CD40 is expressed on rat peritoneal mesothelial cells and upregulates ICAM-1 production

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Abstract

Background. CD40 has been identified on a variety of cell types, and it plays an important role in adaptive immunity and inflammation. Peritoneal mesothelial cells (PMCs) are the main cell layer that line the peritoneal membrane. Previously we found that CD40 ligand (CD154) is functionally expressed on peritoneal macrophages during continuous ambulatory peritoneal dialysis peritonitis. However, there are few studies that have examined both CD40 expression on PMCs and the function of CD40 signalling in peritoneal local defence. The purpose of this study was to determine whether PMCs express CD40 and to investigate potential mechanisms of CD40–CD154 interactions that may be involved in the inflammation of the peritoneal membrane.

Methods. Rat PMCs were harvested from the peritoneal cavity and maintained under defined in vitro conditions. We examined expression of CD40 on PMCs under normal culture or stimulation with interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) or interleukin (IL)-1 by reverse transcription–polymerase chain reaction and fluorescence-activated cell sorting (FACS) analysis. After activation with CD40 monoclonal antibody (mAb), the expression of intercellular adhesion molecule-1 (ICAM-1) on PMCs was analysed by FACS.

Results. A portion of rat PMCs cultured in vitro expressed CD40 constitutively. The expression of CD40 mRNA and protein was upregulated markedly following stimulation with IFN-γ, but not following IL-1 or TNF-α. The expression of ICAM-1 on PMCs was significantly increased after activation of CD40 with IFN-γ and with CD40 mAb.

Conclusion. PMCs functionally express CD40. The interaction between CD40 on PMCs and CD154-positive cells in the peritoneal cavity may play an important role in peritoneal local defence and may be involved in the inflammation process of the peritoneum.

Keywords: CD40; ICAM-1; immunity; peritoneal mesothelial cells; peritonitis

Introduction

CD40 and its ligand, CD154, are members of the tumour necrosis factor receptor (TNFR) and tumour necrosis factor (TNF) families. Until recently, it was thought that expression of CD40 was restricted to B cells. However, recent reports demonstrated that CD40 is also expressed on monocytes, dendritic cells, endothelial cells, epithelial cells, fibroblasts and smooth muscle cells [1]. The CD40 ligand, originally described on activated CD4-positive T cells, is also expressed on non-lymphoid cells. Aside from the importance of CD40–CD154 interactions during appropriate immune responses, ligation of CD40 has also been reported to upregulate the production of adhesion molecules, cytokines and chemokines. The discovery of the broad distribution pattern of CD40 and CD154 has indicated that their interaction plays an important role not only in cell immunity and humoral immunity, but also in inflammatory processes [1–6].

Peritonitis is still a major cause of treatment failure in peritoneal dialysis patients treated for end-stage renal failure. Peritoneal mesothelial cells (PMCs) are the main cell layer that line the peritoneal membrane. It is now clear that in addition to their structural function, PMCs play an important role in peritoneal inflammatory and immune responses [7–10]. To mount an effective immune response against invading pathogens, a large number of leukocytes from the blood are recruited into the peritoneum. An important part of this recruitment is the expression of adhesion molecules on the mesothelial cell surface [11,12]. In addition, functional intercellular adhesion molecule-1 (ICAM-1)
expression has been identified on PMCs where it plays an important role in the attachment of different leukocyte subpopulations [11,12].

The peritoneal cavity normally contains a variable number of macrophages and it has been assumed that these cells, together with mesothelial cells, control host responses to peritoneal infection [9,10]. We recently reported that CD154 expression on macrophages from peritoneal dialysate was significantly increased in continuous ambulatory peritoneal dialysis (CAPD)-related peritonitis patients [13]. However, only a few reports have examined CD40 expression and the function of its signal on PMCs during peritoneal local defence. The purpose of the present study was to determine whether PMCs express CD40 and to investigate potential involvement of CD40–CD154 in the inflammation of the peritoneal membrane. These studies showed that a portion of rat PMCs cultured in vitro express CD40 constitutively. In addition, the expression of CD40 mRNA and protein was markedly upregulated following stimulation with interferon-γ (IFN-γ). The expression of ICAM-1 on PMCs was significantly increased after activation of CD40 with CD40 monoclonal antibody (mAb).

These data provide additional evidence for a role for mesothelial cells in the control of peritoneal inflammatory processes.

