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Atherosclerosis, Oxidation and Endometriosis*

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Endometriosis affects younger women of childbearing age. Atherosclerosis is considered as a disease of the old and increases with the ageing process. Both diseases are characterized by the increased presence of activated macrophages and associated increases in growth promoting activity and the production of inflammatory cytokines. In this review, we propose that oxidative stress and the presence of forms of oxidized low-density lipoprotein (LDL) might contribute to both Atherosclerosis and Endometriosis.

Keywords: Endometriosis; Atherosclerosis; Low-density lipoprotein; Peritoneal cavity

Endometriosis is defined as the presence of endometrial glands and stroma outside the endometrial cavity. Endometriosis has been noted in over 20% of patients undergoing gynecologic laparotomies. The pathophysiology of this disease still remains elusive. The presence of menstrual blood in the peritoneal cavity (PC) is a common occurrence and retrograde menstruation has been suggested to be the source of endometrial tissue in the PC.[1,2] Epithelial cells of menstrual origin have been noted in the PC[3,4] and endometrial cells have been cultured from the peritoneal fluid PF.[5] The presence of menstrual blood in the PC is not restricted to women with endometriosis. Thus it is unlikely that the mere presence of menstrual blood or endometrial cells contribute to an antigenic response or to endometriosis.

MACROPHAGES RECRUITED IN THE PC

The presence of macrophages in the PC of rodents is well known. Their presence is assumed to be a normal physiological occurrence. Usually about 7% of monocytes in circulation are constantly drained, though not exclusively into the PC. The introduction of irritants such as thioglycollate, talc, bacteria and others result in a greater recruitment of these cells into the PC. The presence of macrophages in human PC however, is commonly associated with an inflammatory process. For example, the presence of pathogens or the growth of tumors in the PC is often accompanied by an increase in the presence of macrophages in the ascites fluid.[6,7] The well-documented increased presence of macrophages in women suffering from endometriosis[8–10] is unexpected, as no pathogens have been implicated in the disease. Obviously the ectopic endometrium itself represents an alien presence that results in the influx of monocytes into the PC. Macrophages in the PC can serve a variety of functions. The immunological association of macrophages and T-lymphocytes may demand that macrophages process the foreign invading endometrial cells and present suitable antigenic targets for the generation of antibodies. A corollary to the immune theory of endometriosis is the obligatory recognition, uptake and processing of endometrial cells by macrophages. In other words, the scavenger function of macrophages may be synonymous with their immunological role. However, the *a priori* assumption that

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"normal" endometrial cells may be recognized by macrophages in the PC as an alien entity is not supported by the observation that macrophages are normal constituents of the endometrium.[11,12] Macrophages and endometrial cells coexist in the endometrium and there are cyclic variations in the presence and number of macrophages in the normal uterus.

The increased number of PC macrophages in women with endometriosis may indicate that for some reason the presence of endometrial tissue in the PC represents a foreign entity and needs to be removed. A number of studies have documented that normal women have fewer mononuclear cells in the PF[9,10,13] and most of these cells may represent a less differentiated monocytic phenotype. In contrast, cells in the PF of endometriosis subjects are more differentiated macrophages. The PC macrophages are non-adherent and can be easily lavaged. The increased number of mononuclear cells in the PF of endometriosis subjects may be due to increased chemotactic activity of the PF.[14] A number of studies have documented an increase in specific chemotactic agents such as monocyte chemotactic protein-1 (MCP-1),[15,16] Rantes,[17,18] and lyso phosphatidylcholine (lyso PC) in the PF of endometriosis subjects.[19] Conversely, the decreased lavageble mononuclear cells in the PC of normal women could be due to their increased efflux from the PC or increased presence of adherent cells.

To our knowledge no one has ever speculated the basis for altered immunogenicity or the need for removal of the endometrial cells in the PC. A number of possibilities exist for endometrial cells in the PC to represent a foreign entity resulting in the recruitment of monocytes, which differentiate into scavenger macrophages, into the PC of endometriosis subjects. These include: (a) the very presence of the endometrial tissue outside of the uterine endometrium; (b) changes in the cellularity (undigested tissue pieces as opposed to individual cells); and (c) alterations in cell phenotype (apoptotic endometrial cells and "damaged" red blood cells) may represent a foreign presence and account for their chemotactic recruitment. For example, it is suggested that the endometrial cells become apoptotic before menstruation as a result of increased TNF-α action. Yet another possibility, that these cells are normal when they arrived at the PC but are somehow altered in the PC to elicit a monocyte chemotactic response should also be considered. The possibility that acellular components in the menstrual blood, for example, the menstrual blood plasma may contain increased amounts of chemokines or might be capable of generating chemotactic activity for monocytes also can't be ignored.

