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Use of immunomodulators to treat endometriosis

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A B S T R A C T

Both animal and human studies have demonstrated that endometriosis involves numerous levels of immune dysfunction. From aberrant cytokine signaling to shifts in immune cell populations, it is clear that endometriosis develops in the setting of an elevated pro-inflammatory state. This elevated level of inflammation could exacerbate the morbidity seen in this chronic disease. Consequently, numerous immunomodulating therapies have been tested in both animal models and limited human trials. This review seeks to summarize the in vitro and in vivo studies used to test these agents for the treatment of endometriosis. These agents include small-molecule and antibody-based disease-modifying antirheumatic drugs (DMARDs), cytokines, mTOR inhibitors, nucleotide analogs, and various other small molecules. Although many of these agents have had promising results in in vitro and animal studies, few of them have been tested in humans. For the agents that were studied in women with endometriosis-associated pain, little benefit has been seen in symptom control to date. Nevertheless, there remains the potential that these agents may offer a new pathway in the treatment of the chronic, costly, and debilitating disease.

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Introduction

Endometriosis affects approximately 5–10% of reproductive-aged women [1]. This condition leads to not only significant loss of quality-of-life but also substantial economic costs to the patients [2,3]. One of the prevailing theories of the pathogenesis of endometriosis includes Sampson's theory of retrograde menstruation. However, more than 90% of reproductive-aged women experience retrograde menstruation, yet only a small fraction develop endometriosis [4]. Hence, there is either a factor that enhances implantation or a defect in clearing or eliminating these menstrual remnants. A defect is suspected in the clearance of menstrual remnants by the immune system, which may be part of the pathogenesis of endometriosis.

Abnormal immune cell activity has been observed in patients with endometriosis. This activity has been shown to involve both immune cell signaling and cellular function. Studies of peritoneal fluid and serum of patients with endometriosis have shown consistent elevation in several pro-inflammatory cytokines such IL-6, TNF-alpha, IL-8, and MCP-1 compared to those in patients without surgically confirmed endometriosis [5–7]. LPS stimulation of peritoneal macrophages of patients with endometriosis has also shown increased levels of IL-6, TNF-alpha, and lower levels of IL-10 than that in controls [8,9]. The increased cytokine milieu in patients with endometriosis indicates that the immune system is skewed toward a pro-inflammatory state in patients with endometriosis.

In addition to the enhanced pro-inflammatory cytokine production, there is significant evidence that endometriosis also involves aberrant immune cell activity. Numerous studies have shown polyclonal T and B cell activation, abnormalities in T and B cell function, increased immune cell apoptosis, and multi-tissue damage [10]. In addition, antibodies to a variety of phospholipids, polynucleotides, anti-endometrial, and anti-ovarian have been detected in patients with endometriosis [11–14]. Although no association has been found between endometriosis and certain autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, and Sjogren syndrome, a weak association has been found with inflammatory bowel disease [15,16]. There remains the question as to the exact underlying immune dysfunction, leading to the persistence of endometriosis. Currently, there is little understanding of which branch(s) of the immune system could be involved in this dysfunction. There is some evidence that the cytotoxic activity of NK cells is decreased [17]. Multiple studies have shown that immune surveillance is undermined and that the innate immune system appears to be unable to sufficiently react to endometriotic implants inside the peritoneum [18,19]. In addition to this difficulty in reacting to and destroying the endometriotic implants, these immune cells are still able to produce cytokines and other pro-inflammatory factors that can enhance the growth of endometriotic implants. This relationship suggests that immune suppression could play a role in not only suppressing the spread of endometriosis but possibly reversing the damage caused by the disease.

During the previous two decades, numerous immunomodulating agents have been tested for efficacy in the treatment of endometriosis. This review seeks to summarize the results of human and animal studies evaluating these immunomodulators.

