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Infertility

Reduced Expression of $\alpha v\beta 3$ Integrin in the Endometrium of Unexplained Infertility Patients with Recurrent IVF-ET Failures: Improvement by Danazol Treatment[1]

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***Purpose*:** To determine whether there is any association between the expression of endometrial integrin $\alpha v\beta 3$ and repeated IVF-ET failure and to examine the effect of danazol treatment on $\alpha v\beta 3$ expression.

***Methods*:** This prospective study was performed using a semiquantitative immunohistochemical analysis on the staining intensity of $\alpha v\beta 3$ in the mid-secretory endometria derived from 10 fertile women and 57 infertile patients with a history of repeated IVF-ET failures. Nine patients randomly selected from these 22 patients with unexplained infertility were then treated with oral danazol administration for 12 weeks and reexamined at the first mid-secretory phase after the danazol treatment.

***Result(s)*:** The levels of endometrial $\alpha v\beta 3$ expression were lower in 22 patients with unexplained infertility than in the fertile control and 35 patients with explained infertility. The 9 patients treated with danazol showed a significant increase in the $\alpha v\beta 3$ staining.

***Conclusion(s)*:** The significantly decreased expression of endometrial integrin $\alpha v\beta 3$ suggested that functional, but not morphological, endometrial defect may be one of the causes for the patients with unexplained infertility. Danazol may have a therapeutic potential in improving endometrial function together with up-regulation of $\alpha v\beta 3$.

KEY WORDS: Danazol; implantation failure; integrin $\alpha v\beta 3$; IVF-ET; unexplained infertility.

INTRODUCTION

Despite many dramatic advances in IVF-ET treatment, pregnancy rates per embryo transfer still remain around 30% (1). Implantation failure is believed to be the most likely cause of unsuccessful IVF-ET, and therefore the development of novel treatments of implantation defects has been urgently required. Although the precise mechanisms underlying implantation failure are still poorly understood, it is obvious that successful implantation and placentation critically depend on the endometrial receptivity. Morphological endometrial dating (2) has been widely used to examine the endometrial maturation, which enables clinicians to estimate the endometrial receptivity to some extent; however, a discrepancy between morphological dating and functional maturation has been raised (3,4). To overcome this limitation of the morphological assessment, many studies have focused on a search for biochemical implantation markers as clinical tools to evaluate the receptivity of the endometrium. To date, several candidate molecules

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including Muc 1 mucin, cyclooxygenase-2, heparin-binding epidermal growth factor-like growth factor, calcitonin, leukemia inhibitory factor, HOXA10, HOXA11, and integrins have been proposed on the basis of their spatiotemporal expression during the implantation window and/or the phenotype of mice lacking the corresponding gene (5,6).

One of the promising implantation molecules may be the integrin, which is a family of cell surface $\alpha\beta$ heterodimeric transmembrane receptors that mediate cell–cell and cell–substratum interactions (7). Among those integrins, endometrial $\alpha v\beta 3$ has been proposed as a useful implantation marker on the basis of the following findings: 1) spatiotemporal expression of endometrial integrin $\alpha v\beta 3$ in the luminal and glandular epithelium during the implantation window (8,9), 2) an association between the aberrant expression of integrin $\alpha v\beta 3$ and certain types of female infertility (4,10–12), and 3) impairment of implantation by blockade of the endometrial $\alpha v\beta 3$ using intrauterine injection of various bioactive compounds in mice (13). Although the usefulness of integrin $\alpha v\beta 3$ as an implantation marker still remains controversial (14–18), these accumulating bodies of evidence prompt us to examine the expression of endometrial $\alpha v\beta 3$ in the infertile patients in particular with repeated IVF-ET failures despite the transfer of the morphologically normal embryos.

Establishment of an effective treatment for the implantation failure is the most important theme in assisted reproductive technology (ART). We have previously reported the efficacy of danazol treatment for the repeated failure of IVF-ET with morphologically normal embryos (19). Danazol, an isoxasol derivative of 17 α-ethinyltestosterone, has been widely used to treat patients with endometriosis and adenomyosis. In addition to its hormonal activity, danazol is also known to have a variety of immunoregulatory effects on the eutopic endometrium (20–22). However, the biological effect of danazol on endometrial receptivity has not been extensively studied. It also prompted us to examine the effect of danazol on endometrial integrin $\alpha v\beta 3$ expression. Here we demonstrated the aberrant expression of endometrial $\alpha v\beta 3$ in IVF-ET patients with unexplained infertility and the effect of danazol on its expression.