Materials and methods

Monoclonal antibodies and reagents

We used mAbs with specificities for the following rat antigens: anti-mouse CD40 mAb (clone HM40-3, hamster IgM) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-hamster IgM mAb (clone G188-9) purchased from PharMingen (San Diego, CA). Mouse anti-rat ICAM-1 mAb (Clone: 1A29) was purchased from Chemicon Inc. (Temecula, CA). FITC-conjugated rabbit anti-mouse IgG mAb and anti-factor VIII antibody were from Dako A/S (Glostrup, Denmark). The OneStep RT–PCR Kit was purchased from Qiagen (Glostrup, Denmark). The expression of ICAM-1 on PMCs was measured.

Flow cytometry

Cells were suspended for fluorescence-activated cell sorting (FACS) analysis by washing monolayers twice with phosphate-buffered saline (PBS) followed by incubation for 2 min with 0.25% trypsin-0.02% EDTA-Na2. Suspended cells (100 μl 1 × 10^6) were washed once in PBS containing 1% bovine serum albumin (BSA) and incubated with either 10 μl of specific unconjugated primary antibody, anti-CD40 mAb or anti-ICAM-1 mAb for 30 min at 4°C. Cells were washed twice with PBS/2% BSA and incubated with FITC-conjugated secondary antibody for 30 min at 4°C. PMCs were washed again and the resuspended cells were then analysed by FACS (Coulter Corp, Hialeah, FL). The results are expressed in total mean fluorescence intensity (MFI) and percentage of positive fluorescent cells (%PFC). Mean values from six measurements and standard deviations (SDs) were determined. PMCs incubated with an isotype control mAb served as negative controls.

Semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis

Isolation of RNA. Total RNA from PMCs was isolated according to the manufacturer’s instructions from the TRIZOL kit. The RNA concentration in each sample was determined using a UV/visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech, USA).
Primers. The sequences of primers in this study were as follows: CD40 (442 bp) sense, 5'-GTGTGTTACGTGCAGTGACAA-3'; and antisense, 5'-ATCCTCACAGCTTCCAATGAAATCAC-3'; and GAPDH (230 bp) sense, 5'-ACCACAGTCCAGGCGGCATG-3'. The primers were obtained from Shenggong Bioengineering Technological Co. Ltd. (Shanghai, China).

RT–PCR. RT–PCR was performed according to instructions in the QIAGEN RT–PCR kit. A Perkin Elmer 9600 Thermal Cycler (Perkin Elmer, Weiterstadt, Germany) was programmed as follows: 50°C for 30 min, initial PCR activation step: 95°C for 15 min; denaturation 94°C for 45 s; annealing 57°C for 45 s; extension 72°C for 1 min. Thirty-five cycles were carried out. The final extension was 72°C for 10 min. Negative controls consisting of the reaction mixture without cDNA that were not processed for reverse transcription were included in all reaction series. Aliquots of the PCR products (10μl) were run on 1.7% agarose gels and visualized by UV transillumination. Quantifications of signal intensity were done using a specific computer program (IBAS2.5 Auto Image analysis, Kontron, Germany). Data were expressed as the ratio of specific CD40 mRNA normalized to GAPDH mRNA amplified from the same RNA sample to correct for any error in spectrophotometric RNA quantitation or in pipetting.

Statistical analysis
Data were expressed as means±SD from six separate experiments. Statistical differences among groups were assessed by analysis of variance (ANOVA), followed by Bonferroni (post hoc) tests for continuous variables distributed normally and by Mann–Whitney test for continuous variables not having a normal distribution. A P-value <0.05 was considered statistically significant. The statistical calculations were performed using SPSS for Windows 10.

Results
Culture of rat peritoneal mesothelial cells
Within 3–5 days following culture initiation, >95% of the cells exhibited a polygonal, cobble-stone epithelioid morphology typical of PMCs. These cells were cytokeratin-positive and failed to express factor VIII, which is consistent with a mesothelial cell phenotype (data not shown). These findings suggested that these proliferative cells were of peritoneal mesothelial origin.

