(intake of protein at 20% or 10% of energy, respectively).[17] The 12 participants in their study included two men, and the participants had a wide range of BMI at the time of hospitalization, from 11.9 to 18.9 kg/m^2. Considering these facts, it might be useful to examine the effect of added protein to the diet of AN patients who have an extremely low FM.

We used the Schebendach formula, which was derived from a small sample of patients, to estimate REE. Also, only PAL = 1.2 was used because patients were not allowed to over-exercise and no patient presented with the exclusion criteria hyperactivity. There is a possibility that hyperactivity cannot entirely be prevented in AN patients. Overexercise is one of the pathologies of AN, and hyperactivity was recently reported to be associated with low leptin levels in the acute stage of AN.[18] Therefore, the excess energy that we calculated is still within the estimated range. However, the use of the predictive formula in the present study was meaningful for the comparison of excess energy between the two groups that had different FM and BMI values.

There are three limitations to the present study. One is that we did not evaluate the endocrine effect on REE. Although REE decreases along with hypothyroidism in AN,[19–22] AN patients during refeeding have been reported to require more REE than healthy participants.[23] Not all the interactions among various hormones are obvious, so we focused on the body weight and body composition changes in this time. The second limitation is the method used to assess the energy intake. Despite efforts for the discovery of the violation action enough, we cannot ignore the fact that the food intake may not have been accurately measured due to some abnormal eating behavior. The third limitation is the frequency of measurement of body composition. We measured the body composition only at the time of admission, so it was not clear if there is a difference in the amount of synthesis of FM and FFM between the initial stage of refeeding and the later stages of the treatment. To accurately quantify these changes, further research will be necessary to measure the REE and the body composition periodically, in addition to the measurement of daily energy intake and body weight. By the data from these studies becoming clear evidently, we get possible to perform the initial treatment of the physical factor of the patient with AN effectively in the general hospitals as well as special hospitals.

According to the results of the present study, we conclude that the energy requirement for body weight gain in AN patients might be related to the malnutrition state. Therefore, it would be very useful in the treatment of AN patients to estimate the energy requirement for weight gain based on the initial BMI.

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Predictors of menstrual resumption by patients with anorexia nervosa

Running title: Menstrual resumption by an patients

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Introduction

Anorexia nervosa (AN) is an eating disorder commonly developed at puberty and can cause numerous debilitating symptoms. Amenorrhea is one of the Diagnostic and Statistical Manual of Mental Disorder 4th ed. (DSM-IV) criteria for a diagnosis of AN (1) and is classified as primary or secondary. AN patients often remain amenorrheic even after returning to the normal range of body weight. Persistent amenorrhea is the highest risk factor for irreversible osteopenia and osteoporosis (2). Therefore, resumption of menstruation is an important indicator of recovery for AN patients (3) and its prediction is clinically meaningful for both patients and clinicians.

Several studies have been done to identify the factors associated with the resumption of menstruation. Frisch (4) demonstrated that fatness might be related to normalization of the menstrual cycle. Golden et al. (5) reported that body weight might be related to the resumption of menstruation. Since the discovery of leptin in 1994 (6), abnormalities in the leptin levels of AN patients have been reported as one of the factors related to sustained amenorrhea (7, 8). For example, the leptin level remained low in a study of AN patients with prolonged amenorrhea (8). Kopp et al. (9) also suggested that low leptin levels predicted amenorrhea for underweight and eating disordered females. All these findings support the import role of leptin in the resumption of menstruation. However, other hormones such as cortisol (10) and insulin (11) have also been reported to be associated with the recovery of menstruation. Thus, which factors predict prolonged amenorrhea and the resumption of menstruation by AN patients have as yet

not been fully clarified.

Thus, we compared the hormonal factors of AN patients who resumed menstruation during inpatient treatment with those of AN patients who continued in an amenorrheic state after weight gain.

