Peritoneal macrophage depletion by liposomal bisphosphonate attenuates endometriosis in the rat model

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Background: Activation of macrophages is central to the implantation of endometriosis (EM). We examined the hypothesis that macrophage depletion by intraperitoneal (IP) injection of liposomal alendronate (LA) could result in EM attenuation in a rat model, thus supporting the notion of the pivotal role of macrophages in EM pathology.

Methods: In this study, 90 rats were subjected to an EM model and were divided randomly into seven groups: five groups were treated by 4 x once-weekly IP injections of LA (0.02, 0.1, 1, 5 or 10 mg/kg) and the other two groups received saline injections (control) or empty liposomes. Sham-operated rats also received empty liposomes. Depletion of circulating monocytes was determined by flow cytometry analyzes of blood specimens. Four weeks after the initial surgery, the number, size and weight of implants were recorded, adhesions were graded, macrophage infiltration was assessed and the peritoneal fluid was analyzed for monocyte chemotactic protein 1 (MCP-1) and tumor necrosis factor alpha (TNFα).

Results: Monocyte depletion following IP LA administration resulted in an inhibitory effect on the initiation and growth of EM implants, as expressed by implantation rate, adhesion scoring, implants' size and weight (>0.1 mg/kg LA, P < 0.05). Reduced numbers of infiltrating macrophages were observed in implants of the 1 mg/kg LA group. Peritoneal fluid MCP-1 levels were negatively correlated with LA dose (P < 0.001), whereas no significant correlation could be found for TNFα.

Conclusions: Macrophage depletion using IP LA has been shown to effectively inhibit the initiation and growth of EM implants, in a rat EM model. The clear dose–response effect may be viewed as a confirmation of the validity of the concept and encourages further study.

Key words: endometriosis / rat model / liposomal alendronate / macrophages

Introduction

The prerequisites for endometriosis (EM) formation include retrograde menstruation into the peritoneal cavity, inability of available mechanisms to deal with the refluxed cells and subsequent activation of cellular and humoral inflammatory processes leading to attachment, persistence and progression of implanted cells to active EM tissue (Vinatier et al., 1996; Lebovic et al., 2001, 2004). The immunobiology of EM is extremely complex and as yet not fully understood. The initial survival of ectopic endometrial tissue is dependent on a number of factors. These include defective immunosurveillance of peritoneal scavenger mechanisms (Izumiya et al., 2003), development of endometrial cell resistance to...
apoptosis (Dmowski et al., 1998; Braun et al., 2002), suppression of natural killer cell capability (Furuya et al., 2003), activation of macrophages by abnormal peritoneal cell production of chemotactic and angiogenic cytokines (Keenan et al., 1995; Braun et al., 1996; McLaren et al., 1996; Akoum et al., 2002; Bruner-Tran et al., 2002; Lebovic et al., 2004) and promotion of angiogenesis and lesion growth by dendritic cells (Fainaru et al., 2008). Recent research suggests that defective immunosurveillance in women who are destined to develop EM may support the attachment, persistence and progression of ectopic endometrial tissue in these women (Lebovic et al., 2001; Dmowski and Braun, 2004).

Activated macrophages perpetuate the immune dysfunction by secreting lymphocyte activating factors, cytokines and angiogenic factors which encourage macrophage activation, endometrial stromal proliferation and vascularization (Keenan et al., 1995; Braun et al., 1996; McLaren et al., 1996; Senturk and Arici, 1999; Akoum et al., 2002; Bruner-Tran et al., 2002; Lebovic et al., 2004; Lin et al., 2006). Not less importantly, EM implants interfere with fertility by enhancing other cytokines (Fakih et al., 1987; Verghese et al., 1995; Bruner-Tran et al., 2002; Sharpe-Timms, 2002; Cao et al., 2004; Liu and Lv, 2004), such as interleukin (IL)-1β and tumor necrosis factor alpha (TNFα) which hinder uterine and embryonic function. The macrophage, therefore, is pivotal in initiating and maintaining EM. However, to date, there are no specific immunomodulatory drugs or regimens that are specific or selective in their effect on macrophages or that have any impact on the clinical practice of EM.