MATERIALS AND METHODS

Patients

Sixty-nine patients who had experienced repeated IVF-ET failures despite having more than one embryo with optimal morphology were enrolled in this prospective, observational study. Twelve patients were excluded from this study; five whose curettaged specimens were inadequate for evaluation, and seven whose endometrium was morphologically out of phase ($\geq$3 days delayed by endometrial histology). Informed written consent was obtained from all the patients before they participated in this study. The study protocol was approved by the Internal Review Board of Keio University School of Medicine. The clinical indications for IVF of these 57 patients consisted of explained ($n = 35$) and unexplained infertility ($n = 22$). The indications for IVF-ET of the 35 patients with explained infertility included tubal factors ($n = 21$) and male factors ($n = 14$). There was no significant difference in the mean age of the patients among both groups. In our IVF-ET programs, patients were defined as unexplained infertility when they met all of the following criteria: 1) ovulatory menstrual cycle ranging from 26 to 32 days as determined by urinary LH and BBT charts; 2) normal tubal and peritoneal anatomy as determined by hysterosalpingography and/or laparoscopy; 3) normal endometrial biopsy and midluteal serum progesterone >10 ng/mL; 4) no evidence of male infertility; 5) several ($\geq$6–10) failures of artificial insemination with husbands. Nine patients were randomly selected by the distribution of sealed envelopes from the group of unexplained infertility, and they gave their consent for the oral administration of danazol (400 mg per day for 12 weeks) as previously described (19).

Tissue Collection

Endometrial samples were obtained by curettage from all the patients enrolled in the study during the mid-secretory phase of the menstrual cycle between postovulatory days 6–10. As the control, the mid-secretory endometria were obtained from six fertile women with normal menstrual cycles. The proliferative and early-secretory endometria were also obtained from four fertile women to verify the expression pattern of endometrial integrin $\alpha v\beta 3$ during the menstrual cycle as described elsewhere (8,23). Ovulation was confirmed by urinary LH surge, basal body temperature, and transvaginal sonography. To assess the effect of danazol administration on endometrial integrin $\alpha v\beta 3$ expression, subsequent endometrial biopsies from 9 patients randomly chosen from the 22 IVF-ET patients with unexplained infertility were obtained 6–10 days after the first ovulation followed by danazol treatment. Specimens were snap-frozen on dry ice immediately

and maintained at −70°C until immunohistochemical staining.

Immunohistochemistry

The monoclonal antibody LM609 (Chemicon International, Inc., Temecula, CA), which specifically reacts with the $\alpha v\beta 3$ complex of integrin (24), was used in this study. Serial cryostat sections 6 μm thick were mounted onto poly-L-lysine-coated slides and air-dried for 1 h at room temperature. Histological dating of the endometrium was performed according to the standard criteria of Noyes *et al.* (2). Sections were fixed in acetone and methanol (1:1) for 10 min, and labeled by an avidin–biotin peroxidase technique (Vectastain Elite ABC kits, Vector Laboratories, Burlingame, CA). The primary antibody (or control Ig G as a negative control) was placed on cryosections after blocking with 5% bovine serum albumin in phosphate-buffered saline (PBS) and was allowed to bind at room temperature for 1 h. After three times of washing with PBS (pH7.2), the slides were incubated with the secondary antibody (LSAB kit, DAKO, Glostrup, Denmark) for 30 min. They were then washed three times in PBS and incubated with a preformed avidin horseradish peroxidase macromolecular complex for 30 min. After further washes in PBS, the reaction was developed by incubation in 0.01% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in Tris/HCl buffer, pH 7.6, containing 0.3% H_2O_2, for approximately 2.5 min. The sections were counterstained in Mayer's hematoxylin, dehydrated in absolute alcohol, cleared in xylene, and mounted in synthetic resin. Intensity of the staining of the endometrial components was evaluated by the previously established semiquantitative scoring system; H-SCORE (25). The H-SCORE was calculated using the following equation: H-SCORE = $\sum$Pi $(i + 1)$, where i = intensity of staining with a value of 1, 2, or 3 (weak: 1; moderate: 2; strong: 3) and Pi = percentage of stained luminal and glandular epithelial cells for each intensity (0–100%). All sections were stained with the same lot of antibody at a constant dilution; likewise, the DAB reaction conditions were also kept constant. As internal controls, the staining intensity of the vascular endothelial $\alpha v\beta 3$, which was uniform in all sections, was utilized to classify the intensity of luminal and glandular epithelial $\alpha v\beta 3$. The staining intensity of the tissue sections was evaluated and graded by an independent observer (Dr M. M.) in a blinded fashion. Photomicrographs were taken with Kodak 100 ASA film (Kodak, Tokyo, Japan).