Methods

Subjects

The study included eleven AN patients for whom amenorrhea continued throughout inpatient treatment (amenorrheic group), nine AN patients who resumed menstruation during inpatient treatment (eumenorrheic group), and twelve healthy women (control group). All patients met the DSM-IV criteria for AN and were treated at Kyushu University Hospital mainly with cognitive behavioral therapy, a modification of the intensive, multimodal inpatient treatment for AN developed by Nozoe et al. (12, 13). In this inpatient treatment program, patients are hospitalized until they reach and maintain their target body weight, set at 85 ± 5% of their standard body weight (SBW) for age and height. All patients were in secondary amenorrhea at the start of treatment. Age and sex matched controls were recruited from healthy female students of the Department of Health Science, School of Medicine, Kyushu University. None of the participants had taken drugs for birth control. All participants gave informed consent to participate in the study after a detailed description

of the protocol. The study was approved by the ethics committee of the Faculty of Medicine, Kyushu University.

Measurement

Details about clinical and anthropometric characteristics such as duration of illness, menstrual history, including the last menstrual period, body weight before the onset of AN, and height were obtained from the medical records of each patient. The hormonal testing points were as follows: at admission and just before discharge for the amenorrheic group; at admission and after recovery from amenorrhea (15.1±12.3 days after the start of menstruation) for the eumenorrheic group; during the follicular phase (7 to 10 days from the start of menstruation) for the control group. A fasting blood sample was taken early in the morning at each testing point, and leptin, insulin-like growth factor I (IGF-1), follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), estradiol (E_2), thyroid stimulating hormone (TSH), free triiodothironine (FT_3), free thyroxine (FT_4) and cortisol were measured. Body weight was also measured using a digital scale with the participant wearing underwear and a hospital gown or light clothes. The percent of body fat was measured on admission and before discharge using the Dual Energy x-ray Absorptiometry system (QDA4500A, HOLOGIC, Bedford MA). The hormone assays were done by RIA for IGF-1 (TFB, Tokyo, Japan); ELISA for leptin (Linco Research, St. Charles, MO); EIA for FSH, LH, PRL and GH (TOSOH, Tokyo, Japan); ECLIA for E_2, TSH, FT_3, FT_4 and cortisol (Roche, Basel, Switzerland).

Statistical Analysis

The analyses were performed with a commercially available statistical package (SPSS12.0J). Differences between the two groups were evaluated by Student t-test. Statistical differences in the variables among the three groups were determined by analysis of variance (ANOVA) followed by Tukey's post hoc test. Factors related to the resumption of menstruation by the AN patients were determined by stepwise forward logistic regression analysis. Pearson's simple correlation test was done to search for associations between clinical, anthropometric, and hormonal parameters. A "p" value of 0.05 or less was considered to be statistically significant. The results in the tables are presented as mean ± standard deviation (SD).

The SBW was from documents published by the Japanese Ministry of Health, Labor and Welfare (14) and the Ministry of Education, Culture, Sports, Science and Technology (15).

Results

The clinical and anthropometric characteristics of the AN patients at baseline are shown in Table 1. No significant difference was found in age, duration of illness, body weight, percent standard body weight (%SBW), premorbid BMI or BMI at admission between the amenorrheic and eumenorrheic AN groups, although the BMI of both groups was only about

64 % that of controls. The anthropometric data of the AN patients after weight recovery are shown in Table 2. No significant difference in body weight, %SBW, or BMI was found between the two AN groups. All these variables, however, were still significantly lower for the AN groups than for the controls. No significant difference was observed in the percent of body fat between the two AN groups, but that of the amenorrheic group was lower than that of the controls. Neither weight gain nor ΔBMI showed any difference between the two AN groups.

Moreover, no significant difference was found in days hospitalized between the amenorrheic AN group (199 ± 40 days) and the eumenorrheic AN group (225 ± 94 days). The amenorrheic AN patients were hospitalized for an average of 65 ± 40 days even after reaching 85% of SBW, while the eumenorrheic AN patients resumed menstruation on an average 50 ± 33 days after reaching 85% of SBW.

