Original Contribution

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The Effect of Starvation on Blood Stream Cancer Cell Metastasis to the Liver in Rat after Laparotomy

TATSUYA MURAOKA, KAZUO SHIROUZU, HIROYUKI OZASA, YOUSUKE OKA, KAZUYA MOMOSAKI, NOBUTAKA IWAKUMA, KATSUAKI TANAKA AND NOBUYA ISHIBASHI

Department of Surgery, Kurume University School of Medicine,Kueume 830-0011,Japan

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Summary: Background: Preoperative malnutrition worsens the prognosis of cancer patients. However, it is not certain how preoperative malnutrition affects postoperative hematogenous metastasis. We examined the influence of preoperative starvation on liver metastasis in rats using intra-vascular injection of AH109A hepatoma cells.
Methods: Male donryu rats were divided into Fasting and Control groups. Rats received laparotomy and ^{125}I-iodo-deoxyuridine labeled AH109A hepatoma cells were inoculated via superior mesenteric vein. Radioactivity in the organs, macroscopic liver metastasis, white blood cell count, leukocyte count, NK cell activity, endogenous serum corticosterone and ACTH concentration and mRNA expression of cytokine in the liver and brain were evaluated at certain time points.
Results: 48hours preoperative starvation reduced body weight and induced a state of malnutrition. Accumulation of radioactivity in the liver was more than 4 times higher, and the number of liver metastases was 3.5 times higher in the Fasting than in the Control group. Preoperative starvation caused an almost 2 fold increase in plasma endogenous corticosterone levels and a 66% reduction in white blood cell and lymphocyte counts. Postoperative hypothalamus pituitary adrenal axis response was preserved. In addition, inflammatory cytokine expression in the liver was suppressed in the starved animals, suggesting that preoperative starvation led to a state of cellular immunosuppression, which would be an important factor for liver metastasis.
Conclusion: Preoperative malnutrition by 48 hours starvation reduced inflammatory cytokine response and cellular immunity, resulting in an increase in hematogenous liver metastasis.

Key words starvation, preoperative malnutrition, hematogenous metastasis, cellular immunity, HPA axis, cytokine response, surgical stress

INTRODUCTION

It has been well known that advanced gastrointestinal cancer patients often present with severe malnutrition because of appetite loss, obstruction of gastrointestinal tract and cancer related hyper-metabolism. Preoperative malnutrition is associated with suppression of perioperative cellular immunity [1,2], an increased rate of postoperative infectious complications, and an increase in postoperative tumor metastasis as well as recurrence [3]. In addition, preoperative deficiency of dietary intake caused an increase of secretion of endogenous corticosterone and catecholamine which are thought to be important for host immunity and metabolism [4,5]. Recently, the European society for clinical nutrition and metabolism

Corresponding Author: Nobuya Ishibashi Department of Surgery, Kurume University Medical Center Kokubu-machi, Kurume, Fukuoka, Japan 839-0863. Tel: 0942-22-6111 Fax: 0942-22-6533 E-mail:nob@med.kurume-u.ac.jp

Abbreviations: ACTH, adrenocorticotropic hormone; HPA axis, hypothalamic pituitary adrenal axis; IL-1b,interleukin 1 beta; IL-10, interleukin 10; NK cell, natural killer cell; TNFa, tumor necrosis factor alpha.

(ESPEN) recommended postoperative early nutritional intervention and avoidance of preoperative fasting to facilitate early recovery after surgery [6]. Based on the above issues, preoperative nutritional state is thought to have a great influence not only on postoperative outcome but also on prognosis of cancer patients.

Hematogenous cancer metastasis to liver, lung and brain from the intestinal tract can have a severe impact on prognosis, so it is important to prevent or reduce intravascular cancer cell release during surgical procedures. In fact, it has been proved that there cancer cells are released into the blood stream after operation [7]. The mechanisms of hematogenous metastasis are complicated, but are thought to be associated with cancer cell release, microcirculation, adhesion molecules, neo vascularization, host immunity and so on [8-10]. Activation of hypothalamic pituitary adrenal (HPA) axis, cytokines production, secretion of endogenous corticosterone and catecholamine could also modulate hematogenous metastasis 11). However, it is still uncertain how starvation stress, such as preoperative malnutrition, affects metastasis by intravascular cancer cells.