Statistical Analysis

All H-SCOREs are reported as the mean ± SD. The nonparametric Kruskal–Wallis test and Bonferroni method for multiple significance test were used for comparing the H-SCORE between control group and other groups. The H-SCORE before and after danazol treatment in each patient was compared using the Wilcoxon signed-ranks test. A p value of <0.05 was considered significant, or in case of k comparisons, whenever $p < 0.05/k$ (Bonferroni).

RESULTS

Immunohistochemistry of the endometrium derived from control fertile women revealed that integrin $\alpha v\beta 3$ was strongly expressed in the stroma, but very weakly in the luminal and glandular epithelium, during the proliferative and early-secretory phase (Fig. 1(A) and (B), respectively). In contrast, the prominent expression of integrin $\alpha v\beta 3$ was observed in the glandular and luminal epithelium (Fig. 1(C) and (D), respectively), but not in the stroma (Fig. 1(C) and (D)), during the mid-secretory phase. A constant level of integrin $\alpha v\beta 3$ expression was observed in the vascular endothelium throughout the menstrual cycle. Figure 1(E) depicts a normal pattern of $\alpha v\beta 3$ integrin expression in the glandular epithelium of a patient with explained infertility. In contrast, while endometrial vessels were prominently stained (Fig. 1(F), arrowheads), glandular epithelial cells were very weakly stained in the histologically "in-phase" endometrium derived from a patient with unexplained infertility (Fig. 1(F)).

There was no significant difference in the H-SCORE of epithelial $\alpha v\beta 3$ integrin between the control and the 57 patients with repeated unsuccessful IVF-ET patients group (2.32 ± 0.79 and 1.65 ± 1.05, respectively). The expression of epithelial $\alpha v\beta 3$ integrin in control women did not differ significantly from that found in the patients with explained infertility (Fig. 2). However, the epithelial H-SCORE of $\alpha v\beta 3$ integrin was significantly lower in the 22 patients with unexplained infertility compared to those in the fertile controls and explained infertility patients ($p < 0.01$, by Kruskal–Wallis test) (Fig. 2).

As shown in Fig. 3, all the nine patients with unexplained infertility showed increased immunostaining intensity of endometrial integrin $\alpha v\beta 3$ after danazol treatment and the mean H-SCORE was significantly increased after danazol treatment ($p = 0.0076$, by Wilcoxon signed-ranks test). Immunohistochemistry of the mid-secretory endometria derived from two