A comparison of hormonal parameters at baseline among the three groups is shown in Table 3. The cortisol levels of the amenorrheic group were significantly higher than those of the eumenorrheic group and the controls. Leptin, IGF-1, LH, E_2 and PRL levels were significantly lower for both AN groups than for the control group, although they did not show any difference between the two AN groups.

A comparison of the hormonal parameters after weight recovery is shown in Table 4. The cortisol level of the eumenorrheic group was significantly lower than that of the controls after weight gain, but was not different from that of amenorrheic group. The leptin level of the

eumenorrheic group was significantly higher than that of the amenorrheic group. However, the leptin levels of the AN groups were not significantly different from that of the controls. The IGF, FSH and LH levels of both AN groups were increased after weight recovery, but no significant differences were found among the three groups. The E_2 levels of both AN groups were also increased after weight recovery and were comparable with that of the controls. Importantly, the E_2 level of the eumenorrheic group was significantly higher than that of the amenorrheic group. One-hundred percent of the eumenorrheic (9/9) and control groups (12/12) but 27% (3/11) of those who remained amenorrheic had an E_2 level above 22 pg/ml. The FT_3 levels of the AN groups rose after weight recovery and reached the same level as that of the controls. In contrast, the FT_4 levels of the AN groups remained lower than that of the controls, even after weight recovery.

Logistic regression analysis was done to determine which factor at baseline most predicted the resumption of menstruation (Table 5). Anthropometric and hormonal parameters at baseline that were significant in ANOVA were entered into the logistic model. Serum cortisol level at baseline was extracted as the factor most predictive of prolonged resumption of menstruation (β=-0.825, p=.024, OR=0.438).

Discussion

Anthropometric and hormonal factors were assessed before and after weight gain to investigate possible predictors of the resumption of

menstruation using a group of AN patients who resumed menstruation and a group who did not. In a report of anthropometric characters, Frisch (4) reported that the gain of body weight and fat mass were very important for the resumption of menstruation after secondary amenorrhea caused by a reduction of body weight. In our study, however, body weight and the percent of body fat were not different between patients who resumed menstruation after recovery of body weight and those who did not. This is similar to the results for AN patients at 1-year follow-up reported by Golden et al. (5). Jacoangeli et al. (16) also demonstrated that an adequate body fat mass was certainly a necessary, but not sufficient, condition for the return of the menstrual cycle.

Although it has been reported that AN patients resume menstruation 6 months after weight recovery greater than 90% of SBW (5), our eumenorrheic AN patients seemed to recover menstruation more rapidly during hospitalization and at a lower SBW, an average rate of 85% of SBW. This discrepancy, in part, may be due to a racial difference in somatotype: less than 80% SBW is used in the AN diagnostic criteria for Japan. Recently, Dei et al. found that the difference between current and premorbid BMI (ΔBMI) was a significant predictor of the restoration of menstruation. Together with the reports that BMI before the onset of an eating disorder and standard BMI are not always concordant and the weight required for the return of menstruation is highly individual (17), these findings thus suggest that ΔBMI can be predictive of the resumption of menstruation. In our study, however, neither premorbid BMI nor ΔBMI

significantly differed between the eumenorrheic and amenorrheic groups, suggesting that they might not substantially influence the time of menstrual resumption in the Japanese population. Clearly, further research is still called for to evaluate the actual contribution of ΔBMI to the restart of menstruation.