Methods

A search was performed in PubMed, The Cochrane Library, and Ovid Medline. Phrases used in the search were suited for each individual database and included “immunodulators AND endometriosis”, “immune system AND endometriosis”, “DMARDs AND endometriosis”, “methotrexate AND endometriosis”, “hydroxychloroquine AND endometriosis”, “leflunomide AND endometriosis”, “anti-TNFalpha AND endometriosis”, “Tocilizumab AND endometriosis”, and “microRNA AND endometriosis. Our search period spanned from 1946 to 2019. A total of 249 articles were found. These articles were then assessed for relevance and quality by the above authors. Only studies published in English were included. Forty-eight of these papers were included as part of this review. A manual review of the references in each of the cited sources was performed to ensure that any relevant resource was not excluded.

The primary outcome of this review was to determine whether any immunomodulating therapy has been tested for endometriosis and which immunodulators are still being investigated as a possible

therapy. Markers for immunomodulatory efficacy included pain and quality of life (QoL) scores for patient studies. Measures of efficacy in animal models included lesion size and lesion number.

Articles were selected as relevant if they were 1) prospective or retrospective studies involving reproductive-aged women who were treated with immunomodulatory therapies, 2) in vitro or in vivo animal studies in which a model of endometriosis was used to assess the effect of immunomodulatory therapy. Studies were excluded if they 1) were case reports, systematic reviews, abstracts, or expert opinion articles, 2) did not include an analysis of patients who underwent immunomodulatory treatment, 3) included patients who underwent any gonadotoxic therapy such as chemotherapy, 4) included patients who had any pre-existing immunodeficiency.

Treatments

A spectrum of immunomodulatory therapies have been tested in patients with endometriosis [20]. These range from well-studied disease-modifying antirheumatic drugs (DMARDs) used to treat rheumatologic diseases, to cytokines, oligonucleotides, and a range of small molecules.

DMARDs

Leflunomide

Leflunomide is a small molecule approved for use since 1998 for rheumatoid arthritis. Its proposed mechanism of action relies on the inhibition of dihydroorotate dehydrogenase, which is essential for the production of uridine and the downstream production of DNA and RNA. Hence, it affects rapidly dividing cells such as lymphocytes [21]. Yang et al. tested this compound in a murine model of endometriosis, in which mice with transplanted endometriosis lesions underwent peritoneal injection of either saline or leflunomide. These mice showed a decrease in both the size of the endometriotic lesions and the levels of TNF-alpha [9].

Hydroxychloroquine

Hydroxychloroquine is a hydroxylated derivative of chloroquine, an antimalarial drug used to treat Sjogren's disease, rheumatoid arthritis, and systemic lupus erythematosus. The mechanism of action of this medication involves increase in the pH inside lysosomal vacuoles, which then alters protein degradation. It is thought that this leads to interference in antigen processing within antigen-presenting cells (APCs) [22,23]. This agent was tested in a mouse model of endometriosis by Ruiz et al. [24]. Repeated treatment with hydroxychloroquine led to a decreased number of endometriotic lesions compared to that in placebo (treatment with phosphate-buffered saline). In addition, in vitro assessment of samples with human endometriotic lesions exhibited decreased cell survival after treatment with hydroxychloroquine. Given the role of hydroxychloroquine in affecting lysosomal vacuoles and their role in autophagy, further biochemical assays in this study showed downregulation of mRNA and protein levels of autophagy-associated proteins such as ATG7 and beclin-1.

Biologics

Biologics are immunomodulatory drugs that are partially or completely based on the production of monoclonal antibodies. Both etanercept and infliximab have been studied as possible therapies for endometriosis. Etanercept is an artificial fusion protein comprising the extracellular, soluble p75 portion of the TNF-alpha receptor fused to the Fc portion of human immunoglobulin G1. This dual binding site renders etanercept 50- to 1000-fold more capability of neutralizing TNF activity in vitro than a monomeric soluble TNF receptor. It has been approved for the treatment of rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, and a range of local inflammatory conditions [25]. TNF-alpha has been noted to be increased within the peritoneal fluid of patients with endometriosis [26]. In addition, TNF-alpha levels in peritoneal fluid correspond to the severity of endometriosis [27]. The only

study to assess the use of etanercept in endometriosis was a blinded randomized, placebo-controlled trial by Barrier et al., which involved 12 baboons, of which eight were randomized to etanercept treatment and 4 treated with sterile water subcutaneously. Subjects treated with etanercept showed a decrease in the size of red lesions following 8 weeks of treatment. However, the number of the red lesions and the number of white and black lesions did not differ [28].

