitochondrial transmembrane potential, as did sevoflurane, opioids, and muscle relaxants [5, 6].

Increasing attention is being paid to a group of biomolecules called "alarmins" by Oppenhaim. As an alarmin-like protein, HMGB1 (High Mobility Group Box 1) has a molecular weight of 30 KDa. HMGB1 promotes immune cell function and tissue repair [7, 8].

HMGB1 is implicated in nucleosomal structure stabilization and transcription factor regulation [9, 10].

In subsequent studies, HMGB1 was shown to be released as an extracellular cytokine by mononuclear cells of the peripheral blood (PBMCs). HMGB1 release by activated monocytes is delayed by a non-classical secretory pathway mediated by vesicles [11, 12-15]

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Cytokines are released by HMGB1 during various inflammatory processes. HMGB1 binds to the RAGE multi-ligand immunoglobulin receptor to initiate most of these effects. The HMGB1 signaling pathway is also mediated by Toll-like receptors, such as Toll-like receptors 2 and 4 [16-18].

Subjects undergoing traumatic/surgical injury also release HMGB1 in their serum [19, 20]. Neither the kinetics nor the role of the cellular compartment are understood.

In this study, HMGB1 levels were measured in circulating monocytes and serum of surgical trauma patients. In addition to HMGB1, IL-6, which is one of the key mediators of the surgical stress response, is also examined.

METHODS AND MATERIALS

Patients

A prospective study involving 47 adult subjects, physical status I and II according to the ASA, scheduled for major abdominal procedures, was approved both ethically and by the HSRC. Diabetes, chronic pulmonary disease, renal disease, vascular disease, immunologic diseases, neurodegenerative diseases, infectious diseases, or hepatic disorders contributed to the exclusion of study participants.

Additionally, pregnant or breastfeeding women as well as those taking medications that interfere with hormone, metabolic, or immune function were excluded from the study.

A physical examination and medical history were assessed during the screening period with the consent of eligible patients. Seven postoperative days were recorded for postoperative complications. We also enrolled 15 matched control subjects of the same gender, age, and weight. Both control subjects and patients provided informed consent.

Techniques of anesthesia

A standard general anesthesia protocol was administered to all patients after receiving intravenous midazolam (0.025 mg/kg). Fentanyl (1.4 g/Kg) and thiopentone sodium (5 mg/Kg) were used for anesthesia induction. During direct laryngoscopy, 0.08 mg of vecuronium was injected into the patient's trachea in order to facilitate orotracheal intubation.

Sevoflurane, fentanyl, and vecuronium were administered at concentrations of 1 to 2.5% inspired during anesthesia. For continuous arterial blood pressure monitoring, radial artery catheters were inserted in all patients.

During surgery, many standard parameters were measured, including ECG, oxygen saturation, end-tidal carbon dioxide, and hemoglobin. To achieve a level of ETCO2 between 38 and 40 mmHg, the S/5 AVANCE ventilator was used to ventilate all patients' lungs. Infusion rates were adjusted from 6 to 10 ml/Kg/h with normal saline and Ringer Lactate solutions. Using a Bair Hugger and warm fluids, the rectal temperature was maintained at 37°C. Anesthesia and surgery durations were recorded. All operative procedures were performed by the same surgical team.

Antagonizing neuromuscular blockade after surgery was achieved using atropine and intrastigmine in doses of 0.5 to 1.5 mg each. A morphine bolus (0.20 mg/Kg) was administered intravenously 30 minutes prior to surgery and morphine 0.3 mg/Kg was administered via an elastomeric pump for 24 hours following surgery.

Samples

Three different blood samples were drawn from patients: at time zero: before anesthesia and surgery, at time one: immediately after surgery, and at time two: 24 hours later. The serum was separated from the blood using low-speed centrifugation at 4°C, frozen, and stored at -80°C. By Lymphoprep density gradient centrifugation, fresh heparinized blood mononuclear cells were isolated into pH 7.3 phosphate buffered saline (PBS).

Monocyte isolation

PBS, pH 7.4, was used to wash human peripheral blood mononuclear cells three times. Using anti-CD14-coated microbeads and a magnetic sorter, CD14+ monocytes were purified.

Flow cytometry analysis of HMGB1 expression

A direct immunofluorescence assay was used to investigate the cellular localization of HMGB1. The monocytes were collected and washed in PBS before being fixed in 2% paraformaldehyde (PFA). Cold PBS was used to wash the cellular suspension, which was then permeabilized with 60M digitonin for one hour in ambient temperature. At room temperature, goat anti-rabbit IgG conjugated with FITC was incubated for 30 minutes with antibodies against rabbits after washing with cold PBS.

In PBS, 0.1% bovine serum albumin (BSA) was added and centrifuged twice at 5,000 g for three minutes to remove the unbound Ab. An unlabeled isotypic control antibody was used to determine nonspecific binding.