The amenorrheic AN patients were hospitalized for an average of 65 ± 40 days even after reaching 85% of SBW, while the eumenorrheic AN patients resumed menstruation on an average of 50 ± 33 days after reaching 85% of SBW. Therefore, it is unlikely that the amenorrheic group failed to resume menstruation because they were hospitalized for a shorter length of time after reaching 85% of SBW in comparison to the eumenorrheic group. Thus, it seems that some due time after reaching 85% of SBW is needed for menstrual resumption, but that it does not take a lengthy period of time for all AN patients. In accord with this, Golden et al. (5) also reported that 36% of their AN patients began menstruation within 3 months of attaining 90% of SBW.

Of the baseline hormones, the serum cortisol level of the amenorrheic AN group was significantly elevated compared with both the eumenorrheic AN group and controls. Hypercortisolemia has been extensively documented in underweight AN (18), which could be by increased cortisol production, decreased clearance, or by a combination of both factors. Given that cortisol is expressive of the activation of the chronic stress response with a metabolic effect on hepatic neoglucogenesis, hypercortisolemia could indicate a more rapid or more serious weight loss.

In addition, increased CRH levels in the cerebrospinal fluid of AN patients was also reported, thus suggesting the existence of endogenous CRH hypersecretion by AN patients (19). In this study, the difference in the duration of illness at baseline between the two groups of patients was seen, even if it was not statistically significant. Together with the findings that intracerebroventricular administration of CRH to a variety of species including rats and monkeys inhibits pulsatile LH release (20, 21, 22), these findings thus suggest the amenorrheic patients to have had a longer duration of weight loss, which might consequently have an adverse effect on the hypothalamic-pituitary-gonadal (HPG) axis function through the excess of endogenous CRH production.

According to logistic regression analysis, the hormonal predictive factor at baseline related to a prolongation of the resumption of menstruation by AN patients was only serum cortisol. This result was contrary to the finding of Misra et al. (10) who reported that AN patients with higher baseline cortisol levels may gain more fat mass with weight gain, predicting a greater chance of menstrual recovery. The reason for this discrepancy is uncertain at present. Baseline cortisol levels may be associated with greater subsequent increases in body fat, to be sure, but it was not demonstrated that a high cortisol level directly predicted recovery of menstruation. It appears reasonable that cortisol has a rather more adverse effect than an encouraging effect on the resumption of amenorrhea in AN because chronic HPA axis activation has been well established by numerous studies as suppressing gonadal function (23, 24, 25). Interestingly,

Jacoangeli et al. (16) reported that cortisol levels were significantly higher for AN patients with sustained amenorrhea even after weight gain than for those who restored menstruation. Although such elevated cortisol levels were not seen in our study, these findings taken together suggest that the cortisol level might play an important role in regulating the resumption of menstruation. Nonetheless, it should be noted that serum cortisol level alone may not exactly reflect HPA axis activity. Therefore, this result should be confirmed by an alternative measurement of HPA axis activity such as diurnal change in the serum cortisol and urinary cortisol levels.

After weight recovery, the E_2 level was significantly higher for the eumenorrheic group than for the amenorrheic group. Golden et al. (5) demonstrated that the serum E_2 level at 1-year follow-up best estimated the resumption of menstruation by AN patients. Given the critical role of E_2 in hypothalamus-pituitary-gonadotropic function, it seems natural that such an elevated E_2 level is necessary for the recovery of menstruation. A cutoff E_2 level of 21 pg/ml correctly identified 100% of the eumenorrheic group and 73% of the amenorrheic group in the present study , which is similar to the cutoff E_2 level of 30 pg/ml reported by Golden et al. (5). However, these elevated E_2 levels indicate a consequence, not a prediction, of the resumption of menstruation because it was not measured before the periods returned. On the other hand, ovarian volume on pelvic ultrasonography, which is also a marker of ovarian function, was reported normal for 50% of patients who resumed menstruation within a year (26). The ovarian volume may be a useful predictor of the resumption of menstruation. Basal LH

tended to be higher, but not significantly, after weight recovery in the eumenorrheic group than in the amenorrheic group in our study. Considering the critical role of LH in the normal menstrual cycle, it should be clear that normal LH pusatility and an increase in basal LH level are also needed for menstrual resumption.