ARE THERE ALTERATIONS IN THE ENDOMETRIAL CELLS THAT ARE PRESENT IN THE PC?

Substantial quantities of endometrial cells are shed with blood during menstruation. Very little is known regarding the nature of these cells in menstrual blood or in the peritoneal milieu. The uterine endometrium undergoes considerable cyclic variations during the monthly cycle and the endometrial cells are destined for a programmed death at the end of the cycle. During the late proliferative and menstrual phase of the cycle TNF-α and its receptor activity go up and the expression of Bcl-2, a marker gene for protection against apoptosis, is reduced. More apoptotic endometrial cells appear at this phase.[20,21] Digestion of the extracellular matrix and disruption of blood supply to the endometrium would isolate the tissue from the uterus thus depriving the cells of nutrients. Whether the apoptosis is augmented as a result of this nutritional deprivation process and whether the endometrial cells that are drained into the PC include a substantial proportion of apoptotic cells is not known.

MACROPHAGES AS SCAVENGERS OF ENDOMETRIAL COMPONENTS IN THE PC

Even if apoptotic endometrial cells are present in menstrual blood of women with endometriosis, why are they not cleared by the macrophage scavenger receptor(s)? Is it possible that the peritoneal macrophages from women with endometriosis do not express fully functional scavenger receptors?

Suggestion for the potential altered scavenger function in the macrophages of the PF may come from the studies of Hughes *et al.*[22,23] They demonstrated that scavenger receptor may also play a role in cellular adhesion process and an antibody to the scavenger receptor also blocked the adhesion of cells to culture dishes. Accordingly, macrophages that are not adherent and are not attached to extracellular matrix in tissues do not express scavenger receptors.[24] Thus, in contrast to tissue macrophages, the PF macrophages that are not attached to extracellular matrix components, despite their differentiated status may not be competent scavengers.[24] A decreased expression of several integrins, $\alpha_V\beta_3$ in particular, has been noted in the endometrium of women with endometriosis.[25] Whether such low expression is an inherent defect in women with endometriosis is an important question that has to be addressed.

DO MACROPHAGES PLAY A ROLE IN THE ECTOPIC GROWTH OF ENDOMETRIAL CELLS?

Both monocytes and macrophages stimulate the growth of endometrial cells[26,27] by mechanism(s) not known. It has been noted that peripheral blood monocytes from women with endometriosis may support growth of endometrial cells whereas cells from control subjects failed to support endometrial growth.[27] This finding would suggest that there might be factors in the peripheral blood of endometriosis subjects that activate the synthesis of growth factors. In other words, the monocytes from these subjects are already primed to generate growth-promoting activity. The identity of such activity or the factors that control the expression of such activity is not known at the present.

THE PF OF ENDOMETRIOSIS SUBJECTS

The PF of endometriosis subjects has been studied with great interest for a long time, and as a result, a plethora of information is available on the presence and levels of hormones, prostanoids, cytokines, growth factors, chemokines, and various biochemical parameters. There is a long list of components that are present at higher or lower levels in the PF of endometriosis subjects and as a result, some type of association is suggested between their presence and the disease process itself. These components include, prostaglandin products, IL-1, IL-6, glycodelin, MCP-1, Rantes, TNF-α, IFN-γ, M-CSF, various growth-promoting activities, iron, enzymes, hormones, and others.[28–30] The reason these components are elevated or decreased in the PF is not understood. For example, why MCP-1 or M-CSF genes are induced has not been studied.

IS THERE A CONNECTION BETWEEN ENDOMETRIOSIS AND ATHEROSCLEROSIS?

Endometriosis affects younger women whereas atherosclerosis is a disease of "old age". In fact, younger women are protected from coronary artery disease. These two diseases though seemingly unrelated, are remarkably alike. First, both have tissue macrophages that express scavenger receptors and these macrophages are exposed to lipoproteins. More importantly, both atherosclerotic lesions and the peritoneal fluid of women with endometriosis are characterized by the presence of inflammatory cytokines, chemokines and growth factors. For example, Lipoproteins [low-density lipoprotein (LDL), high density lipoprotein HDL], Macrophages and cytokines derived from macrophages, T-cells and cytokines derived from T-cells, chemotactic factors for T-cells and macrophages, growth factors and mildly oxidized LDL (m-LDL) have been noted both in the atherosclerotic lesion and in the PF of women with endometriosis. More importantly, these are suggested to play similar roles, e.g. recruitment of monocytes, growth promotion, and generation of localized inflammation, at these sites.