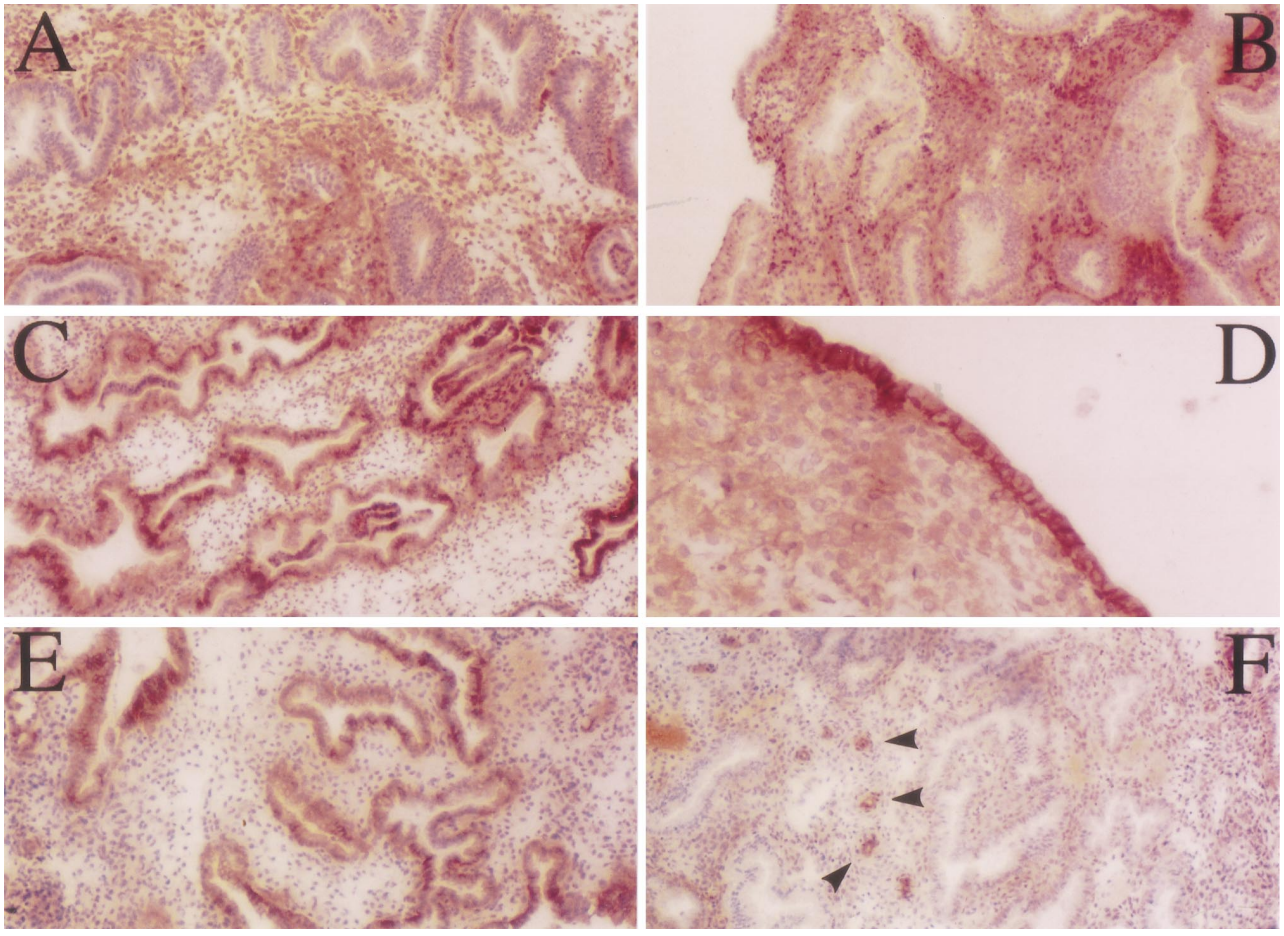

Fig. 1. Immunohistochemical staining of $\alpha v\beta 3$ integrin in the endometrium of control fertile women (A-D) and the expression of $\alpha v\beta 3$ in two contrasting cases of patients with repeated failures of IVF-ET (E and F). Endometrial specimens were obtained at the proliferative phase (A), early-secretory phase (B), and mid-secretory phase (C-F) of the menstrual cycle (Magnification, A-F except D, ×100; D, ×200). Integrin $\alpha v\beta 3$ is expressed on the stroma during the proliferative (A) and the early-secretory endometrium (B), while the glandular and luminal epithelium was very weakly stained (A and B). During the mid-secretory phase a dramatic increase in staining was observed around the entire circumference of the glandular (C) and luminal (D) epithelium. A similar pattern of epithelial $\alpha v\beta 3$ expression was found in an unsuccessful IVF-ET patient with explained infertility (E), whose H-SCORE was designated as 2.5. In contrast, while the endometrial vessels were intensively stained (F, arrowheads), glandular epithelial cells were very weakly stained in the histologically "in-phase" endometrium derived from a patient with unexplained infertility (F).

representative unsuccessful IVF-ET patients with unexplained infertility (shown as open circle and closed circle in Fig. 3) revealed that the intensity of glandular $\alpha v\beta 3$ staining was enhanced after danazol treatment (Fig. 4(A) and (C) vs. (B) and (D)).