We hypothesized that EM can be attenuated by depleting peritoneal macrophages. Depletion of macrophages and circulating monocytes in vivo can be achieved by particulated delivery systems (liposomes or polymeric nanoparticles) enabling intracellular delivery of bisphosphonates (BP), which inactivate and kill these cells following effective phagocytosis, while being non-toxic to non-phagocytic cells (van Rooijen and Sanders, 1994; Danenberg et al., 2002; Danenberg et al., 2003a,b; Cohen-Sela et al., 2006a; Epstein et al., 2007). The BP are a family of drugs of bone-seeking agents utilized clinically in calcium-related disorders such as osteoporosis and tumor-induced bone osteolysis (Lin, 1996; Rogers et al., 2000). Free BP do not permeate cells and have a short half-life in the systemic circulation. The nanoparticulated delivery system is formulated to be of suitable size and charge to enable safe delivery after in vivo administration, while ensuring subsequent intracellular delivery of the drug exclusively to endocytosing cells (Danenberg et al., 2002; Danenberg et al., 2003a,b; Cohen-Sela et al., 2006a; Epstein et al., 2007).

Previous studies in several animal models have established the biological validity of the concept of transient macrophage depletion by exposure to liposomal BP. Both circulating monocytes and infiltration of macrophages after tissue injury are significantly suppressed after treatment with liposomal BP, in vascular injury models in rats and rabbits (Danenberg et al., 2002, 2003a,b; Cohen-Sela et al., 2006a,b; Epstein et al., 2007).

In this work, we examine the hypothesis that macrophage depletion by intraperitoneal (IP) injection of liposomal alendronate (LA) could result in EM attenuation in a rat model, thus supporting the notion of the pivotal role of macrophages in EM pathology.

**Materials and Methods**

**Preparation of LA**

LA was prepared by a modified thin film hydration method as described previously (Epstein et al., 2007). Phospholipids and cholesterol (DSPC:DSPG:CHOL, 3:1:2 molar ratio) were dissolved in tert-butanol and lyophilized to produce a cake. An aqueous solution of alendronate (200 mM) was added to the cake; the liposomes thus obtained were homogenized to <200 nm by means of an extruder. To remove non-encapsulated drug, the liposomes were passed through a Sephadex G-50 column and were eluted with MES/HEPES buffer pH 7.2 (50 mM MES, 50 mM HEPES, 75 mM NaCl). The formulation volume was adjusted to 8.0 ml. Drug-free liposomes were prepared by the same procedure omitting the drug. Drug and lipid concentrations were determined as described previously (Epstein et al., 2007).

**Rat EM model**

Animal care and procedures were in accordance with the standards for care and the use of laboratory animals of the Hebrew University of Jerusalem conforming to NIH regulations. In total, 90 adult (8–10 weeks old) female Sabra strain rats (Harlan, Israel) were subjected to an EM model in four sessions. All animals had a 4 day estrous cycle documented before initiation of the experiment, were housed in an environmentally controlled area and maintained with water and rat chow ad libitum.

A mid-ventral incision was performed aseptically under anesthesia by an IP injection of ketamine (80 mg/kg, Fort Dodge Animal Health, USA) and xylazine (5 mg/kg, V.M.D. NV, Belgium) solutions. Resection of one uterine horn was achieved after ligation at the utero-tubal junction and the utero-cervical junction. The excised horn was immersed in sterile medium and the endometrium was exposed by lengthwise incision, and six squares of 4 mm² (2 x 2 mm) of open uterus were prepared; these were sutured endometrial side-out to the mesentry in each rat by 5/0 nylon suture under a dissecting microscope, with constant saline irrigation. Three additional rats were subjected to a sham operation, which included identical anesthesia, laparotomy and resection of the uterus, whereas six nylon sutures were affixed to the mesentry with no uterine squares.