Flow cytometry was performed using an Epics XL-MCL Cytometer equipped with a 488 nm argon-ion laser. A total of 10,000 cells were counted for each histogram. As a measure of antibody reactivity, mean fluorescence intensity was used. To determine whether or not the monocyte population was pure, FITC-conjugated monoclonal antibodies (MoAbs) anti-CD14 were used. As controls, 15 healthy volunteers provided blood samples.

Extracting nuclear and cytosolic material

The monocytes were reconstituted in buffer A and vortexed. The samples were centrifuged for 30 minutes at 4°C after 30 minutes on ice. We resuspended

the pellet with buffer A and 0.1% Nonidet P-40. The cytosolic extracts of the supernatants were frozen after centrifugation at 10,000 g for five minutes at 4°C in the presence of ice.

Using buffer B as a resuspension medium, the pellets were then vortexed. On ice for one hour, tissue extracts were cleared at 10,000 g for one hour at 4°C, and supernatants were transferred to new vials. Samples were frozen at -80°C and protein content was determined by Bradford assay using BSA as a standard.

Nuclear and cytosolic extracts were separated using SDS-PAGE at 15% without reducing. Electrophoretically transferred proteins were probed with monoclonal anti-HMGB1, following 1% albumin blocking in PBS. The bound antibody was detected using HRP-conjugated anti-mouse IgG and immunoreactivity was assessed using Western blotting. We used monoclonal antibodies against a-tubulin and polyclonal antibodies against laminin B as purity controls.

Using immunoblotting

Bradford assay was used to measure serum and plasma protein concentrations. Diluted serum samples were electrophoresed on sodium-dodecyl sulphate polyacrylamide gels. Proteins were electrophoretically transferred to PVDF membranes. HMGB1 monoclonal antibody was probed on membranes incubated in 5% defatted dried milk in Tris buffered saline (TBS). ECL Western blocking detection system was used to detect bound antibodies using anti-mouse IgG conjugated with HRP and chemilumination reaction. NIH Image 1.62 software was used for densitometric scanning analysis, developed by the National Institutes of Health in the United States.

HMGB1 was measured in serum and plasma and found to be almost identical.

IL-6 assay

A commercially available enzyme-linked immunosorbent assay (ELISA) kit was used to test serum samples for IL-6 levels. In preliminary experiments, detection limits, linearity and range of the ELISAs were determined, essentially in accordance with the Q2A and Q2B guidelines of the International Conference on Harmonisation. Variations within and between assays ranged from 3% to 6%. The detection limit was 0.7 pg/ml. Recombinant histidine-tagged HMGB1, 100 ng/ml lipopolysaccharide (LPS) or 100 ng/ml LPS and 100 ng/ml HMGB1 for 24 h at 37°C. ELISA was used to detect levels of IL-6 in the supernatant.

Analyses of statistics

A summary statistic is presented in the form of a mean and standard deviation (SD). Monocytes and serum HMGB1 concentrations were analyzed using an analysis of variance with repeated measures in a one-way design. In an ANOVA setting, Bonferroni post hoc tests were used to determine whether groups differed significantly. In order to consider a difference statistically significant, a p value of 0.05 was reached.

RESULTS

Patients

Table 1 shows the characteristics of patient groups and the types of surgical procedures. Table 2 reports the average dosage and time of anesthesia. No patients received blood transfusions because blood transfusions may modulate the immune system and may increase or suppress HMGB1 production. Throughout the study period, no serious post-operative complications were observed.

Expression of HMGB1 in cells

Flow cytometry was used to determine the expression level of HMGB1 in monocytes. CD14 staining was used to identify and gate the monocyte population. Due to the underlying diseases, patients had higher HMGB1 levels than healthy donors, but the difference did not reach statistical significance. In monocytes of the patients, HMGB1 fluorescence intensity increased over time. At t1, HMGB1 staining is significantly higher than at t0 (P or t2. In surgical/anesthesia trauma, the presence of HMGB1 is one of the first signs of injury.

Western blots were performed on monocyte cytosolic and nuclear extracts to determine whether the elevated expression of HMGB1 was derived from the nucleus. HMGB1 expression was increased at T1. We speculate that neo-expression of HMGB1 in the cytoplasm might be due to translocation from the nucleus, since HMGB1 expression in the nucleus was decreased.

Concentration of serum HMGB1

By using Western Blot, HMGB1 levels were detected at the same time points in sera from patients. Based on densitometric analysis, it was found that the concentration of HMGB1 significantly increased at 24 hours (t2) (P 0.001). As compared to samples collected before surgery (t0), HMGB1 levels did not change significantly after surgery (t1). HMGB1 overexpression on monocytes precedes HMGB1 overexpression in patients, based on these findings.