LIPOPROTEINS IN THE PERITONEAL FLUID

The PF is derived from follicular fluid and secretions from the cells of fallopian tubes, uterus, and the peritoneal wall. Follicular fluid lipoproteins have been well studied.[31,32] Follicular fluid is rich in HDL and very little, if any, LDL and VLDL are present. Liver and intestine are the major lipoprotein producing organs and it is very unlikely that the cells of the uterus, fallopian tubes, or those of the PC generate VLDL and LDL. We recently observed that the PF of endometriosis subjects contain substantial quantities of LDL, suggesting the infiltration of plasma components into the PC, thus mimicking components that are present in inflammatory, interstitial fluid. Very little, if any, LDL was present in the PF of control subjects. The PF lipoproteins are distinct from the plasma in having several major nutrients depleted during their passage through the endothelial cell barrier of the blood vessels. Besides, large lipoproteins (VLDL and chylomicrons) cellular components of blood do not cross the endothelial barrier.

OXIDIZED LDL

The importance of oxidative modifications of LDL (Ox-LDL) in the pathogenesis of atherosclerosis is now well recognized[33] and treatments based on antioxidant properties are already in clinical trials. The general properties of Ox-LDL have been reviewed in a number of publications and we will briefly review information pertaining to the current review. Its detection primarily involves marginal increases in electrophoretic mobility and bioassay for the induction of MCP-1 and M-CSF genes, and the chemical identification of peroxidized phospholipids.

OX-LDL AND MACROPHAGES

The presence of increased number of monocyte/macrophages in the PF of endometriosis subjects as compared to normal women has been well documented.[34] There is a growing body of evidence suggesting that the presence of activated macrophages and LDL together, may lead to oxidation.[35]

Specific enzymatic oxidations of lipoproteins by 15-lipoxygenase and myeloperoxidase, either by direct interactions or by seeding mechanisms have been suggested.[36,37] Monocytes, which are precursors of macrophages do not posses 15-lipoxygenase and upon differentiation into macrophages readily express this enzyme. Cytokines such as IL-4 and IL-13 activate this enzyme and the presence of this enzyme activity has been observed only in mouse peritoneal and tissue macrophages.[38] We reported the presence of this enzyme in mouse peritoneal macrophages[39] suggesting that PF macrophages, at least in this animal, is present in an already differentiated form. Two important diseases, atherosclerosis and endometriosis, with no obvious connections to each other represent conditions that would place activated macrophages and lipoproteins together. In both diseases, the commonalties include recruitment and retention of monocyte/macrophages, differentiation and growth of monocytes and smooth muscle cells (or endometrial cells), activation of inflammatory genes, and cytotoxicity.[40]

IS THERE ANY EVIDENCE TO SUGGEST THAT THE FACTORS THAT MAY BE INVOLVED IN ENDOMETRIOSIS MAY BE INDUCED BY AN OXIDATIVE STRESS?

A number of components have been identified to be present at increased levels in the PF of endometriosis subjects. Some of these may or may not play a role in endometriosis. As mentioned earlier, the factors that influence and induce the expression of the genes for these proteins in the PF of endometriosis subjects are not known. However, these genes have been well studied in other diseases and it has now been recognized that oxidants may be responsible for the induction of these genes. Many of them have NFκB or AP-1 response elements in their genes, which are known to be associated with oxidative stress.[40,41] In Table I, we present suggestions from literature that would suggest that oxidized lipids and lipoproteins might enhance the formation of some of these components.

IS THE PERITONEAL ENVIRONMENT IN THE PATIENT WITH ENDOMETRIOSIS CONDUCIVE TO AN OXIDATIVE PRO-INFLAMMATORY STATUS?

The presence of oxidized lipids and oxidatively modified proteins has been demonstrated in the atherosclerotic artery wherein macrophages and LDL interact to generate the foam cells of the fatty streak lesion. Studies by Ball *et al*.[35] have also shown that the mere presence of macrophages with lipoproteins is sufficient to generate oxidized lipids and ceroid-like lipid–protein complexes. The PF is rich in lipoproteins, particularly in LDL. Our preliminary results show that the concentration of LDL can be as much as 40% of its plasma concentration. In fact, if one compares it to arterial LDL concentration, it can be several folds higher! Considering the co-occurrence of activated macrophages and LDL in the PF it is likely that oxidation is a major event in the peritoneum. Further more, the PF-LDL may be already depleted of its antioxidants during its passage through the capillary endothelium thus becoming increasingly susceptible to lipid peroxidation.

IS THERE ANY EVIDENCE TO SUGGEST THAT PF LIPOPROTEINS MAY REPRESENT AN OXIDIZED LIPOPROTEIN?

Raymond *et al.* have used a sponge implantation model to document the "oxidized nature" of the sponge-associated lipoproteins.[42] In this model

TABLE I Can oxidized lipids/lipoproteins induce components associated with endometriosis?

