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ORIGINAL ARTICLE

Role of Metalloproteinases in the Pathogenesis of Unexpected Poor Ovarian Response with a Possible Genetic Predisposition

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ABSTRACT

Aim: To study the role of matrix metalloproteinase (MMP-1,2,3), inhibitor tissue inhibitors of metalloproteinase (TIMP)-2, and specific gene polymorphisms in unexpected poor ovarian responders (un-PORs).

Materials and methods: Group I consisted of 44 un-PORs, group II of 42 subfertile, normal ovarian responders (NORs), and group III of 66 fertile women in a prospective study. Matrix metalloproteinase-1,2,3 and TIMP-2 were assessed in 40 patients from groups I and II. Specific polymorphisms (SP; MMP-1 –519 A/G, MMP-2 –1575 G/A, MMP-3 –1171 5A/6A, and TIMP-2 rs55743137T/G) were investigated in group I, II, and III patients.

Results: Group I required similar amount of gonadotropins compared with group II, with fewer oocytes retrieved, lower fertilization rates, embryos/embryo transfer, clinical pregnancies/cycle, and "take-home babies" (p = 0.900, 0.001, 0.002, 0.001, 0.031, and p = 0.128) respectively, Table 1). Group I had lower MMP-2 with higher TIMP-2 (p = 0.002, 0.037 respectively; Table 2). In the same group, MMP-1 was higher in women with GG genotype of the MMP-1 polymorphism, *vs* GA genotype (p = 0.047; Table 3). The MMP-2, MMP-3, and TIMP-2 polymorphisms did not affect MMP-2, MMP-3, and TIMP-2 respectively. The same applied for MMP-1,2,3 and TIMP-2 in group II. Comparing frequencies of different genotypes of the MMP-1,2,3 and TIMP-2 polymorphisms, they did not differ between the three different groups: A, B, and C (Table 4).

Conclusion: Impaired MMP-2 activity, associated with significantly higher TIMP-2 detected, could be involved in un-POR pathogenesis. There was no strong association between MMP polymorphisms and un-POR susceptibility. However, women with A/G polymorphism (MMP-1 –519) had lower MMP-1 compared with GG homozygotes.

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Clinical significance: Identification of patients with poor ovarian response in a pretreatment environment would help improve their ongoing fertility plan and manage their expectations. Also by having the ability to investigate if one belongs to that group, it could provide important family planning information for the patient.

Keywords: Infertility, Metalloproteinases, Unexpected poor ovarian response.

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INTRODUCTION

Poor Ovarian Responders

One of the biggest difficulties and disputatious topics in assisted reproductive medicine is the management of poor ovarian responders (PORs), as defined by the European Society of Human Reproduction and Embryology (ESHRE) Consensus (Bologna criteria, 2011).[1-3] Even more so are the relatively rare cases of normogonadotropic, unexpected PORs (un-PORs) of younger age, a heterogeneous and understudied population, less than 20% of the total PORs.[4] In view of the fact that no single regimen will benefit all PORs, a variety of regimes have been proposed to improve the unsatisfactory reproductive performance of this particularly challenging group of patients.[5-13] As a complex condition, the precise molecular mechanism of un-PORs is still unknown. A genetic predisposition has been proposed.

Matrix Metalloproteinases and their Tissue Inhibitors: Structure and Function

Matrix metalloproteinases (MMPs), also called matrixins, are major enzymes involved in the timely degradation of the extracellular matrix (ECM). They play a central role in

many biological processes, such as angiogenesis, follicular development, implantation, and embryogenesis. They are also found intracellularly and may act on intracellular proteins.[14-17] Currently, 23 MMP genes have been identified in humans. Loss of activity control may result in impaired wound healing, tissue repair, and remodeling in response to injury, e.g., after myocardial infarction and cancer. Their activities are regulated by tissue inhibitors of metalloproteinases (TIMPs).[18,19]

The balance between MMPs and TIMPs is critical for the eventual ECM remodeling in the tissue. Further studies have also indicated that members of the family called a disintegrin and metalloproteinase (ADAM or adamalysin family) participate too.[20] Most proMMPs are secreted from cells and activated extracellularly.[21]