Recently, leptin has been proposed as a factor that impacts E_2 secretion. Leptin stimulated the ovarian function in animal experiments (27) and recombinant human leptin was reported to be effective for recovery from hypothalamic amenorrhea (28). In fact, the leptin level after weight recovery was significantly higher in the eumenorrheic group than in the amenorrheic group in our study. Audi et al. (29) reported that the leptin level of patients who resumed menstruation did not differ from that of patients who remained amenorrhectic after recovery of body weight. These discrepant results might be due to differences in the testing periods in which serum leptin levels were measured. We did the hormonal testing soon after the resumption of menstruation, while they tested it after three menstrual periods. Hence, fluctuation of the leptin level after weight recovery may have led to the different results. In fact, Hebebrand et al. (30) demonstrated that the leptin level fluctuated during weight recovery by AN patients: i.e. leptin levels became higher than those of controls for a time after recovery, and then returned to the normal range. The present study found that the leptin level of the eumenorrheic group after weight recovery was higher, but not significantly, than that of the controls, suggesting a fluctuation of the leptin level.

Misra et al. (10) reported that the leptin level of patients who remained amenorrhectic was lower than the normal range even after weight recovery. In our study, however, the leptin level of patients who remained amenorrhectic after weight recovery was lower than that of patients who resumed menstruation, but it was within the normal range. It has been hypothesized that a leptin threshold level must be maintained to have normal menstrual function (31). Although this threshold may be the lowest level necessary to keep menstruation, it does not seem to be an index of the resumption of menstruation. Because the E_2 level of the amenorrheic group was lower than that of the eumenorrheic group after weight recovery and, even if the leptin level increased to the normal range, it might not be sufficient to raise E_2 to the level necessary for the resumption of menstruation. Because AN patients usually have ovarian dysfunction, a higher leptin level might be necessary to promote E_2 secretion, which is an index for the recovery of ovarian function. Thus, it is possible that the effect of leptin on E_2 is reduced in AN patients who are recovering ovarian function, possibly due to resistance of the response to leptin. In regard to this, obese patients were often reported to have a resistance to leptin. A similar pattern of leptin resistance may occur in AN patients on weight recovery because of their rapid gain of body weight by dietary treatment.

Leptin is also known to affect the HPA axis and the HPG axis as a signal of nutritional status. Leptin administration blunted food-deprivation hypercortisolemia in animals (32). Herpertz et al. (33) revealed a strong inverse relationship between leptin and cortisol after refeeding in AN. In the

present study, such an inverse relationship between the leptin level and cortisol after weight recovery was not found; however, our small sample size makes it difficult to draw clear conclusions. Further studies will be needed to investigate the relation between cortisol hypersecretion and leptin in AN patients.

Peripheral thyroid hormones such as T_3 and T_4 usually normalize after weight recovery in AN. The lower FT_4 and normal FT_3 after weight gain by our AN patients may at least partly be explained by hypothalamic·pituitary·thyroidal dysfunction (34) and by normalized peripheral conversion of T_4 to T_3 with nutritional restoration, although the precise reason why T_3 and T_4 were differentially affected after weight recovery remains to be clarified.

This study has the following limitations. First, the number of subjects was so small that the results may not represent all types of AN patients. Second, only a single point of measurement of the fasting hormone levels may not reflect the actual function of the targeted organs because it is well known that leptin, E_2, LH and FSH have a pulsating secretory pattern, Third, ovarian size was not assessed using ultrasound. Fourth, we did not investigate the influence of psychopathology on hypercoltisolism, although Lawson et al. (35) revealed that cortisol levels were positively associated with anxiety and depression in AN patients with hypothalamic amenorrhea.

In conclusion, a higher serum cortisol level at low weight body was suggested to be predictive of the prolonged inhibition of menstrual resumption by AN patients.

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