In this study, we examined whether starvation stress affected hematogenous metastasis after laparotomy by utilizing a liver metastasis model with intraportal injection of radio labeled AH109A hepatoma cells.

MATERIALS AND METHODS

Animal preparation and experimental protocol.

Male donryu rats (n=80, 5 weeks of age) were purchased from the Shizuoka animal center and housed in the animal facility of Kurume University under 12 hours light-dark conditions. The animals were fed standard rat chow (Clea Inc., Tokyo) and water ad libitum for 7 days prior to the start of the experimental protocol. The experimental protocol was approved by the Kurume University Ethics Committee.

On day 0, the rats were anesthetized with an intramuscular injection of pentobarbital (50 mg/kg, Dainippon Pharmaceutical Co., Tokyo) and randomly assigned into either the Fasting group (F group), which was starved for 48 hours before starting experiments, or the Control group (C group) which had free access to food and water. Body weight was measured just before and 48 hours before surgery. Laparotomy was achieved by vertical mid incision (5 cm) and the inferior mesenteric vein was isolated. We injected 2×10^5 radiolabeled or not radiolabeled AH109A ascites hepatoma cells into the portal vein. During laparotomy the abdominal cavity was exposed to the room air for 30 minutes before being closed by interrupted sutures of 4－0 Nylon (Ethicon Japan Inc., Tokyo). The rats were returned to individual metabolic cages after operation.

The first set of animals (n=12) were sacrificed preoperatively and the blood was harvested. The second set of rats (n=36) was sacrificed 30 minutes or 3 hours after the surgery. The whole brain, liver, spleen and blood were harvested for measurement of white blood cell count, lymphocyte count, NK cell activities, serum cytokines levels, serum corticosterone levels, serum ACTH levels and tissue cytokine mRNA expression levels. The tissues and serum were immediately frozen in liquid nitrogen, and stored at －80°C until analysis. The third set of rats (n=16) were sacrificed 5 hours after the surgery and liver, lung and thyroid were harvested and used for measurement of radioactivity. The fourth set of animals (n=16) was decapitated 3 weeks after surgery and the liver was harvested for evaluation of macroscopic liver metastases.

AH109A ascites hepatoma cell preparation.

The rat AH109A ascites hepatoma cells were cultured in RPMI1640 (Gibco Life Technologies Inc., Grand Island, NY) for two weeks, harvested from the culture dish and injected into the rat abdominal cavity for subculture. Three days after subculture, AH109A cells were withdrawn from the abdominal cavity and counted. AH109A cells were radiolabeled according to the method of Isaiah [12] as follows. Briefly, AH109A cells were cultured with ^{125}I-iodo-deoxyuridine in CO2 incubator at 37°C and 95% humidity. Cells were harvested and divided into 2×10^5 per 0.5 mL PBS, and radioactivity was measured by gamma counter. Around 20,000 cpm per 0.5 mL of cell suspension was used for intra-superior mesenteric vein injection.

Analytical procedures.

Blood cell count and NK cell activity assay.