Tissue Inhibitors of Metalloproteinases

Matrix metalloproteinase activities in the tissue are regulated by endogenous specific inhibitors (TIMPs) that bind MMPs in a 1:1 stoichiometry.[22] The balance between MMPs and TIMPs is critical for the eventual ECM remodeling of tissues and its disruption perturbs tissue homeostasis. Pathological changes of TIMP levels are considered to be important because they directly affect the level of MMP activity. Four TIMPs are found in humans (TIMP-1, TIMP-2, TIMP-3, TIMP-4). Although they are separate gene products, each TIMP inhibits most members of the MMP family.[23]

MMPs as Mediators of Reproductive Function

Remodeling of the ECM is a prerequisite for many physiological and pathological processes. Examples of ECM remodeling by MMPs during normal physiological processes within the reproductive system include follicular growth and ovulation, corpus luteum formation and regression, endometrial remodeling at menstruation, blastocyst outgrowth, trophoblast invasion, uterine involution, and mammary growth and involution.[24,25]

Tissue remodeling and angiogenesis in the follicular wall with continuous and steadily increasing blood flow are essential for proper follicular development. They may be prevented by inhibition of certain key MMPs.[26] Initial attempts, however, to detect MMP activity within periovulatory ovarian tissues were unsuccessful owing to the presence of TIMPs, including a2-macroglobulin.[27] More recently, experimental studies have indicated that excessive TIMP-1 is associated with ovulatory dysfunction. Rats with endometriosis treated with the TIMP-1 function-blocking antibody had significantly more follicles and corpus luteals (CLs) than the rest or in comparison to the TIMP-1-treated endometriosis-free rats.[28]

The aim of this study was to investigate possible involvement of certain MMPs (1,2,3) and their inhibitors (TIMP-2) in the understudied pathophysiology of un-PORs. In view of the absence of relevant data and with a view to distinguish potential genetic variants associated with un-PORs, attempts have been made to explore their relevance with specific MMP-gene polymorphisms.

MATERIALS AND METHODS

Three groups of women were studied: Group I consisted of 44 *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI)-treated patients with un-POR, and group II (controls) of 42 normal ovarian responders (NORs) on the same treatment (Table 1). All were normogonadotropic under 38. Group III (controls) consisted of 66 women of proven fertility with spontaneous conception and delivery.

Serum MMP-1,2,3 and TIMP-2 levels were assessed in groups I and II using enzyme-linked immunosorbent assay method (R&D Systems, Inc, Minneapolis, MN, USA). In addition, women of all three groups were subjected to polymerase chain reaction-restriction fragment length polymorphism genetic analysis following deoxyribonucleic acid extraction from the peripheral blood. The following genetic polymorphisms were studied in relation in to Adenine (A) Guanine (G) Thiamine (T) Cytosine (C): –519 A/G for MMP-1, –1575 G/A for MMP-2, –1171 5A/6A for MMP-3, and rs55743137 T/G for TIMP-2.

The restriction enzymes used were KpnI for MMP-1 –519 A/G, Tsp45I for MMP-2 –1575 G/A, TthlllI for MMP-3 –1171 5A/6A, and AluI for TIMP-2 rs55743137T/G polymorphisms.

RESULTS

Patients in both groups I (un-PORs) and II (NORs) were normogonadotropics with no significant differences in basal follicle-stimulating hormone (FSH), luteinizing hormone (LH), and anti-mullerian hormone (AMH) levels ($p = 0.075$, $p = 0.883$, and $p = 0.084$ respectively). Final E2 were significantly higher in group II, as expected ($p = 0.001$) (Table 1). The amount of gonadotropins administered was similar ($p = 0.900$). The oocytes retrieved, embryos/embryo transfer (ET), per-cycle clinical pregnancies, and fertilization rates were lower in group I ($p = 0.001$, $p = 0.001$, $p = 0.031$, and $p = 0.002$ respectively).

The take-home babies/ET rate was almost two times higher in group II than in group I [47.5 *vs* 26.6% respectively; p = not significant (NS)]. Group I had lower MMP-2 and higher TIMP-2 compared with group II ($p = 0.002$ and $p = 0.037$ respectively; Table 2), with a trend for MMP-1 and MMP-3 levels to be higher in group I ($p = 0.051$ and $p = 0.088$ respectively).