Expression of CD40 protein on the surface of cultured rat PMCs
PMCs in log phase growth were treated with and without IFN-γ, IL-1 or TNF-α for 24 h and subsequently were analysed by flow cytometry using an anti-CD40 mAb. As shown in Figure 1 and Table 1, part of the PMCs (12.67±1.60%) expressed low but detectable CD40 surface protein. Treatment with IFN-γ for 24 h caused a marked upregulation of CD40 expression (P<0.001). In contrast, IL-1 or TNF-α at the selected dose had no effect on CD40 expression in PMCs (P>0.05).

Expression of CD40 mRNA by cultured rat PMCs
Representative levels of CD40 442 bp mRNA expression in PMCs cultured in the various cytokines for 24 h are shown in Figure 2. Low constitutive CD40-specific mRNA expression was seen in PMCs cultured with medium alone, with IL-1 or with TNF-α. In contrast, CD40 mRNA expression was significantly upregulated after 24 h treatment with IFN-γ (P<0.001) (Figure 2).

Fig. 1. Flow cytometry analysis of CD40 proteins on PMCs. PMCs were labelled by a primary antibody to CD40 and then stained with FITC-labelled secondary antibody. (A) The background MFI of cells labelled with an isotype-matched control antibody (control). (B) The MFI of PMCs incubated in medium alone. (C) The MFI of PMCs stimulated with IFN-γ (100 U/ml). (D) The MFI of PMCs stimulated with TNF-α (100 U/ml). (E) The MFI of PMCs stimulated with IL-1 (100 U/ml). The figure is a representative from six experiments.
CD40 mRNA levels on PMCs. Total RNA was isolated and RT–PCR was performed to amplify CD40 and GAPDH cDNA. (A) Aliquots of the PCR products (10 μl) were run on 1.7% agarose gels and visualized by UV transillumination. (B) Data were expressed as the ratio of specific CD40 mRNA normalized to GAPDH mRNA amplified from the same RNA sample. (1) PMCs incubated in medium alone (control); (2) PMCs stimulated with IFN-γ (100 U/ml); (3) PMCs stimulated with TNF-α (100 U/ml); (4) PMCs stimulated with IL-1 (100 U/ml). The figure is a representative from six experiments. ***P < 0.01 compared with group 1.

**Table 1.** Expression of CD40 protein on the surface of cultured rat PMCs (n = 6, mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>MED</th>
<th>MED plus IFN-γ</th>
<th>MED plus TNF-α</th>
<th>MED plus IL-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFI</td>
<td>0.50±0.01</td>
<td>0.66±0.05**</td>
<td>0.52±0.03</td>
<td>0.52±0.04</td>
</tr>
<tr>
<td>%PFC</td>
<td>12.67±1.60</td>
<td>18.50±3.23*</td>
<td>11.80±1.55</td>
<td>12.53±2.27</td>
</tr>
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</table>

MED = medium.

*P < 0.05; **P < 0.01 compared with the MED group.

Fig. 2. CD40 mRNA levels on PMCs. Total RNA was isolated and RT–PCR was performed to amplify CD40 and GAPDH cDNA. (A) Aliquots of the PCR products (10μl) were run on 1.7% agarose gels and visualized by UV transillumination. (B) Data were expressed as the ratio of specific CD40 mRNA normalized to GAPDH mRNA amplified from the same RNA sample. (1) PMCs incubated in medium alone (control); (2) PMCs stimulated with IFN-γ (100 U/ml); (3) PMCs stimulated with TNF-α (100 U/ml); (4) PMCs stimulated with IL-1 (100 U/ml). The figure is a representative from six experiments. ***P < 0.01 compared with group 1.
Primers specific for GAPDH (230 bp), used as internal controls for RT–PCR and GAPDH bands, were present in each of the tested groups. There was no amplified PCR product in the reactions that did not receive reverse transcriptase, ruling out the possibility that false-positive PCR product formation was due to contaminating DNA in the RNA preparation.

Effects of CD40 activation on ICAM-1 expression

Anti-CD40 mAb most closely resembles the CD154 in terms of functional properties, since both of these reagents are capable of stimulating proliferation of small resting B lymphocytes in the absence of other cofactors [2,16]. We examined whether CD40–CD154 interactions induce PMCs to produce ICAM-1, a product that may play a key role in the development of peritoneal inflammation. For this purpose, PMCs were pre-treated with IFN-γ for 24 h to optimize CD40 surface expression and these were stimulated with anti-CD40 mAb for 24 h. As shown in Table 2, PMCs expressed low but detectable ICAM-1 protein. Stimulation with IFN-γ by itself stimulated expression of ICAM-1 to levels higher than that of unstimulated PMCs (\(P < 0.05\)). Furthermore, stimulation of PMCs with both anti-CD40 mAb and IFN-γ caused the most pronounced increase in ICAM-1 expression, resulting in higher levels than in unstimulated PMCs (\(P < 0.001\)) and in IFN-γ-treated PMCs (\(P < 0.001\)). Incubation of PMCs with the anti-CD40 mAb caused a slight but non-significant increase in the expression of ICAM-1 (\(P > 0.05\)). A representative ICAM-1 MFI from six experiments is shown in Figure 3.