**LA effects on EM**

After the operations, the rats were divided randomly into five treatment groups (10, 5, 1, 0.1 or 0.02 mg/kg per injection) or two control groups (treated with saline or empty liposomes). Treatment with IP injections started on Day 0 (immediately after surgery) and was repeated with an additional 3 injections after 7, 14 and 21 days (a total of 4 x once-weekly injections). Each experimental session included one control group and 2–5 treatment groups of 3–6 animals per group. The sham-operated rats were treated with empty liposomes.

At 4 weeks after the initial surgery (1 week after the last IP injection), the rats were sacrificed by anesthetic overdose. All analyses were done by an investigator blinded to the experimental group. The number of implants was recorded, the size of the implants was measured in two diameters by calipers and the implants were dissected out of surrounding tissue and weighed on an analytical scale. The implantation rate was calculated (number of implants on sacrifice/number of implants induced), and adhesions were independently graded by a 0–10 scoring, by two blinded observers: 0, no adhesions, no fibrin; 1–2, slight adhesions, little fibrin, hardly identifiable; 3–4, slight-moderate adhesions, some fibrin around implants, lysis hardly necessary; 5, moderate adhesions, some fibrin, adhesions around implants, lysis relatively easy; 6–7, moderately severe adhesions and fibrin mostly around implants but also between intestinal loops, lysis of adhesions difficult; 8–9, severe adhesions, much fibrin mostly between intestinal loops, lysis of adhesions very difficult; and 10, severe adhesions between intestinal loops, around the mesentery and abdominal wall, much fibrin and no ability to lyse adhesions without damage to organ. The adhesion score was calculated as the average of the score given by each observer. Since no significant difference (P < 0.01) was found...
between the control groups (saline, n = 11, and empty liposomes injections, n = 7), the results were combined with one control group.

Histopathological sections and immunohistochemical staining of macrophages in the implants were undertaken in the control, 1 and 10 mg/kg groups. Formalin-fixed tissue sections were embedded in paraffin and stained with hematoxylin–eosin. Immunohistochemical staining was done using the avidin–biotin method with mouse anti-rat macrophage antigen ED1 (CD68, Serotec, UK), followed by counterstaining with goat anti-mouse Ig-biotin (Jackson Immunoresearch, USA). Slides incubated with the goat anti-mouse Ig-biotin without pretreatment with anti-ED1 antibody served as negative controls to rule out non-specific binding. The slides were then scored by two observers blinded to the treatment group, and the density of macrophage infiltration in two implants per rat was scored by counting stained macrophages in a high-power field and averaging scores of 10 fields per case. The score was expressed as the average number of stained macrophages counted per 800 background cells.

Peritoneal fluid was collected at the time of sacrifice from individual animals by rinsing the open peritoneal cavity before resection of the EM implants with 2 ml of saline; the collected fluid was centrifuged at 4 °C, and the cell-free supernatant was frozen at −20 °C. These samples were subsequently analyzed in duplicates by an investigator blinded to the experimental group, for concentrations of TNFα and monocyte chemotactic protein 1 (MCP-1) using commercially available ELISA kits (Bio-source International, CA, USA).