Infliximab is an IgG monoclonal anti-TNF-alpha antibody. In contrast to etanercept, this antibody has been tested in humans after promising results in nonhuman primates with peritoneal endometriosis; a proof-of-concept study demonstrated that TNF-α inhibitors prevent the development of endometriosis in baboons [29,30]. A placebo-controlled, randomized, controlled trial (RCT) carried out by Koninckx et al., which is the only human trial, involved 21 women with severe pelvic pain and a rectovaginal nodule >1 cm. Surgery was then performed three months following infusion of either infliximab or placebo. At the conclusion of the study, no difference was found in the level of dysmenorrhea, dyspareunia, and nonmenstrual pain [31].

The lack of infliximab efficacy in humans can possibly be explained by the fact that the clinical phenotype of women treated with infliximab (severe and deep endometriosis with fibrotic component and dense adhesions, resistant to previous medical/surgical treatment) (28) was completely different from the inflammatory peritoneal endometriosis induced in baboons [29]. In that sense, the clinical study, although placebo controlled and well designed, was done in a very different patient population. All preclinical data showed a beneficial effect on superficial endometriotic lesions, and therefore, the first proof-of-concept clinical study, following preclinical research in rodents and nonhuman primates, should have included symptomatic patients with pain and significant inflammatory and superficial endometriosis, corresponding to ASRM endometriosis stages I, II, and III also including women with small ovarian endometriotic cysts and limited filmy adhesions, and excluding women with deep infiltrative endometriosis, dense adhesions, or large ovarian endometriotic cysts [32].

Cytokines

Interferons are proteins produced to counter pathogen invasion and replication and are approximately classified into two groups: Type 1 interferons such as INF-alpha and INF-beta, which are the best characterized, and Type II interferons such as INF-gamma, which are produced by T-cells [33,34]. Interferon alpha-2beta is the main molecule of this class, which has been studied in endometriosis. This form of interferon alpha has been shown to reduce fibroblast proliferation and reduce proliferation of various cancer cell lines in vitro. Ingelmo et al. used intraperitoneal (IP) injection of interferon alpha-2beta in a rat model of endometriosis in which even a single IP injection led to a 40% decrease in endometriotic implant size. While this decrease in implant size was sustained for up to 120 days after the first injection, the authors did notice a plateau effect on the decrease in implant size [35]. A subsequent study by Badawy et al. on exposure of endometrioma cells to increasing levels of interferon alph-2b in vitro led to a halt in cell proliferation with increasing levels of this interferon and reduced DNA proliferation based on tritylated thymidine uptake. However, cell proliferation resumed with cessation of interferon exposure [36]. This work was followed by another peritoneal endometriosis rat model study by Ingelmo et al. from 2013 looking at prolonged IP dosing of interferon alpha-2beta using 15 injections spaced out by 48 h versus short-term dosing of three injections spaced out by 48 h. Both treatment groups experienced a decrease in endometriotic implant size compared to that in controls. However, the prolonged treatment group had a greater portion of endometriotic cells resorbed compared to the short-term treatment or control group [37]. Ingelmo et al. [37] also looked at the effect of IL-2 in the treatment of a rat model of peritoneal endometriosis showing an increase in the levels of peritoneal NK cells, macrophages, and dendritic cells in addition to decreased endometrial implant size in the IL-2-treated group [38]. This was followed by a randomized trial of 24 human subjects conducted by the same group in 2005, in which patients with endometriomas underwent cyst drainage instillation of either a single dose of IL-2 or two instillations separated by one month. No significant improvement was noted with two instillations of IL-2 versus one; this lack of effect is not entirely surprising given the limited number of subjects [39]. These studies suggest a beneficial role of interferon alpha-2beta and IL-2 in the treatment of peritoneal endometriosis and possibly also ovarian endometriosis. However, further animal studies are necessary to determine whether the IP route

versus a more convenient route of administration such as intravenous infusion can produce a clinically relevant effect before any human studies.