White blood cells and lymphocytes were counted prior to and 3 hours after surgery. The chromium release assay was employed to evaluate NK cell activity. Harvested spleen was cut on a sterilized ice-cold dish with 5 mL of PBS, passed through a stainless steel mesh and centrifuged after adding Percoll (GE Health Care Bioscience, UK) at 2,200 rpm, room temperature, for 15 minutes. The separated mononuclear cells layer was washed in ice-cold PBS and mononuclear cells were poured into 96 well U bottom microplates at 4×10^4 per well. We used T-cell lymphoma YAC-1

cells derived from A/st mouse as target cells. 1×10^6 Yac-1 cells were incubated with 100micro Ci of $Na^{51}CrO_2$ (Japan Radioisotope Association, Tokyo, Japan) for 60 minutes at 37°C in 5% carbon dioxide atmosphere and then washed with culture medium. 1×10^4 Yac-1 cells were then suspended in 4×10^4 mononuclear cells and poured into a 96 well U bottom microplate at an E/T ratio of 40 to 1, and cultured for 5 hours at 37°C in 5 % carbon dioxide atmosphere. The microplate was then centrifuged at 1,000 rpm for 5 minutes. Supernatants were collected and radioactivity was measured by gamma counter. Natural killer cell activity was calculated as follows:

Natural killer cell activity (% cytotoxicity) = (specimen dissociation(cpm) – nature dissociation(cpm)) / (maximum dissociation(cpm) – nature dissociation (cpm)) × 100.

Measurement of serum corticosterone, ACTH concentration and interleukin-6 concentration

Serum corticosterone and ACTH concentration were measured by radioimmunoassay ammonium sulfate precipitation method at 0, 30 minutes and 3 hours after surgery. Serum interleukin-6 concentration was measured by enzyme-linked immune-sorbent assay using a commercially available kit (Rat IL-6 Quantikine ELISA Kit, R&D Systems, USA) according to the manufacturer's instruction at 3 hours after operation.

Tissue cytokine mRNA expression

TNF a, IL-1b and IL-10 mRNA expression levels in the liver and brain were determined by real time RT-PCR using samples at 3 hours after surgery. Total RNA was isolated from liver and brain samples using TRIzol reagent (Life Technologies, Japan) according to the manufacturer's protocol. The extracted RNA was treated with DNase I. The RNA concentration was measured by spectrophotometer and 0.5 microgram of total RNA was used for reverse transcription. RNA was mixed with oligo dT primer, random 6mer primer, PrimeScriptTM RT Enzyme Mix 1 and 5 × PrimeScriptTM Buffer (Takara Bio, Japan) and incubated at 37°C for 15 minutes. Oligonucleotide sense and antisense primers using for this experiment were follows;

rat IL-1b
 sense: 5'-GCTGTGGCAGCTACCTATGTCTTG-3'
 antisense: 5'-AGGTCGTCATCATCCCACGAG-3'
rat TNFa
 sense: 5'-AACTCGAGTGACAAGCCCGTAG-3'
 antisense: 5'-GTACCACCAGTTGGTTGTCTTTGA-3'
rat IL-10
 sense: 5'-CAGACCCACATGCTCCGAGA-3'
 antisense: 5'-CAAGGCTTGGCAACCCAAGTA-3'
rat GAPDH
 sense: 5'-GGCACAGTCAAGGCTGAGAATG-3'
 antisense: 5'-ATGGTGGTGAAGACGCCAGTA-3'.

2 micro-liters of RT product were mixed with SYBR RPremix Ex Taq™ (Takara Bio, Japan) and PCR reaction was performed on a thermal Cycler DiceR Real Time System (Takara Bio, Japan). Initial denaturation was performed for 10 seconds at 95°C, the amplification cycles were 95°C for 5 seconds and 60°C for 30 seconds. Cytokine gene expression was corrected by GADPH expression as an internal control and compared by the relative comparison method using mRNA purified from the same tissues in rats receiving a 1 cm vertical mid incision laparotomy as a control (n=2)

Evaluation of adhesion ratio of radio labeled AH109A hepatoma cells to the tissues

Collected tissues were weighed and 10 mg of tissues were homogenized in 1 ml of PBS. We repeated freezing with liquid nitrogen and thawing three times at room temperature for each specimen, and then samples were centrifuged for 10 minutes at room temperature. Supernatants were collected and radioactivity was counted by gamma counter (1480WIZARD: Wallac Berthold Japan Inc.). For measurement of blood radioactivity, whole blood was counted by gamma counter. Adhesion ratio to the tissues was evaluated as follows;

Adhesion ratio to the tissues (%) = counted tissue radioactivity × tissue weight / 10 / injected AH109A cells radioactivity

Evaluation of macroscopic liver metastases

Macroscopic liver metastases on liver surface were counted three weeks after AH109A hepatoma cells inoculation via superior mesenteric vein.