	Activators	
Component	Non-oxidant	Oxidant
MCP-1	IL-1, TNF-α, LPS	Oxidized phospholipids of mildly Ox-LDL
CSF-1, M-CSF	IL-1, TNF-α	Oxidized phospholipids of mildly Ox-LDL
IL-1	LPS	Lipid peroxides, products derived from lipid peroxides, Ox-LDL, Oxidatively modified proteins
RANTES	IFN-γ, TNF-α	Contains NFκB activated domains suggesting potential activation by oxidants
IL-6	Glycodelin	Oxidized lipids
PDGF, FGF		Ox-LDL, Lyso PC
Prostaglandin's	LPS, Innumerable cell activators	Innumerable cross-reactive PG like products Ox-LDL

a sponge was implanted subcutaneously and after some time was taken out and squeezed to obtain the "inflammatory interstitial fluid." Lipoproteins of the atherosclerotic lesion have been well studied and characterized.[43] Studies from Hoff *et al.* and from others have provided evidence to suggest that LDL isolated from the interstitial fluid of lesion is different from plasma LDL.[44] The lesion LDL showed characteristics of Ox-LDL, showed evidence of aggregation and increased electrophoretic mobility, and cross-reacted with antibodies generated against Ox-LDL. Immunohistochemical evidence also has provided evidence documenting that modified lipid-protein adducts resembling those in Ox-LDL are present in the atherosclerotic artery and may be localized near the macrophage.[45] Metal ions significantly enhance lipid peroxidation.[46] Increased amounts of iron have been noted in the PF of women with endometriosis perhaps indicative of the release from macrophages that took up damaged red blood cells.[47] The presence of increased amounts of iron in endometriotic tissue (with the characteristic rustic appearance) has been known for a long time. Despite these lines of indirect, circumstantial evidence, there has been no direct evidence for the occurrence of lipid peroxidation in the PF. In fact, the TBARS assay (a test for the terminal, water-soluble degradation products of lipid peroxidation) failed to find any differences in their amounts in the PF of normal and endometriosis subjects. This could be due to rapid removal of these products from the peritoneum or the poor sensitivity of the assay or more importantly the presence of not extensively Ox-LDL, but mildly Ox-LDL in the PF. Such LDL does not possess increased levels of malondialdehyde MDA or F_2-isoprostanes. This review is the first to suggest and evaluate more complex products of lipid peroxidation and its potential as a causative factor in the generation of the pro-inflammatory environment of the PC of women with endometriosis.

It has been hypothesized that growth promoting substances in peritoneal fluid may enhance the ectopic growth of endometrial cells.[48,49] However, little data is known about which factors within the peritoneal fluid compartment promote endometrial cell proliferation, which is presumed to be the essence of disease initiation and progression. Hammond *et al.* showed an increase in endometrial stromal cell proliferation with the addition of cell free peritoneal fluid of endometriosis patients when compared to fertile controls with similar peritoneal fluid estradiol concentration.[50] Halme *et al.* have shown that conditioned media of activated macrophages of women with endometriosis induces a significantly greater degree of fibroblast growth than fertile controls or infertile patients with tubal abnormalities.[51] This "macrophage derived growth factors" has been shown to be distinct from IL-1 and may well be composed, at least partially, of platelet derived growth factor (PDGF) and fibroblast growth factor (FGF). Furthermore, Olive has shown that macrophage conditioned media from patients with endometriosis appears to function as a competence growth factor.[26] A competence factor confers on the cell the ability to respond to specific mitogens and a progression factor causes a "competent" cell to progress through the G_1 phase to the S phase of cell growth. Macrophage derived growth factors stimulate stromal growth in the presence of a known progression factor (insulin) in serum-free media but cannot promote growth with insulin-free serum.[52]

Products of lipid peroxidation have profound effects on cell growth. Recent studies have shown that lipid peroxides induce cellular oncogene expression and promote protein phosphorylation reactions involved in cell signaling and growth.[53] Ox-LDL promotes smooth muscle cell proliferation at low concentrations presumably as a result of induction of FGF-2 gene. The biochemical mechanisms involved in Ox-LDL mediated proliferation of smooth muscle may involve the presence of lyso PC. Both m-LDL and Ox-LDL have increased levels of lyso PC. The incubation of aortic endothelial cells with Ox-LDL results in a seven-fold increase in the secretion of GM-CSF and a several fold increase in mRNA and protein levels of basic FGF.[54] The induction of the latter has also been attributed to the presence of lyso PC. The role of lipid peroxidation products in promoting proliferation of endometrial cells is unexplored.

While the above discussion presents an intriguing picture, the question whether promotion of macrophage adhesion and prevention of inflammatory response would alleviate pain and infertility associated with endometriosis, cannot be answered easily. More basic research into the factors that prevent macrophage adhesion and endometrial cell growth is needed to generate criteria that can be used to further test the hypothesis.

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