Furthermore, in group I patients (un-PORs) subjected to genetic analysis, MMP-1 levels were higher in those

Table 1: Clinical characteristics and IVF/ICSI results in young normogonadotrophic: (a) poor ovarian responders (PORs – group I) and (b) normal ovarian responders (NORs – group II)

	Group I (un-PORs)	*Group II (NORs)*	*p-value*
Number of patients (n)	44	42	
Age (years)	34.0 ± 4.2	32.7 ± 3.6	0.112
Body mass index (kg/m^2)	22.9 ± 2.8	23.6 ± 2.6	0.197
FSH (basal) mIU/mL	8.1 ± 2.6	7.6 ± 2.4	0.075
LH/(basal) mIU/mL	6.7 ± 2.3	6.6 ± 2.3	0.883
AMH (any day) mIU/mL	10.8 ± 6.1	12.2 ± 7.3	0.084
E2 (hCG day) pg/mL	573.5 ± 363.9	2,328.4 ± 1,202.1	<0.001
Gonadotropines required units/patient pg/mL	2,704.9 ± 501.2	2,684.6 ± 915.2	0.900
No. of oocytes retrieved/patient	2.3 ± 1.2	11.7 ± 4.6	<0.001
Number of embryos/ET (average)*	1.1 ± 0.7 (30 ETs)	2.1 ± 0.8	<0.001
Infertility duration (years-average)	5.0 ± 2.9	3.8 ± 2.4	0.044
Previous IVF attempts (average)	2.4 ± 1.2	1.2 ± 1.2	<0.001
Clinical pregnancy			
Per cycle	29.5% [13/44]	54.76% (23/42)	0.031
Per ET	43.3% (13/30]	57.5% (23/40)	0.351
Cancellation rates	31.8% (14/44)	7.14% (3/42)	0.009
Fertilization rates	67.3% (68/101)	82% (406/496)	0.002
Implantation rates	30% (15/50)	35.95% (32/89)	0.599
Miscarriage rates/Clin. pregnancies	23.0% (3/13)	7.14% (2/28)	0.348
Take-home-babies/ET	26.6% (8/30)	47.5% (19/40)	0.128

*There were no significant differences in embryo quality between groups I and II; hCG: Human chorionic gonadotropin

Table 2: Matrix metalloproteinase and TIMP levels in women in groups I (un-PORs) and II (NORs)

	Group I (n = 40)	*Group II (n = 40)*	*p-value*
MMP-1	3.6 ± 3.2	2.3 ± 2.2	0.051
MMP-2	62.3 ± 17.8	73.8 ± 14.6	0.002
MMP-3	13.9 ± 8.0	11.2 ± 5.9	0.088
TIMP-2	189.1 ± 106.0	150.2 ± 43.4	0.037

with the GG genotype of the MMP-1 polymorphism compared with women with the GA genotype (p = 0.047; Table 3). Otherwise, the MMP-2, MMP-3, and TIMP-2 polymorphisms did not affect the levels of MMP-2, MMP-3, or TIMP-2.

In group II patients (NORs), unlike group I, the MMP-1,2,3 and TIMP-2 polymorphisms did not seem to exert any influence on the respected MMPs and TIMP-2 studied.

Finally, comparing the three main groups studied (I, II, and III) in total, there was no difference found in the frequencies of the different genotypes of the MMP-1,2,3, and TIMP-2 polymorphisms (Table 4). To our knowledge, similar studies on these rather understudied, un-POR patients have not been reported.

All data were analyzed with the statistical package Statistical Package for the Social Sciences (version 17.0; SPSS, Chicago, Illinois, USA). Data are presented as percentages for categorical variables and as mean and standard deviation for continuous variables. Differences in categorical variables between groups were assessed with the chi-square test. Differences in continuous variables between groups were assessed with one-way analysis of variance and *post hoc* tests were performed with the Holm–Šídák test. In all cases, a two-tailed $p < 0.05$ was considered significant.

DISCUSSION

The pathophysiology of un-PORs appears heterogeneous and unresolved. A variety of etiological mechanisms have

Table 3: MMP-1, MMP-2, MMP-3, and TIMP-2 in women with different genotypes in group I (un-PORs)