mTOR inhibitors

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase involved in numerous signaling pathways such as apoptosis, transcription, translations, and ribosome biogenesis [40]. Its activity has been studied in the regulation of various types of cancer and have been shown to be associated with the occurrence of endometriosis [41]. In cancer cells, rapamycin has been shown to induce apoptosis, cell proliferation, and limit angiogenesis [42,43]. Leconte et al. showed that treatment of deep-infiltrating endometriosis cells with Temsirolimus (another inhibitor of mTOR) led to decreased cell proliferation both in vitro and in vivo in mice [44]. Consequently, Laschke et al. [45] used a hamster model of endometriosis placed into a dorsal skin fold in which the animals were randomized to treatment with daily dose of rapamycin versus DMSO. Subsequent assessment of the endometriotic lesions showed a decrease in lesion size, vascular bed size, endothelial cell outcomes, and VEGF level. Ren at al [46] used a model of peritoneal endometriosis in severe combined immune deficiency mice (SCID) to test the effect of rapamycin. After injecting endometriotic lesions once a week for two weeks with rapamycin, saline, or placebo, the authors noted a significant decrease in lesion volume, level of VEGF, and mean vessel density in the lesions. However, thus far, no studies have been carried out using mTOR inhibitors in women with endometriosis.

Miscellaneous

As previously described, there is increasing evidence of defects in cell-mediated immunity in endometriosis. Loxoribine is a guanosine analog. This drug class, as a group, has been shown to enhance NK cell activity, stimulate proliferation of B-cell activity, and consequent antibody-mediated cytotoxicity, in addition to stimulating macrophages and T-cell activity. Loxoribine itself has been shown to increase interleukin-1α, tumor necrosis factor-α, tumor necrosis factor-β, interleukin-6, interferon-α, and interferon-γ [47]. Levamisole is an antihelminthic drug and is used as an adjuvant in treating colorectal adenocarcinomas. Keenan et al. performed a study, in which rats were treated with IP injections of saline, loxoribine, or levamisol. Regression of both epithelial and stromal elements of the endometriotic implants was seen only with loxoribine ($p < 0.004$). In addition, loxoribine led to a significant elevation in dendritic cell infiltration ($p < 0.05$) and a decrease in NK cell levels within the endometriotic implants ($p < 0.05$) [48]. Xu et al. [49] addressed the issue of preventing endometriotic implant proliferation by limiting the effects of vascular endothelial growth factor (VEGF). They focused on the use of lipoxin A4 (LXA4), which is an endogenous eicosanoid that helps limit inflammation and block VEGF-mediated angiogenesis and also helps block matrix metalloproteinases (MMPs) [50]. In addition, the endometrium of rats with experimentally induced endometriosis and that in patients' samples exhibited higher levels of lipoxin receptors [51]. Consequently, Xu et al. [49] tested the effects of IP injection of lipoxin versus saline each day for three weeks. There was an overall decrease in lesion size as compared to controls (13.58 ± 4.01 mm in the LXA4 group and 23.20 ± 7.49 mm ($P = 0.0002$). In addition, they showed decreased levels of various pro-inflammatory cytokines such as IL-6 and IFN-gamma ($p < 0.005$), VEGF ($p = 0.0006$), and suppressed activity of MM9 ($p = 0.0005$).

Pentoxifylline is a small molecule that inhibits phagocytosis and the production of TNF-alpha and the actions of both TNF-alpha and IL-1. Several clinical trials have demonstrated the effect of this agent to reduce postoperative adhesion formation, address male-factor infertility, and enhance IVF fertilization rates with positive results [52,53]. Kamencic and Thiel [54] first attempted to assess the effect of PTX in endometriosis by randomizing patients to conservative surgery for endometriosis (CSE) versus CSE and PTX twice daily for three months after surgery. There was a lower visual analog scale (VAS) score in the PTX-treated group, which sustained for three months; the study was not blinded and did not account for any placebo effect. Three additional RCTs have been performed by groups in Iran and Spain looking at PTX following laparoscopy in patients with endometriosis and with or without infertility. None of these trials showed any