Statistical procedure

Unpaired t-test was employed for comparisons between two groups and paired t-test was used for comparison of change over time in each group. If necessary, Mann-Whitney U test and Wilcoxon signed-rank test were employed as nonparametric tests. P value less than 0.05 was judged statistically significant.

RESULTS

Changes of body weight after starvation

Pre-operative 48 hours starvation caused a significant body weight loss in the Fasting group (138.0±5.0 g) compared to the controls (160.3±13.7 g) ($p < 0.01$,

TABLE 1.
Changes of body weight after fasting period.

	48 hours before surgery (n=8)	0 hours before surgery (n=8)
C group	161.0±17.3	170.5±20.2*
F group	159.8±14.0	143.1±13.4*, **

Values were expressed as mean ± SD.
*: p<0.01 (paired t-test) vs BW at 48 hours before surgery
**: p<0.001 (unpaired t-test) vs BW at 0 hours before surgery in C group

Table 1).

Adhesion ratio of radio labeled AH109A cells to the tissues

There was no difference in adhesion ratio to the lung, thyroid and blood between groups, and the ratio was so small in lung and blood that it seems AH109A hepatoma cells were not able to implant to the lung, and radio-labeled hepatoma cells did not seem to remain in the blood stream 5 hours after cell inoculation. In the thyroid, adhesion ratio was significantly higher than in the lung. AH109A cells were labeled with ^{125}I-iodo-deoxyuridine and some of the cells might have been destroyed in blood stream by immune cells. We speculate that ^{125}Iodine from the destroyed cells was taken into the thyroid. In contrast to the lung, thyroid and blood, the adhesion ratio in the liver was about 4 times higher in the Fasting group compared with the controls (Table 2).

Analysis of metastases on liver surface

The average number of metastatic liver nodules in the Fasting and Control groups was 7.1±2.7 and 2.0±1.7, respectively, and this difference was significant (p<0.01). Counted metastatic lesions were microscopically confirmed by a pathologist (data not shown).

TABLE 2.
Adhesion ratio of the cancer cells to the tissues 5 hours after surgery (%).

	C group (n=8)	F group (n=8)
Liver	3.63±1.42	13.13±5.31#
Lung	0.23±0.10	0.21±0.17
Thyroid	1.23±0.69	0.76±0.31
Blood	0.21±0.20	0.24±0.17

Values were expressed as mean ± SD.
#: p = 0.0011 (Mann-Whitney U test) vs C group

Changes of white blood cell and lymphocyte count and NK cell activity

Preoperative starvation caused a significant decrease in white blood cell and lymphocyte counts (p<0.01, Table 3a). White blood cell count was significantly increased at 3 hours after laparotomy in both groups and there was no difference between groups. On the other hand, lymphocyte count was also significantly increased at 3 hours after operation in both groups compared to the preoperative value, but lymphocyte count in the Fasting group was significantly less than in the Control group (p<0.05, Table 3b). There was no difference in NK cell activity between groups at 3 hours after laparotomy (Table 3c).

Peripheral TNFa and IL-1b reaction and central IL-10 reaction after laparotomy

We measured serum levels of TNFa and IL-1b by ELISA, but those were undetectable. We employed laparotomy as a surgical stress for this experiment, but it might not be enough stress to affect serum levels of TNFa and IL-1b. TNFa and IL-1b

TABLE 3a.
Preoperative WBC and Lymphocyte count (/ul).