Flow cytometry (fluorescence-activated cell sorting, FACS) analyzes were performed by means of an FACSCan Flow cytometer (Becton Dickinson, USA) on blood specimens of an additional 10 female Sabra rats that were subjected to the EM model as above. Animals were randomly assigned to the treatment (1 mg/kg LA) or control (saline) group, which were injected on day 0, 7, 14 and 21. Blood specimens were taken prior to and 48 h after injections on: Day 0 (prior to EM surgery, baseline), 2, 14, 16, 21, 23 and 28 (sacrifice). No sampling was done in the second week following injury to allow recovery. At the specific time points, blood specimens (500 μl) were drawn from the retro orbital sinus by capillary tube under isoflurane anesthesia (Minrad International, USA) and collected in EDTA tubes (Vacutainer, BD, USA). Each blood sample (100 μl) was incubated for 30 min (4 °C, in the dark) with mouse anti-rat FITC-conjugated anti-CD3, and mouse anti-rat PE-conjugated anti-CD4 (BD). A red blood cell lysing solution (Erythrolyse, 1:20 dilution, AbD Serotec) was added to the mixture, which was incubated for additional 10 min. The residual cells were washed twice (>1500 rpm, 5 min, 4 °C) in the FACS medium (PBS, 1% BSA and 0.02% sodium azide) and were suspended in 1 ml FACS medium for flow cytometry. The population of white blood cells (WBC) was gated according to forward and side scattering. In order to distinguish monocytes from other WBC, the gated WBC were analyzed using anti-CD3 and anti-CD4 staining; the proportion of monocytes was determined by an investigator blinded to the experimental group, based on the expression of CD4 and lack of CD3 (CD3−CD4+).

Statistical analysis

Data are expressed as mean ± SD. Comparisons of implantation rate between the control and treatment groups were made with χ² and Fisher’s exact test. Comparisons of implant size and weight, adhesions score and cytokine activation between the control and treatment groups were made with ANOVA and Dunnett post hoc test analysis, after confirming normal distribution of the data by the Kolmogorov–Smirnov test (P > > 0.10, Graphpad, Instat software, USA). Correlation was determined by second-order polynomial trendline and the r² correlation coefficient (Excel software, Microsoft, USA). Comparison of blood monocyte results between the control and the treatment groups was made by the unpaired Student’s t-test after confirming normal distribution by the Kolmogorov–Smirnov test. Differences were termed statistically significant at P < 0.05.

Results

Rat EM model

In total, 67 rats were available for evaluation: four animals did not survive the anesthesia, and six animals died during the 4-week post-operative injection period (three animals died 12–18 h post-surgery, in the saline, 0.1 mg/kg and 1 mg/kg groups, respectively; one animal of the 1 mg/kg group died in the second week; and two animals of the 10 mg/kg group died in the third and fourth weeks). The deaths could be attributed to the surgery (initial insult, inadvertent perforation of the intestine during surgery, necrosis of the intestine from suturing near mesenteric blood vessels, bleeding due to inadvertent puncture of blood vessels not identified during surgery) or due to the subsequent iP injections (inadvertent perforation of the intestine) or to drug toxicity. It is unlikely that the death of animals was due to the treatment, rather than to the procedure, since it was random among the various groups and the numbers were small.

Macro- and histopathology demonstrated typical features of EM (Figs 1 and 2). Four weeks after initiation of the EM model (Fig. 1, left), the implants retrieved were cystic, and some had brown-bloody or sero-sanguinous fluid (Fig. 1, right). Photomicrographs of typical endometriotic implants resected 4 weeks after initial EM-inducing surgery can be seen in Fig. 2. Cystic dilatation of endometriotic gland, surrounded by characteristic endometriotic spindle cell stroma and stroma infiltrated by chronic inflammatory cells, including lymphocytes and macrophages were observed (Fig. 2a and b). In addition, endometriotic implants showing deposition within the stroma of hemosiderin pigment, a classical hallmark of EM, were also observed (Fig. 2c). Mesothelial cells, lymphocytes, neutrophils and macrophages were observed in peritoneal fluid cytology (Fig. 2d). The rat endometriosis model was characterized by adhesions, a mean adhesion score of 7.1 ± 2.2 (scale of 0–10) was observed in the control group (Table I).
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**LA effects on EM**

**Implantation rate, implant size and weight and adhesions**

The detailed data obtained after harvesting the implants 28 days post-implantation are summarized in Table I. Only treatment with the high-dose alendronate liposomes (10 mg/kg) significantly reduced the implantation rate: 59.5% (25/42) and 96.2% (104/108) for the 10 mg/kg and control groups, respectively (P < 0.005, χ² with Fisher’s exact test).