	AA (n = 14)	*GA (n = 19)*	*GG (n = 8)*	*p-value*	*AA vs GA*	*AA vs GG*	*GA vs GG*
MMP-1	3.9 ± 3.4	2.8 ± 2.4	7.8 ± 6.0	0.049	0.720	0.167	0.047
	GA (n = 20)		GG (n = 16)		p-value		
MMP-2	62.2 ± 16.0		64.3 ± 21.4		0.735		
	5A5A (n = 7)	5A6A (n = 20)	6A6A (n = 9)	p-value	5A5A *vs* 5A6A	5A5A *vs* 6A6A	5A6A *vs* 6A6A
MMP-3	12.4 ± 6.1	12.7 ± 7.5	15.8 ± 9.8	0.593	0.999	0.785	0.723
	TG (n = 14)		TT (n = 22)		p-value		
TIMP	168.6 ± 59.4		198.8 ± 133.1		0.432		

Table 4: Frequencies of the different genotypes of the MMP-1, MMP-2, MMP-3 and TIMP-2 polymorphisms in groups I (subfertile un-PORs), II (subfertile NORs), and III (women of proven fertility)

	Group I (n = 44)	*Group II (n = 37)*	*Group III (n = 66)*	*p-value*
MMP-1				0.776
A/A	34.1	45.9	40.9	
G/A	54.5	48.7	51.5	
G/G	11.4	5.4	7.6	
MMP-2				0.198
A/A	0	5.4	3.0	
G/A	52.3	35.1	33.3	
G/G	47.7	59.5	63.7	
MMP-3				0.488
5A/5A	18.2	24.3	18.2	
5A/6A	54.5	40.6	59.1	
6A/6A	27.3	35.1	22.7	
TIMP-2				0.305
G/G	0.0	5.4	1.5	
T/G	36.4	43.2	31.8	
T/T	63.6	51.4	66.7	

been proposed, such as decreased number of FSH receptors in granulosa cells,[29] defective signal transduction after FSH-receptor binding,[30] anti-FSH immunoglobulin (Ig)A and IgG potentially exerting a local FSH-antagonizing effect in maturing follicle,[31] the presence of a specific FSH receptor-binding inhibitor in the follicular fluid,[32] and possibly underlying occult theca cells deficiency.[12] Some are well established, some are still controversial, and some novel candidates may be identified in the near future.[3]

The balance between MMPs and TIMPs is decisive for the appropriate maintenance of tissues, and its disruption perturbs tissue homeostasis. Growth of an ovarian follicle culminating in ovulation or atresia and the development, maintenance, and regression of a corpus luteum are cyclic events that depend upon extensive remodeling of the ECM. The TIMP-1 contributes to ovarian anomalies in both MMP-dependent and -independent manner in a rat model.[28]

With regard to their selectivity, although TIMP-1,2,3,4, are separate gene products, each one inhibits most members of the MMP family. However, TIMP-1 shows higher affinity to MMP-1 and TIMP-2 binds most tightly to MMP-2.[23]

This study focuses on the expression of certain MMPs and their inhibitors on ovarian function and the evidence supporting a role for these enzymes in the process of impaired folliculogenesis in un-PORs, with a possible genetic predisposition. The significantly higher levels of the MMP-2 inhibitor TIMP-2 could be the reason for the compelling lower levels of MMP-2 seen in group I patients (PORs), resulting in impaired MMP-2 activity, a possible cause of poor ovarian response (Table 2). With regard to the trend for higher MMP-1 and MMP-3 levels recorded in the same group ($p = 0.051$ and $p = 0.088$ respectively; Table 2), no adequate explanation could be attempted as their relevant inhibitors TIMP-1 and TIMP-3 were not assessed in this study. However, MMP-3, when not appropriately controlled, is involved in pathologically increased breakdown of ECM in various physiological processes, such as the complex implantation process and early embryonic development.[24,25]

The home-take babies/ET rate was almost two times higher in group II than in group I, but this difference did not reach significance, probably due to the small sample size (47.5 *vs* 26.6% respectively; p = NS) (Table 1).

The activity of MMPs is dependent on the gene encoding them. The existence of gene polymorphism determines the different expression level of these genes among individuals, which ultimately result in different phenotype of disease in a population. In the past decade, considerable efforts have been made to find out the relationship between MMP single-nucleotide polymorphism and the risk of various diseases.[33]

In this study, in PORs subjected to genetic analysis (group I), MMP-1 was higher in patients with the GG genotype of the MMP-1 polymorphism than in patients with the GA genotype ($p = 0.047$; Table 3) with a possible involvement in the complex mechanism of un-POR due to the loss of balanced MMP-1 activity required in various physiological processes including folliculogenesis.[25,26] In the same group, MMP-2, MMP-3, and TIMP-2 polymorphisms did not affect the levels of MMP-2, MMP-3, or TIMP-2 respectively, neither the clinical outcome as a consequence.