DISCUSSION

Since the first report on the endometrial integrins (8), several studies have demonstrated that some types of integrins cyclically undergo spatial and temporal changes in expression throughout the menstrual cycle, implicating its important role in the process of implantation (7,8,26,27). For instance, integrin $\alpha 4$ and $\beta 3$ subunits are specifically co-expressed only during the time of maximum uterine receptivity (9,23). Integrin $\alpha v\beta 3$, the vitronectin receptor, has been shown to be specifically expressed in the glandular and luminal epithelial cells during the implantation window, which suggests its importance in endometrial receptivity (9,10,17). In addition, it has been reported that some infertile women exhibit abnormalities in the expression pattern of endometrial integrins such as $\alpha 4\beta 1$ and $\alpha v\beta 3$ (4,23).

The present report showed that endometrial integrin $\alpha v\beta 3$ is specifically expressed in the glandular

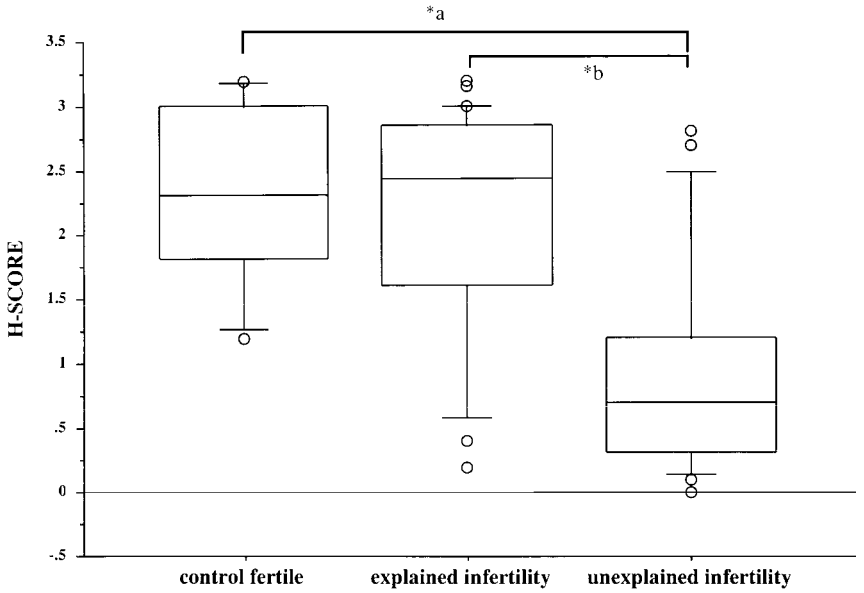

Fig. 2. The comparison of H-SCORE of endometrial integrin $\alpha v\beta 3$ expression in the control and the IVF-ET patients with repeated failures. The intensity and distribution of staining (H-SCORE) was calculated as described in Materials and Methods. In each plot, the horizontal lines mark the 10th, 25th, 50th, 75th, and 90th percentile points of the data. The box encompasses the 25th through the 75th percentiles. All H-SCORE values above the 90th percentile or below the 10th percentile are shown as circles. The patients with unexplained infertility showed a significant decrease in H-SCORE when compared with either the control group^{*a} or patients with explained infertility^{*b} ($p < 0.01$ by Kruskal–Wallis test and $p < 0.05/3$ by Bonferroni method for multiple significance test).