Concentration of serum IL-6

Local tissue sites produce IL-6, which is then released into the bloodstream. IL-6 is released when tissue homeostasis is disrupted in almost all situations, and it is also released during surgical stress. Thus, we examined whether HMGB1 treatment of monocytes induced the release of IL-6 in vitro. HMGB1, LPS or LPS plus HMGB1 were incubated with monocytes from patients under test. It was found (Figure 3a) that all treatments increased IL-6 (P 0.001), showing that

HMGB1 can trigger monocytes to release IL-6 in vitro. Accordingly, cells treated with LPS produced less IL-6 than that following LPS plus HMGB1 treatment, indicating a synergistic effect between the two agents [23].

We then tested serum samples for IL-6 levels using ELISA. Based on the results, IL-6 release correlates with an increase in HMGB1 concentrations in patient serum at t2 compared to t0 and t.

Table 1: Surgical procedures and patient demographics

No . of patients	94
Male	52
Female	42
* Age, yr	65 ± 13
* Weight (Kg)	72 ± 18
ASA (I/II)	30/64
Surgical procedures	
Emicolectomy	36
Isterectomy	26
Gastrectomy	18
Hepatic resection	14

Table 2: Duration of surgery/anesthesia and total doses of anesthesia drugs

Duration of surgery/anesthesia in minutes	175 ± 24/187 ± 18
Drugs used in anesthesia	
(mg) Tiopenthal	360 ± 19
(mg) Fentanyl	0.4 ± 0.010
(mg) Vecuronium	12 ± 5

DISCUSSION

This study examined the kinetics of HMGB1 production after major elective surgery and how monocytes may be involved. Serum IL-6 levels were measured to examine the potential interaction HMGB1 has with IL-6, an indicator of surgical stress [20, 24]. In this study, HMGB1 expression in monocytes was significantly higher than preoperative values, according to the results obtained in this study; b) 24 hours after surgery, HMGB1 levels were found to be significantly higher in the sera of patients; and c) IL-6 levels were significantly higher than preoperative baselines.

Previous studies have demonstrated that activated monocytes release HMGB1, while damaged cells passively release it following injuries [19, 20, 25, 26]. Additionally, HMGB1 in patient serum may be influenced by endotoxin translocation and protein release from damaged cells during surgery. [27]

Additionally, our findings suggest that increased levels of HMGB1 in traumatic insults are early phenomena, contrary to reports indicating that HMGB1 mediates human sepsis and endotoxemia [28 -30]. For the first time, monocytes of patients after surgery overexpress HMGB1. HMGB1 is secreted into the bloodstream following surgical stimuli, suggesting that intracellular pathways are rapidly activated. We observed a significant increase in serum HMGB1 levels following surgery and a decrease in cellular HMGB1. The release of such a protein from damaged cells on a passive basis may also contribute to an increase in HMGB1 in patients [8]. In spite of this, monocytes show abnormal intracellular expression of HMGB1 after surgical injury. Surgery and general anesthesia play a role in this phenomenon, as well as the mechanism underlying its regulation. The production of HMGB1 is triggered by hypoxic conditions or oxidative stress induced by reactive oxygen species (ROS).

PBMCs produced excessive ROS during general anesthesia and surgery, and some anesthetics altered mitochondrial redox state to induce oxidative stress [33, 34]. According to our hypothesis, postoperative upregulation of HMGB1 is associated with increased ROS production following anesthesia and surgery.

Apoptotic cells do not release HMGB1, but macrophages engulfing them do because they retain it within their nucleus [12]. During the early postoperative period, lymphocytes exhibited an accelerated rate of apoptosis [35-37]. As a result, patients' massive release of HMGB1 may be caused by an increase in apoptosis following surgery/anesthesia trauma.

We also found that: a) monocytes treated with HMGB1 released IL-6 in vitro; b) circulating IL-6 levels were higher at t2 than with t0. Postoperative HMGB1 increases may induce IL-6 secretion. Toll-like receptor 4 (TLR-4) binds to HMGB1 on monocyte surfaces and triggers signal transduction. Upon activation of the TLR pathway, the interleukin-1 receptor-associated kinase (IRAK) as well as MyD-88 are phosphorylated by TLR

pathway activation, which activates and translocates nuclear factor kB, causing IL-6 to be released [17].

Based on our results, patients with severe injuries have higher levels of IL-6 and HMGB1 [25].

HMGB1 has also been shown to correlate significantly with IL-6 in human cerebrospinal fluid, suggesting that it may induce IL-6 secretion. In addition, HMGB1 administration increases brain IL-6 production [29, 38].

CONCLUSIONS

A new study reveals that surgical/anesthesia trauma induces early intracellular upregulation of HMGB1. A significant rise in serum levels of IL-6 and HMGB1 24 hours after surgery suggests HMGB1 originates from monocytes and triggers IL-6 secretion. HMGB1 production is modulated by surgical/anesthesia stimuli, and the ultimate mechanism is unclear.

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