	C group (n=6)	F group (n=6)
WBC	4403.0±960.4	1459.2±459.6[a]
Lymphocyte	3142.2±896.1	957.8±246.7[b]

Values were expressed as mean ± SD.
[a]: p<0.001 (unpaired t-test) vs C group
[b]: p = 0.0039 (Mann-Whitney U test) vs C group

TABLE 3b.
Postoperative WBC and Lymphocyte count (/ul) at 3 hours after operation.

	C group (n=6)	F group (n=6)
WBC	7379.8±1854.9	7718.2±1984.0
Lymphocyte	2344.0±583.6	1709.5±308.3c

Values were expressed as mean ± SD.
[c]: p = 0.0403 (unpaired t-test) vs C group

TABLE 3c.
Natural killer cell activity (%) at 3 hours after operation.

	C group (n=6)	F group (n=6)
NK(Yac-1)	11.3± 3.0	11.2±2.2

Values were expressed as mean ± SD.

mRNA levels in the liver were significantly up-regulated in both groups compared to samples from rats that received 1 cm vertical mid incision laparotomy, however, TNFa mRNA expression level was two times higher in the Control group than in the Fasting group (Fig. 1A) and IL-1b mRNA expression level was five times higher in the Controls than in the Fasting group (Fig. 1B). On the other hand, IL-10 mRNA expression levels in the brain were significantly higher in the Fasting than in the Control group (p=0.0005, Fig. 1C).

Hypothalamus – pituitary – adrenal axis reaction to starvation and surgical stress (Figs. 2A, B)

Preoperative starvation caused a significant reduction of serum ACTH concentration and an increase of serum corticosterone concentration. Serum ACTH concentration was significantly increased at 30 minutes after laparotomy in both groups. Serum ACTH reacted differentially at 3 hours after operation between groups. In the Control group, serum ACTH concentration remained higher than baseline, while a very low concentration was observed in the Fasting group. Serum corticosterone concentration after laparotomy was significantly increased at both time points compared to the preoperative value in both groups.

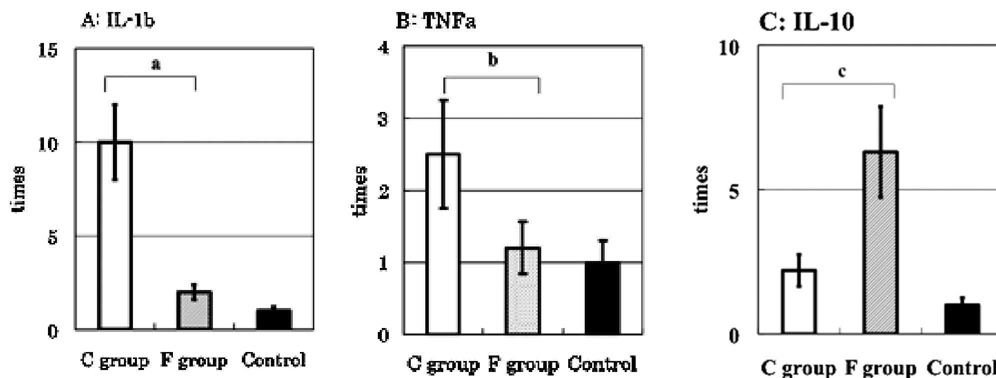

Fig. 1. Analysis of mRNA expression in hepatic IL-1b (panel A), hepatic TNFa (panel B) and cerebral IL-10 (panel C) in each group at 3 hours after operation. Data was presented as mean ± SD. Mann-Whitney U test was used for statistical analysis; a = 0.004, b = 0.036, c = 0.004.