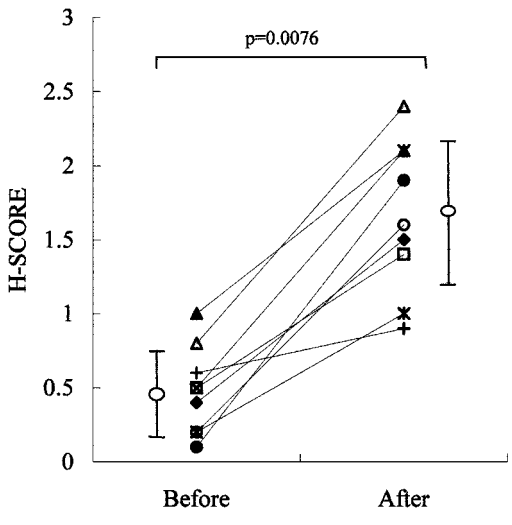

Fig. 3. The H-SCORE values of endometrial integrin $\alpha v\beta 3$ expresson before and after danazol treatment. All the 9 patients randomly selected from 22 patients with unexplained infertility showed an increased intensity of $\alpha v\beta 3$ expression after danazol treatment. The mean H-SCORE after danazol treatment was significantly higher than that before danazol treatment ($p = 0.0076$ by Wilcoxon signed ranks test).

and luminal epithelial cells during the time of maximum uterine receptivity, in agreement with earlier reports (8,9,17). Furthermore, the reduced expression of integrin $\alpha v\beta 3$ on mid-secretory endometrium was observed in the 22 unsuccessful IVF-ET patients with unexplained infertility despite normal male and tubal factors as well as normal morphological endometrial dating and embryo quality. This result is consistent with the previous reports on non-IVF patients with unexplained infertility (4). The definition of unexplained infertility is usually made by exclusion after all the standard investigations have revealed no abnormality (28). Our result suggested that the functional, but not morphological, endometrial defect may be one of the causes for unexplained infertility.

The usefulness of integrin as a biomarker for the implantation window remains controversial (14–18), as discrepancies in expression patterns have been observed possibly because of differences in methodologies or antibodies employed in various studies. Furthermore, since so far non-IVF-ET infertile patients have been mostly studied on endometrial integrin $\alpha v\beta 3$ expression (4,8,9,11,12,14–17), a

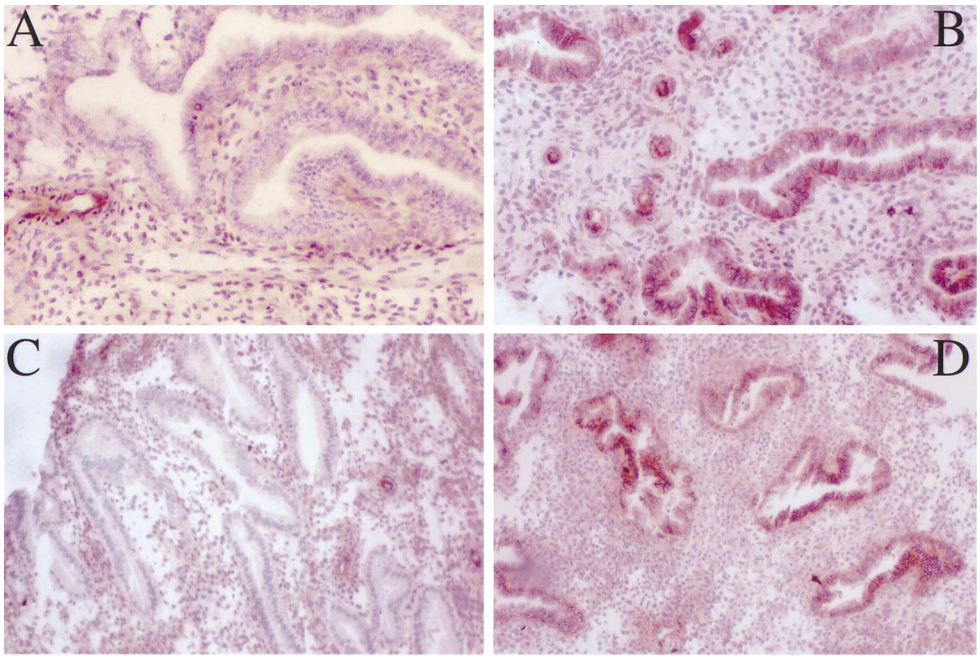

Fig. 4. Immunohistochemical staining of integrin $\alpha v\beta 3$ in the mid-secretory endometria derived from two representative patients (A and B as open circle and C and D as closed circle in Fig. 3) with unexplained infertility. The endometrial specimens were obtained before (A, C) and after (B, D) danazol treatment. Note that histologically "in-phase" endometria showed a weak epithelial $\alpha v\beta 3$ staining before danazol treatment (H-SCORE, 0.2 for A, 0.1 for C), while the staining of $\alpha v\beta 3$ becomes prominent after danazol treatment (H-SCORE: 1.6 for B, 1.9 for D). Magnification A, B $\times$ 200, C, D $\times$ 100.