CD40 is a 50 kDa glycoprotein that is a member of the TNFR family. CD40 was identified initially on the surface of B cells, where it binds to a surface-expressed ligand, gp39, or CD154, on activated CD4-positive T lymphocytes and plays a key role in T cell-dependent activation, proliferation and differentiation of B cells [1]. CD40 is also constitutively expressed on a variety of other leukocytes, such as macrophages, dendritic cells and eosinophils, and on the surface of cells of mesenchymal and epithelial origin, such as endothelial cells, fibroblasts, keratinocytes and vascular smooth muscles cells [1–5,15]. Activation of CD40 induces the secretion of cytokines and chemokines and upregulates the expression of adhesion and accessory molecules to increase the efficiency of antigen presentation [1]. Consequently, CD40 signalling has been associated with pathogenic processes of inflammatory diseases including autoimmune diseases, atherosclerosis and arthritis [1]. Together, these data support the concept

![Flow cytometry analysis of ICAM-1 proteins on PMCs.](image)

**Table 2. Effects of CD40 activation on ICAM-1 expression by PMCs (\(n = 6\), mean ± SD)**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFI</td>
<td>6.48±0.33</td>
<td>6.78±0.40</td>
<td>7.18±0.42*</td>
<td>8.51±0.38***+</td>
</tr>
<tr>
<td>%PFC</td>
<td>98.75±0.23</td>
<td>97.54±0.17</td>
<td>97.86±0.19</td>
<td>98.42±0.26</td>
</tr>
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</table>

A = medium; B = medium + CD40 mAb; C = medium + IFN-γ; D = medium + IFN-γ + CD40 mAb. *\(P < 0.05\) and ***\(P < 0.001\) compared with group A; +\(P < 0.001\) compared with group C.
that interactions between CD40 and its ligand not only play an important role in cell immunity and humoral immunity, but are also importantly involved in inflammatory responses.

Peritoneal mesothelium, a simple squamous epithelium, is of mesodermic origin and continuously lines serosal membranes within the peritoneal cavity. It is now clear that in addition to a structural function, this cell layer plays an important role in peritoneal inflammatory and immune responses [7–10]. Immune responses that occur in the peritoneum are of special relevance in CAPD, which is an established therapy for end-stage renal failure. Patients treated with CAPD carry a transcutaneous catheter inserted into the peritoneum, and peritonitis is a frequent complication. Furthermore, the non-physiological nature of the majority of currently available dialysis solutions may hinder effective functioning of the peritoneal immune system [10]. PMCs have been shown to secrete the proinflammatory cytokines IL-1 and IL-6 as well as the chemokines IL-8, monocyte chemoattractant protein 1 and RANTES, thereby amplifying the inflammatory signals [10,17]. However, there has been little study of CD40 expression on PMCs and on the function of the CD40 signal during peritoneal local defence. For these reasons, we investigated whether mesothelial cells acting as antigen-presenting cells can participate in specific immune and inflammatory responses by expressing CD40 or CD154. We found that CD40 was present on PMCs cultured in vitro. The expression of CD40 on PMCs was markedly upregulated following stimulation with the proinflammatory cytokine IFN-γ, which is abundant at the site of inflammation. These results suggest that CD40 expression on PMCs is regulated by mediators of inflammation. Phagocytosis performed by macrophages represents one of the main defences of the peritoneal cavity against invading pathogens. We previously reported that CD154 expression on macrophages from peritoneal dialysate was significantly increased in peritonitis. The CD154-positive cells were also significantly increased. We had found additionally that successful treatment significantly reduced the expression of CD154 [13]. In this study, CD154 expression on PMCs was not detected under defined in vitro conditions or following stimulation with IFN-γ, TNF-α or IL-1 (data not shown). Given these data, we propose that CD40 on mesothelial cells may interact with its ligand on peritoneal macrophages to take part in the peritoneal local inflammatory response during CAPD peritonitis.