significant improvement in pain levels or pregnancy rates [55–57]. Subsequent work has focused on the effect of PTX in small mammals. Vlahos et al. used a rat model of endometriosis in which the animals were randomized to intraperitoneal injections of PTX. They showed a decreased in endometrial lesion size, lesion number, and VEGF and flt-1 levels [58]. A recent study from Spain looking at PTX treatment in nude mice showed a no significant decrease in lesion volume or number. However, the study did show a decreased in microvascularity based on CD31 staining. Interestingly, they did not show any difference in lesion cytokine levels, which may be explained by the T-cell deficiency present in these mice [59]. Despite numerous promising data in small mammal models on the effect of PTX on peritoneal endometriosis, a clear benefit has yet to be seen in human trials. Future studies should seek to isolate the effect of PTX in patients with endometriosis and without infertility.

Recently, statins have been tested as possible treatments for endometriosis [60–64]. Owing to their known anti-proliferative and anti-inflammatory effects, Taylor et al. tested statins in a baboon model of endometriosis [65]. Sixteen baboons were randomly assigned to simvastatin treatment or control group. Following three months of treatment, each baboon was evaluated by laparoscopy. Those treated with simvastatin showed a 78% reduction in red, orange-red, and white lesions. In addition, the authors showed decreased expression of proliferating cell nuclear antigen (PCNA) and estrogen receptor alpha in red lesions. Their findings suggest that blocking the mevalonate pathway may be a possible method for arresting the growth of endometrial lesions [65].

One unique form of treatment that has been tested in animal models is an adenovirus-induced immune-conjugate molecule called Icon, which targets tissue factor. Tissue factor is a receptor for Factor VII/VIIa, which is abnormally expressed in the endothelium of blood vessels supplying endometriotic lesions. Icon leads to destruction of any epithelium-containing tissue factor by inducing a natural killer (NK) cell cytolytic response. In an athymic mouse model of endometriosis, Icon led to the widespread destruction of endometriotic lesions without significant toxicity, infertility, or teratogenic effects [66]. Icon was then tested using an adenovirus vector in baboon by Hufnagel et al. [67] Fifteen baboons underwent surgical induction of endometriosis. Of the seven who were treated with Icon, all exhibited a significant decrease in red lesion size compared to the eight subjects in the control group. This provides an example of not only the utility of targeting tissue factor but also the use of adenoviral vectors as a method for endometriosis therapy.

MicroRNAs

Relatively recent additions to the class of agents seen as possible immunomodulators are microRNAs. MicroRNAs are 18–23 nucleotide (nt) fragments of RNA used to modulate gene expression [68]. Over the past several years, evidence has been accumulating implicating these RNA fragments in the pathogenesis of endometriosis. In addition, they have been found to be differentially expressed in the serum of patients with endometriosis [69–72]. These miRNAs, such as miR-155a, have been shown to influence cytokine levels and B and T cell function [73–75]. Our group looked at miR-125b-5p and let-7b-5p, which have been shown to be upregulated and downregulated in patients with severe endometriosis, respectively, in macrophage cytokine production. Transfection in macrophages of an miR-125 or Let-7b mimic led to the upregulation of TNF-alpha, IL-1-beta, IL-6, and IL-8. This correlated closely with macrophage cytokine production and with the results of sera of patients with endometriosis [76].

Future directions

New additions to our immunomodulatory repertoire remain to be tested. A new class of DMARDs, the Janus Kinase (Jak) inhibitors, has recently been studied in the treatment of rheumatic disease, and one of them, tofacitinib, is now approved for the treatment of rheumatoid arthritis resistant to other DMARDs. There is emerging preclinical evidence that kinase inhibitors may be relevant as immunomodulators in the treatment of endometriosis. In baboons with induced endometriosis, a treatment with the JNKI bentamapimod (60 days) for a short duration was as effective as treatment with JNKI combined with MPA or with the GnRH antagonist cetrorelix in reducing the surface and volume of

induced endometriosis in the baboon but without any significant effect on cycle length or on serum reproductive hormones [77]. These results confirm earlier data demonstrating that the JNKI bentamapimod caused regression of experimentally induced endometriosis in the nude mouse model for endometriosis as well as in the surgically induced model of endometriosis in the rat but require confirmation in human studies [78].