A significant reduction in implant size, as expressed by mean implant cross-section, and implant weight was achieved by treatment with LA. The mean area (cross-section) of implants in the control group was 28.7 ± 9.5 (mm² ± SD), whereas the mean area in the treatment groups of 0.1 mg/kg and above was between 8.5 ± 9.1 and 13.2 ± 11.1 mm² (P < 0.01, Table I). As can be seen from Fig. 3, a dose–response effect was demonstrated for both implant size and weight. Implant size followed an exponential second-order polynomial trend line of \( y = 1.38 x^2 - 13.1 x + 39.3, \ r^2 = 0.888; \) and implant weight followed a line of \( y = 6.6 x^2 - 63.7 x + 171.7, \ r^2 = 0.927. \)

Adhesion scores were also correlated with the LA treatment. Mean adhesion score was 7.1 ± 2.2 (grade 0–10) in the control group in comparison to 5.5 ± 1.9 and 4.5 ± 3 in the 0.1 and 10 mg/kg groups, respectively (P < 0.05, for differences between treatment versus control, ANOVA and Dunnett post hoc test). Adhesion score correlated with drug dose with a second-order polynomial trend line of \( y = 0.1089 x^2 - 2.654 x + 8.21; \ r^2 = 0.9908 \) (Fig. 4).

**Cytokines and immunohistochemistry**

Peritoneal TNFα levels after treatment were significantly reduced in the high-dose group as opposed to all other dosages (0.5 versus 4.1–10.2 pg/ml, P < 0.01, ANOVA, Fig. 5). TNFα levels in the sham-operated animals were comparable with the levels found in the high-dose treatment group (10 mg/kg), 0.5 ± 1.0 and 0.91 ± 1.43 pg/ml, respectively. The dose–response effect for this cytokine was non-equivocal (\( r^2 = 0.26, \) Fig. 5).

Peritoneal MCP-1 levels showed a clear positive dose–response to the treatment with LA, from 22.3 ± 7.6 pg/ml in the control group increasing to 61.5 ± 25.8 pg/ml for the highest dose (Fig. 5; P < 0.001, ANOVA). This was also expressed as a correlation coefficient of 0.98 (P < 0.001). It should be noted that in sham-operated animals (n = 3), the concentration of TNFα and MCP-1 were 0.91 ± 1.43 and 26.2 ± 4.02 pg/ml, respectively. Multivariate analysis revealed that each individual rat’s peritoneal TNFα concentrations were significantly correlated to implant size (r = 0.24, \( F = 5.78, \ P = 0.02, \) ANOVA) and to the degree of macrophage infiltration in implants per each animal (see below): infiltration score and TNFα levels behaved similarly, to a significant degree, over all treatment groups (\( F = 23.5, \ P < 0.001, \) ANOVA). Conversely, MCP-1 concentrations in the peritoneal fluid were significantly negatively correlated with the degree of macrophage infiltration per subject, regardless of treatment group (r = −0.65, \( F = 18.1, \ P < 0.001, \) ANOVA).

Immunohistochemistry for the rat macrophage marker (Fig. 6) demonstrated significantly reduced numbers of macrophages infiltrating the endometriotic (implant) stroma in the low-dose (1 mg/kg) LA.

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**Table I The effect of LA dose on implantation, implants weight, cross-sectional area and adhesion score 28 days after implantation in the rat EM model (4 × once-weekly, IP injections)**