possible heterogeneity of the patients enrolled in those individual studies may generate a controversy on a correlation between the reduced expression of integrin $\alpha v\beta 3$ and infertility. In this study, although there was not a significant difference in the expression of endometrial $\alpha v\beta 3$ between the control fertile women and the infertile 57 patients with repeated IVF-ET failures, an association between the decreased expression of endometrial $\alpha v\beta 3$ and the patients with unexplained infertility supports an idea that $\alpha v\beta 3$ might possess a potential to reflect the intrauterine milieu responsible for endometrial function. Together with an increasing body of knowledge about implantation biomarkers, we stress that $\alpha v\beta 3$ may be at most amongst many molecules participating in implantation and not the sole determinant of endometrial receptivity. Further studies are required to determine whether possible implantation markers other than $\alpha v\beta 3$ are also dysregulated in the endometrium of the unsuccessful IVF-ET patients with unexplained infertility.

The diagnosis and treatment of abnormal endometrial function may pose a major advance for ART and may ultimately decrease the economical and physical burden placed on women undergoing infertility treatment. At present, there are no established treatment guidelines for functional endometrial defects (3). Our previous clinical study (19) showed an improvement in pregnancy rates among repeated unsuccessful IVF-ET patients using danazol treatment; this prompted us to determine whether danazol has a positive effect on the expression of integrin $\alpha v\beta 3$. In this study, 9 patients randomly chosen from the 22 patients with unexplained infertility showed a significant increase in the $\alpha v\beta 3$ immunostaining by subsequent danazol treatment. The mechanism underlying the effect of danazol on the $\alpha v\beta 3$ expression remains to be elucidated. Endometrial expression of integrin $\alpha v\beta 3$ depends on the progesterone exposure (29). We examined the serum progesterone levels of several patients when endometrial biopsy was performed, although we could not find any significant differences in their levels between before and after danazol treatment (data not shown). In addition to progesterone, endometrial epithelial $\beta 3$ subunit has been demonstrated to be upregulated in vitro by epidermal growth factor (EGF) or a blastocyst-derived complete interleukin-1 (IL-1)

system consisting of IL-1α and IL-1β/IL-1ra (30,31). Human endometrium is known to produce a large number of growth factors and cytokines including EGF and IL-1 under the influence of ovarian steroid hormones (32,33). Thus, although low levels of progesterone may result in reduced expression of integrin, a normal level of progesterone does not necessarily warrant the proper integrin expression. On the other hand, danazol possesses a potential to regulate ovarian steroid hormone-induced growth factors and cytokines including IL-1 system in the endometrium (34,35). Taken together, it is possible that endometrial adequacy for implantation may be impaired in the specific subgroup of unsuccessful IVF-ET patients, which could be ameliorated by danazol treatment possibly through improvement of the orchestration of those endometrial factors including EGF and IL-1 system. The usefulness of danazol for infertility requires further corroboration as disparities exist among published studies because of differences in patient selection and treatment methodology (19,36–38). On the basis of our previous (19) and present results, we suggest that danazol might be beneficial for unsuccessful IVF-ET patients with unexplained infertility, particularly those with low levels of endometrial integrin $\alpha v\beta 3$ expression.

In conclusion, we showed an association between repeated IVF-ET failures with unexplained infertility and the reduced expression of endometrial $\alpha v\beta 3$ and demonstrated a significant increase in the level of $\alpha v\beta 3$ expression after danazol treatment. It is interesting that an aberrant endometrial function existed in the subgroup of repeated IVF-ET failure patients. The results suggested that further diagnostic methodology and treatment for the clinical implantation failure would be one of the important themes in ART. Further studies are required to elucidate the precise molecular basis for implantation and more reliable implantation biomarkers of endometrium are expected.

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