MicroRNAs, while having a clear linkage to the biology of endometriosis, are still in their infancy of testing. One major drawback of them is ensuring effective, specific, and safe delivery of the miRNAs to their target tissues. Recent phase I and phase II clinical trials have been attempted in patients with diseases as varied as Hepatitis C infection, various cancers, and scleroderma. Delivery systems have ranged from using locked nucleic acids, which mimic the antisense strand of a target microRNA, to encapsulating micrRNAs in liposomes or lipid nanoparticles [79].

Challenges in translation from preclinical research to clinical practice

There are still major translational challenges in drug development for endometriosis, with an urgent need for standardization of the design and reporting of preclinical endometriosis models, taking into account the following needs: unambiguous and transparent control measures to address the potential for investigational, observer or other bias, model standardization to enable replication and build to a consensus on clinical research priorities based on these observations, and incorporation of additional pharmacological end-points in all efficacy studies to confirm that the target activity is expressed and modulated in a desired, dose-dependent manner by the investigational agent under study [80]. Recently, a menstruating mouse model for laparoscopic induction of endometriosis has been developed and is being standardized [81–83]. In our opinion, the baboon model for endometriosis induction has been sufficiently characterized and validated by different research groups worldwide with approximately 100 papers thus far published in the international peer-reviewed literature [84,85]. From a purely scientific point of view, the baboon model (very close to humans in terms of reproductive physiology, endocrinology, anatomy, genetics, and similarity of both spontaneous and inducted endometriosis in baboons to human endometriosis) is superior to any rodent model for endometriosis and is at present the best preclinical model for efficacy studies on novel therapeutic agents for endometriosis [32]. Nevertheless, rodent models are often preferred for preclinical research in the Western world because of ethical and/or economic reasons. Although the importance of these ethical and economic reasons should not be minimized, it is important to realize that different ethical opinions and economic realities exist outside of Europe/North America and that preclinical research in nonhuman primate models may be undertaken outside the Western world in AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International)-accredited facilities in Asia, Africa, or South America.

Conclusions

Immunomodulation remains a tantalizing avenue for the treatment of endometriosis. Numerous small molecules and antibodies have already been tested with promising results in animal models. However, there have been major problems in translating the results from preclinical animal studies to clinical studies. This could be solved by resolving issues related to the safety of new nonhormonal treatment modalities, by expanded testing of new treatments in the baboon model. Data from these studies could then be used to design Phase I clinical trials to assess safety of any immunomodulating agent. An essential part of this design is careful selection of women with pelvic pain and laparoscopically confirmed inflammatory peritoneal endometriosis but excluding women with deep endometriosis, fibrotic disease, or large ovarian endometriotic cysts. Rather than large randomized trials, Phase II proof-of-concept studies should be done in limited placebo-controlled fashion, i.e., in 50 women. Only if these trials show a clear trend toward effectiveness, larger, multi-institution RCTs with adequate blinding can be considered to further determine the efficacy of these immunomodulatory agents in the treatment of endometriosis. This approach will reduce the risk of all parties involved in developing these potentially effective medications and harnessing them to treat this profoundly debilitating disease.

Conflict of interest statement

Thomas D'Hooghe, MD, has been Vice President and Head of Global Medical Affairs Fertility, Research and Development, Merck KGaA, Darmstadt, Germany, since October 2015. His participation in this publication is part of his academic work. There is no conflict of interest, as Merck KGaA is not involved.

Professor Hugh Taylor has been a consultant to DotLab, AbbVie, Bayer, and ObSeva.

Alexander Kotlyar has no conflicts of interest.

Practice point

- Despite extensive preclinical research in in vitro and in animal models, immunomodulatory drugs for endometriosis treatment are not available at present.

Research agenda

- Promising immunomodulatory drugs should be tested in the baboon model with induced peritoneal endometriosis shortly after proof of concept has been established in rodent models
- After immunomodulatory drugs have shown a reduction in peritoneal endometriosis in the baboon model, a pilot placebo-controlled study should be considered in women with pelvic pain and laparoscopically confirmed inflammatory peritoneal endometriosis but excluding women with deep endometriosis, fibrotic disease, or large ovarian endometriotic cysts, as this latter group is less likely to respond to immunomodulatory treatment.

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