<table>
<thead>
<tr>
<th>LA dose (mg/kg)</th>
<th>n</th>
<th>Implanted/total attempts (%)</th>
<th>Mean implant cross-sectional area (mm²) (± SD)</th>
<th>Mean implant weight (mg) (± SD)</th>
<th>Mean adhesion score (0–10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>18</td>
<td>104/108 (96.2)</td>
<td>28.7 ± 9.5</td>
<td>116.5 ± 61</td>
<td>7.1 ± 2.2</td>
</tr>
<tr>
<td>0.02</td>
<td>8</td>
<td>45/48 (93.7)</td>
<td>18.3 ± 11.7</td>
<td>73.9 ± 48</td>
<td>6.0 ± 2.3</td>
</tr>
<tr>
<td>0.1</td>
<td>9</td>
<td>54/54 (100)</td>
<td>10.1 ± 6.5*</td>
<td>28.4 ± 18*</td>
<td>5.5 ± 1.9*</td>
</tr>
<tr>
<td>1.0</td>
<td>16</td>
<td>87/96 (90.6)</td>
<td>9.1 ± 8.7*</td>
<td>20.6 ± 18*</td>
<td>4.8 ± 2.0*</td>
</tr>
<tr>
<td>5.0</td>
<td>9</td>
<td>53/54 (98.1)</td>
<td>13.2 ± 11.1*</td>
<td>36.1 ± 22*</td>
<td>4.7 ± 1.0*</td>
</tr>
<tr>
<td>10.0</td>
<td>7</td>
<td>25/42* (59.5)</td>
<td>8.5 ± 9.1*</td>
<td>18.8 ± 20*</td>
<td>4.5 ± 3.0*</td>
</tr>
</tbody>
</table>

* Treatment versus control, ANOVA and Dunnett post hoc test; P < 0.05.
**10 mg/kg dose versus other dosages, χ² with Fisher’s exact test, P < 0.005.
The relationship between adhesion score (scale of 0–10) and LA dose (IP, 4× once-weekly) 28 days after implantation in the rat EM model (second-order polynomial trend-line).

The relationship between implant average cross-sectional area and weight to LA dose (IP, 4× once-weekly) 28 days after implantation in the rat EM model (second-order polynomial trend-lines).

The effect of LA (IP, 4× once-weekly) on cytokine activity in the peritoneal fluid 28 days after implantation in the rat EM model (second-order polynomial trend-line). In sham-operated animals (n = 3), the concentrations of TNFα and MCP-1 were 0.91 ± 1.43 and 26.2 ± 4.02 pg/ml, respectively.
The rat EM model has been used to evaluate the pathogenesis and pathophysiology of EM, including infertility and adhesive disease (Vernon, 1990; Sharpe-Timms, 2002; Uchiide et al., 2002). Surgically transplanted uterine tissue in the rat grows and behaves in a similar manner as human endometriotic lesions, it is responsive to steroid treatment, and histologically it contains endometrial glands and stroma (Vernon and Wilson, 1985; Vernon and Hodgen, 1987). It has been noted that rat endometriotic implants have epithelial cell regression and stromal fibroblastic transformation similar to histologic findings in human endometriotic lesions (Keenan et al., 1999). This model was successfully implemented in the present study. The implants were cystic and were filled with brown-bloody or serosanguinous fluid, as seen in human EM (Fig. 1). The rat EM model was associated with significant adhesions (Table I) and a characteristic histopathology of endometriotic spindle cell stroma infiltrated by chronic inflammatory cells, including lymphocytes and macrophages and deposition of hemosiderin pigment within stroma, a classical hallmark of EM (Fig. 2).

This work examined the hypothesis that macrophage depletion will result in EM attenuation. To that end, endometriotic rats were treated by 4x once-weekly IP injections of LA. Treatment with high-dose alendronate liposomes (10 mg/kg) significantly reduced the implantation rate (Table I). A significant reduction of both implant size (cross-sectional area) and weight, in a dose–response relationship, was achieved following treatment with all doses of LA examined (Table I, Fig. 3). In addition, a significant dose–response inhibition of adhesions, a hallmark of both human (Defrere et al., 2005) and animal models of EM (Vernon, 1990; Sharpe-Timms, 2002; Uchiide et al., 2002), was achieved (Table I, Fig. 4). Although the growth of endometriotic implants in rats (Vernon and Wilson, 1985) and SCID mice (Aoki et al., 1994) was reported to be unaffected by the estrous cycle stage, an apparent limitation of our study is the lack of experimental evidence that all the rats were operated on and sacrificed on the same day of estrous cycle. It should be also noted that the rats were sacrificed on the same day of the week as they were initially operated on (after 28 days, seven cycles), and rats that live together synchronize their estrous cycles (McClinztco, 1981).