To mount an effective immune response against invading pathogens, a large number of leukocytes are recruited to the peritoneum from the blood. During this process, ICAM-1 is a key adhesion molecule that mediates leukocyte attachment and migration [11,12]. It has been reported that CD154 activation induces the expression of ICAM-1 on human vascular and umbilical vein endothelial cells, dendritic cells and keratinocytes as well as on synovial membrane or dermal fibroblasts [1,4,5]. Recently, it was reported that platelets constitutively express surface CD40. Activated platelets cause upregulation of ICAM-1 and vascular cell adhesion molecule-1 expression as well as production of IL-8 by human intestinal microvascular endothelial cells in a CD40-dependent fashion, which represent important actions in the recruitment of leukocytes to sites of thrombosis or inflammation by enhancing platelet–leukocyte adhesion [18]. These findings suggest that CD40 may serve as a crucial signalling receptor in the development of T lymphocyte-mediated inflammatory reactions [1]. In vivo constitutive expression of the ICAM-1 has been detected on mesothelial cells [11,12]. In the present study, we found that PMCs constitutively express ICAM-1 in vitro. Stimulation of PMCs with IFN-γ resulted in expression of ICAM-1 by PMCs that was significantly higher than in control PMCs. Stimulation of PMCs with anti-CD40 mAb plus IFN-γ caused the most pronounced effect on ICAM-1 expression. We therefore propose that CD40 ligation on PMCs is functionally synergistic with IFN-γ to cause induction of ICAM-1, which plays an important role in leukocyte adhesion and migration.

In the current experiment, IFN-γ stimulation increases both the cell surface expression and mRNA expression of CD40. This finding indicates that the increased protein expression of CD40 in response to IFN-γ is likely to be the result of an increase in CD40 mRNA transcription. However, we found that the increase in CD40 protein expression was not comparable with the larger increase in CD40 mRNA levels. Nguyen et al. [20] demonstrated that transforming growth factor-β (TGF-β), which was induced to higher levels from pro-inflammatory cytokines by peritoneal mesothelial cells, can inhibit CD40 protein expression and does so at the post-transcriptional level by destabilizing IFN-γ-induced CD40 mRNA [20]. This result may offer a possible explanation for the difference in expression of CD40 mRNA and protein in PMCs. However, a mechanistic understanding of these actions will require further study.

In previous reports, stimuli for CD40 expression included cytokines, such as IFN-γ, IL-1 and TNF-α. However, little is known about the regulation of CD40 expression [20]. The expression of CD40 on fibroblasts is enhanced by IFN-γ, while IL-1α and TNF-α alone have no effect; however, IL-1α and TNF-α significantly augment IFN-γ induction of CD40 in these cells [4]. In contrast, IL-1α, TNF-α and IFN-γ significantly upregulate CD40 on thymic epithelial cells [2]. Studies examining CD40 expression by endothelial cells have been conflicting, with one group reporting an upregulation of CD40 expression by IFN-γ, but not by IL-1α or TNF-α [3], whereas Karmann et al. [5] demonstrated that all three cytokines as well as IFN-β increased CD40 expression. Basok et al. [6] demonstrated that human PMCs express CD40 mRNA and protein. After stimulation with IFN-γ (5 U/ml) or TNF-α (1 ng/ml), there was a small increase in CD40 mRNA and protein levels; when both cytokines were applied, the increase in CD40 levels was >3-fold [6]. In our study, the most
effective stimulator of CD40 expression on rat PMCs was selected doses of IFN-γ. Although TNF-α and IL-1 increased CD40 expression on mesothelial cells, those increases were not statistically significant. The different types of cells and cytokine doses used in these studies may have contributed to these discrepancies.

In summary, we found that PMCs functionally express CD40 and this process is regulated by IFN-γ. Activation of CD40 on PMCs with CD40 mAb was highly synergistic with IFN-γ to cause ICAM-1 secretion. The interaction between CD40 on PMCs and CD154-positive cells such as macrophages and T cells in the peritoneal cavity may play an important role in peritoneal local defence and may be involved in the inflammation process in the peritoneum.

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Conflict of interest statement. The authors declare that they have no competing financial interests.

References

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