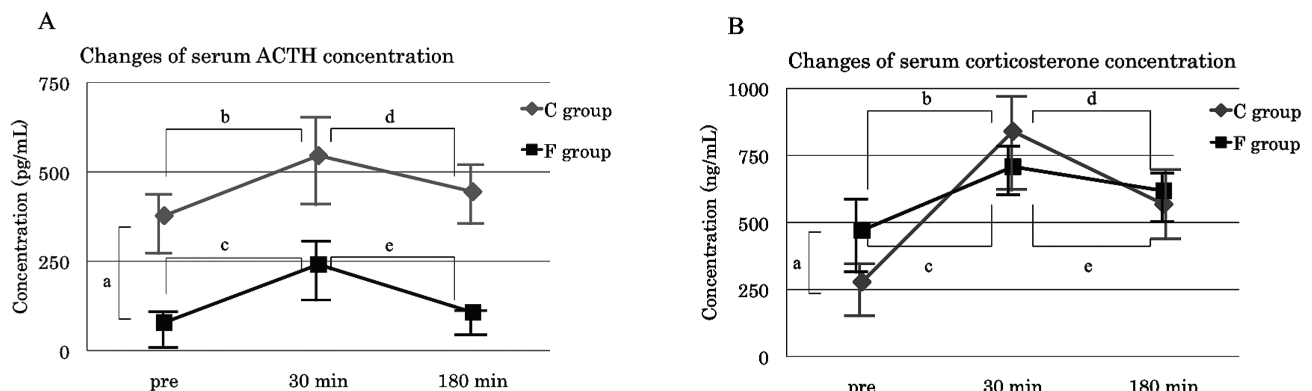

Fig. 2. Changes of serum ACTH (Panel A) and Corticosterone (Panel B) concentration. Data was presented as mean mean ± SD. 48 hours preoperative fasting significantly reduced preoperative serum ACTH concentration (unpaired t-test, a: $p < 0.0001$), but ACTH secretion at 30 minutes after operation was significantly increased in both group (paired t-test, b: p = 0.005, c = 0.0193), then the ACTH concentration decreased 180 minutes later in both groups (Wilcoxon signed-rank test, d: p = 0.0277, e: p = 0.0273). Serum corticosterone concentration was significantly increased by 48 hours fasting stress (unpaired t-test, a: p = 0.0134). Endogenous corticosterone secretion was significantly increased at 30 minutes after surgical stress in both groups (paired t-test, b: p = 0.0006, c = 0.0161), then decreased 180 minutes later (Wilcoxon signed-rank test, d: p = 0.0464, e: p = 0.0431).

DISCUSSION

One interesting result of this study was that in the starvation state, intravascular cancer cells accumulated more easily in the target organ than under normal conditions. Many factors, such as adhesion molecules, tumor neovascularization and immune function, are known to be associated with metastasis and tumor growth, and it has been reported that modulation of those factors could reduce cancer metastasis[13-15]. In this study we primarily focused on accumulation to the target organ of radiolabeled AH109A hepatoma cells injected into the intra superior mesenteric vein. Our study showed a significant difference in accumulated radioactivity in the liver. On the other hand, there were no differences in accumulated radioactivity in blood and lung between the groups. Moreover, we demonstrated that injection of AH109A hepatoma cells via the superior mesenteric vein could produce macroscopic metastases to liver but not to other organs, suggesting that AH109A hepatoma cells have a characteristic affinity for metastasis to the liver, thus this model was suitable for examining the accumulation and metastasis of intravascular cancer cells to the liver.

The most important finding of this study was that preoperative malnutrition caused an increase not only of cancer cell accumulation in the target organ, but also in the number of liver metastases. Unfortunately we could not pathologically evaluate extravasation nor adhesion to the vascular endothelium of the injected cancer cells (data not shown) in the liver. Electron microscopic observation [16] revealed that most of the intravascular injected cancer cells were destroyed and disappeared from the vessels. Only 0.1 percent of cells could be engrafted into target organs so it would be very difficult to show the pathological process of the tumor metastasis.