The inflammatory nature of the induced EM was manifested in a steady increase of circulating monocytes (Fig. 7). LA treatment negated the increased number of monocytes suggesting that the mechanism of the desired therapeutical effect is the partial and transient depletion of circulating monocytes. A diminished inflammatory response as expressed by a decrease in arterial wall macrophage infiltration resulted from the partial and transient depletion of circulating monocytes following systemic LA treatment in the rat restenosis model (Danenberg et al., 2002, 2003a; Epstein et al., 2007). Nevertheless, a direct inhibition of peritoneal macrophages could also contribute to attenuating EM since animals were treated by local administration of LA in this study. However, a reduced number of implant macrophages was demonstrated only in the 1 mg/kg treatment group but not in the 10 mg/kg treatment group (Fig. 6). This could be attributed to an unknown local toxic effect of the higher dose. Although circulating monocytes have been shown to be the significant marker following liposomal BP treatment by various routes of administration in a local pathology (Danenberg et al., 2002, 2003a,b; Cohen-Sela et al., 2006a,b; Epstein et al., 2007, 2008), and IP treatment has been shown to deplete macrophages in several organs.
Figure 7  Peripheral blood monocytes in the rat EM model and the effect of LA treatment. Representative flow cytometry analysis demonstrate the gated monocytes population (CD3^+ CD4^+), 48 h after implantation and IP injection of 1 mg/kg LA in comparison with the saline injected control group (a). The effect of LA treatment (1 mg/kg, IP, 4× once weekly) on circulating monocytes number (±SD) over time in endometriotic rats was expressed as the percentage of total WBC (b), and as the change from baseline (c). Animals were injected with saline or LA on Day 0, 7, 14 and 21. Monocytes number was analyzed prior to and 48 h after each injection: Day 0 (prior to EM surgery, baseline), 2, 14, 16, 21, 23 and 28 (sacrifice). A significant depletion of circulating monocytes following LA treatment in comparison with the control group was obtained 28 days post-implantation (n = 5 in each group, P < 0.01).
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(Soestayo et al., 1991; Biewenga et al., 1995), further studies should examine peritoneal fluid macrophages as well as peritoneal cells. Macrophage depletion by liposomal BP has been examined in recent years as a treatment modality in the inhibition of monocyte/macrophage mediated disease processes (van Rooijen and Sanders, 1994; Danenberg et al., 2002, 2003a,b; Pipp et al., 2003; van Rooijen and van Kesteren-Hendriks, 2003; Cohen-Sela et al., 2006a,b; Zeisberger et al., 2006; Epstein et al., 2007, 2008). After phagocytosis, lysosomal action disrupts the fatty bilayers of the liposome, biodegrading the polymeric shell and the free BP is released into the cell, causing irreversible functional damage and apoptosis. BP leaking from dead macrophages and the delivery system does not accumulate to a significant extent in tissues other than bone (Cohen-Sela et al., 2006a). Thus, no effect was seen on somatic growth or on serum mineral content after liposomal BP treatment, since the outcome of the liposomal formulation, as with most other liposomes and particulate drug delivery systems, was in the mononuclear phagocytic system. Long-term inactivation of macrophages carries the danger of immunosuppression and infection. However, as in studies on Mac-1-deficient mice and rats, no overt infection was observed with transient macrophage depletion (Danenberg et al., 2002; Cohen-Sela et al., 2006a). Circulating blood monocytes recover fully 6 days after injection and IL-1β concentrations return to basal levels. The effects of nanoparticulated BP are limited to cells with phagocytic capacity with no effect on other cells, such as smooth muscle cells or endothelial cells. Decreased levels of macrophages in the arterial wall, following the partial and transient systemic depletion of circulating monocytes, results in the inhibition of restenosis (Danenberg et al., 2002, 2003a,b).