In normal responders (NORs; group II), polymorphisms in all three MMP genotypes analyzed, unlike in PORs, did not seem to exert any influence on MMP-1,2,3, and TIMP-2 respectively, and on the end reproductive outcome.

Despite the markedly different profile and clinical outcome between the three groups analyzed (A, B, and C), the frequency of their polymorphisms did not differ (Table 4).

Genetic analyses using transgenic mice that have gain and loss of function of MMPs or of their endogenous inhibitors, the TIMPs, and pharmacogenetic studies with chemical inhibitors have begun to elucidate the roles that they play. It has been suggested that the expression of TIMP variants directed to specific metalloproteinases in a targeted tissue may be a potential therapeutic. Tissue inhibitors of metalloproteinase-1-treated rats had fewer follicles while CL- rats treated with the TIMP-1 function-blocking antibody had significantly more follicles and CLs.[28] However, the results of different researches are

not consistent. The function of MMPs is much more complex and subtle than straightforward demolition and the complexity of the role of MMPs in health and disease is evident.[34] Evidence existed that therapeutic inhibitors can potentiate therapy against cancer and other conditions with disrupted MMP/TIMP enzymatic milieu.[35] Several potent, orally available MMP inhibitors have been developed by a number of pharmaceutical companies and some were clinically tested for the treatment of arthritis or cancer, but none were found to be efficacious probably due to nonselective inhibition of metalloproteinases.[36] In addition, there are general concerns about the safety of synthetic MMP inhibitors mentioning as an example, the musculoskeletal problems caused by the broad-spectrum MMP inhibitor Marima-stat (British Biotech Pharmaceuticals, Oxford, UK).[37] However, there is a clear potential for the application of TIMPs as endogenous inhibitors with altered specificity, to allow targeting of specific proteinases. The fact that there are more than 50 similar metalloproteinases in humans (23 MMPs, 13 ADAMs, and 19 ADAMTSs) presents an additional challenge. As scientific understanding of the MMPs has advanced, therapeutic strategies focusing on regulating these enzymes by their specific TIMPs including gene transfer technologies have rapidly developed as a new therapeutic approach for the treatment of individual types of malignant as well as nontumorous conditions.[34,38-41]

Experimental studies showed that increased levels of TIMP-1 were deleterious to ovulation and embryo development and that "neutralizing" TIMP-1 restores fecundity. Thus, novel TIMP-1-modulating therapies may be developed to alleviate infertility,[28,42-44] with a potential to include cases with unexplained infertility and poor ovarian response. As already stressed, ECM as a whole has been found to cause regrowth and healing of tissue. In human fetuses, for example, the ECM works with stem cells to grow and regrow all parts of the human body, and fetuses can regrow anything that gets damaged in the uterus. In the light of pioneering regenerative medicine, the therapeutic potential of the ECM is being researched further as a device for tissue regeneration in humans.[45]

This study presents evidence for the involvement of MMPs and their inhibitors in ECM remodeling, supporting a role for these enzymes in the process of impaired folliculogenesis in un-PORs, with a possible genetic predisposition. To our knowledge, similar studies referring to PORs and un-PORs in particular have not been published. However, a more complete understanding of the expression, production, and regulation of follicular MMPs and TIMPs and of connective tissue remodeling, with a possible genetic predisposition, awaits further investigation.

CONCLUSION

Women with un-POR had significantly lower MMP-2 due most probably to the significantly higher TIMP-2 levels detected. The MMP-2, MMP-3, and TIMP-2 polymorphisms did not affect MMP-2, MMP-3, or TIMP-2 respectively, and the same applied with the NORs in all three MMPs (1,2,3) and TIMP-2. The three main groups studied (I, II, and III), although with markedly different profile and clinical outcome, did not differ in the frequency of their polymorphisms analyzed (Table 4). However, subgroup I analysis showed that patients with A/G polymorphism (MMP-1 –519) had lower MMP-1 levels compared with GG homozygotes, indicating a possible higher genetic risk for certain un-PORs.

CLINICAL SIGNIFICANCE

Identification of patients with poor ovarian response in a pretreatment environment would help improve their ongoing fertility plan and manage their expectations. Also by having the ability to investigate if one belongs to that group, it could provide important family planning information for the patient.

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