To elucidate mechanisms for an increase in liver metastases by 48 hours starvation, we measured NK cell activity, lymphocyte count and mRNA expression of TNFa and IL-1b. We found no difference in NK cell activity between groups. On the other hand, a significant reduction of lymphocyte count was seen in the Starvation group both preoperatively and postoperatively. Malter M et al. [17] evaluated NK cell activity and macrophage activity in rat with or without fasting and concluded that those cytotoxic immune activities were highly preserved against fasting stress. This is similar to our result. It had been reported that fasting stress reduced lymphocyte count and functions [18]. Although we did not perform functional examination for lymphocytes, it had already been reported that acute starvation caused a reduction of white blood cell count and PHA response [19], lymphocyte count and Con-A response [20], and suppression of T cell mediated immunity[21]. From these previous reports, we would expect the lymphopenia caused by 48 hours starvation to facilitate liver metastasis. On the other hand, TNFa and IL-1b expression were significantly reduced in the Fasting group. It was well known that tumor adhesion molecules such as E-selectin are stimulated by TNFa and IL-1b [22,23].Thus, we think that the starvation related enhancement of postoperative liver metastasis is more strongly associated with cellular immunity than with adhesion molecules.

We were also interested in the response of stress hormones such as corticosterone with or without preoperative fasting stress. Corticosterone was known to suppress cell mediated immunity as well as inflammatory cytokine responses [24-27], thereby impairing the ability to remove intravascular malignant cells [28,29]. In our study, preoperative serum corticosterone level was significantly increased in the fasting group compared to the control group. Similar to our result, endogenous down regulation of corticosterone by starvation had been reported at the serum hormone level [30] as well as the transcription level [31], suggesting that preoperative activation of hypothalamus pituitary adrenal axis by fasting impaired the cell mediated immunity and inflammatory cytokine responses and could affect liver metastasis. This is very important, because it shows that preoperative malnutrition can facilitate the hematogenous metastasis of cancer. The other interesting finding of this study was a significant increase of cerebral IL-10 mRNA expression in starved animals after laparotomy compared to normally fed animals. Starvation is known to activate the HPA axis and stimulates secretion of corticosterone [32] and caused an increase of endogenous corticosterone secretion [33]. Moreover, IL-10 modulate gene expression profile in cells of HPA axis origin [34] and IL-10 enhances corticotropin releasing factor (CRF) and corticotropin (ACTH) production in hypothalamic and pituitary tissues [35, 36]. We did not measure the preoperative cerebral IL-10 expression, but there was a possibility that preoperative starvation might activate cerebral IL-10 expression and influence preoperative and postoperative endogenous corticosterone secretion. In this study, even though peripheral inflammatory cytokine responses were suppressed in starved animals, postoperative adrenal response was preserved. There might be two reasons for this. First, we have reported that a surgical stress response for the HPA axis could be induced by a very small amount of cerebral TNFa or IL-1b [37,38], so it is possible that the HPA axis response was preserved even if cytokine ac-

tivation was inhibited by starvation. Secondly, as we described above, IL-10 might influence HPA axis response because postoperative cerebral IL-10 mRNA expression was significantly increased in the Fasting group and there was a difference in change of plasma ACTH level between groups. We do not have enough data to explain this phenomenon but cerebral IL-10 expression in malnutrition might be very important to understand the hormonal metabolism after surgery. Further study is needed to understand the relationship between cerebral IL-10 and HPA axis response in the malnutrition state.

In recent years the enhanced recovery after surgery (ERAS) program has attracted attention, and an important factor of this program is shortening of the fasting period [6]. Our result might suggest that perioperative prolongation of the fasting period would not only delay recovery after surgery but also might increase hematogenous metastasis. We think that patients with preoperative malnutrition should receive adequate nutritional treatment and perioperative prolongation of fasting period should be avoided.

CONCLUSION

Preoperative fasting stress was found to increase the attachment of perioperative intravascular cancer cells to the target organ, and to increase liver metastasis.

Our study suggested that reduction of lymphocyte count by an increase in endogenous corticosterone secretion, suppression of the inflammatory cytokine responses and increase of the expression of cerebral IL-10 could be factors associated with an increase of hematogenous metastasis in patients suffering from malnutrition. Preoperative fasting period should be shortened for cancer patients.

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