A positive correlation between the treatment dose and MCP-1 levels was found (Fig. 5). MCP-1 has been suggested to play a role in adhesion formation (Zeyneloglu et al., 1998a,b). MCP-1 is produced by EM tissue (Boucher et al., 2000), and the peritoneal administration of endometrial cells in a mouse model can incite the expression of MCP-1 (Cao et al., 2004). Moreover, macrophages themselves express MCP-1 (Akoum et al., 2002) in EM. Systemic changes related to cytokine expression levels in women with EM remain a subject of controversy. When a group of women with EM was divided with regard to severity of disease, a significantly higher percentage of CD14⁺ cells stained for MCP-1 in advanced EM patients compared with the control group were observed (Gmyrek et al., 2008b). Other studies with MCP-1 in humans showed that serum and peritoneal fluid MCP-1 concentrations at all stages of the disease are higher than in controls, yet show an opposite behavior in both biological fluids. In fact, serum levels of MCP-1 are significantly higher at early stages and decrease with the severity of the disease, whereas significant enhancement of MCP-1 in peritoneal fluid from stage I to stage II and stage III is observed (Pizzo et al., 2002). In another study of women with EM, the fraction of CD14⁺ cells producing MCP-1 were significantly lower in peritoneal fluid than in serum (Gmyrek et al., 2008a). Conversely, MCP-1 levels in our study might have been elevated in response to increased apoptosis of endometrial cells, which were not able to implant, without the support from cytokines from activated macrophages. In addition, MCP-1 is mainly secreted from lymphocytes or lymphocytic cell lines, cells that are not affected by LA. Our results suggest that the mechanism of EM mitigation observed in this study is not mediated by MCP-1. It is possible that since the number of macrophages depleted was higher with increasing dose, more MCP-1 was secreted by cells for recruiting macrophages. Although incompletely explained, the clear dose–response effect of LA treatment on peritoneal MCP-1 levels is another aspect of ‘proof-of-concept’ of efficacy of LA in inhibition of EM implant formation.

We did not find specific correlations between TNFα levels in peritoneal fluid and LA dose; only the high-dose treatment (10 mg/kg) reduced TNFα levels to that of sham-operated animals. TNFα is a thought to be a key cytokine in the activated macrophage-EM cell cross-talk and positive feedback loop (D’Hooghe, 2003). Such discrepancies have been noted in human EM, whereby TNFα levels are elevated in peritoneal fluid of EM patients, but not in serum or follicular fluid, and these levels are not correlated with the severity of the disease (Kalu et al., 2007; Kilic et al., 2007). TNFα and other cytokine levels are elevated in women with EM only during the menstrual phase, and not during other phases of the cycle (Kyama et al., 2008). Moreover, the peritoneal fluid, in addition to macrophages, comprises additional cytokine producing immune cells, such as lymphocytes and leukocytes (Fig. 2d), which are not susceptible to LA treatment (Epstein et al., 2007). The relationship between the peritoneal level of TNFα and EM activity is complex and apparently dependent upon a number of factors, which might be elucidated by further study.

**Conclusion**

Monocytes/macrophages depletion by IP LA treatment had an inhibitory effect on the initiation and growth of EM implants, as expressed by implantation rate, adhesion scoring, implant size and weight and macrophage infiltration of implant stroma. The negative, or lack of, correlation between LA treatment and MCP-1 or TNFα levels, respectively, suggests that the immunobiology of EM is complex and warrants further studies to elucidate the underlying cytokine(s) affected. The clear dose–response effects support the hypothesis of the pivotal role that macrophages play in the initiation and proliferation of EM implants. Further studies are required in order to validate the potential of this new therapeutic strategy.

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