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Molecular Immunology 42 (2005) 773–780
Molecular Immunology
www.elsevier.com/locate/molimm

Requirement of the extracellular cysteine at position six for CD40/CD40 dimer formation and CD40-induced IL-8 expression

Julie Girouard[a,1], Carlos Reyes-Moreno[a,1,2], André Darveau[b], Ali Akoum[c], Walid Mourad[a,*]

[a] *Centre de Recherche en Rhumatologie et Immunologie (CRRI), Centre Hospitalier de l'Université Laval, 2705 boulevard Laurier, T1-49, Quebec City, Que., Canada G1V 4G2*
[b] *Département de Biochimie et Microbiologie, Université Laval, Québec City, Que., Canada G1K 7P4*
[c] *Centre de Recherche de L'Hôpital Saint-François d'Assise, Université Laval, Québec City, Que., Canada G1K 7P4*

Received 7 June 2004; accepted 13 July 2004
Available online 23 November 2004

Abstract

We recently showed that oligomerization of CD40 molecules on cell surface leads to disulfide-linked CD40/CD40 dimer formation, an event that is necessary for CD40-induced B7-2 expression in human B cells. Here, we demonstrate that CD40/CD40 dimers formation also occurs in different cell types such as T24 bladder cancer cells and CD40-transfected HEK 293 cells. Disulfide bonds mediate the formation of CD40/CD40 homodimers in CD40-activated cells. To determine the potential residue(s) involved in disulfide bonds formation and subsequent CD40-induced IL-8 expression, we generated a CD40 mutant in which the extracellular cysteine 6 was replaced by a glutamine (CD40-C6Q). CD40-induced IL-8 mRNA expression and protein synthesis were studied in stably transfected HEK 293 cells that were sorted out along with similar levels of expression of wild type (CD40-WT) and CD40-C6Q molecules. In contrast to cells expressing CD40-WT protein, disulfide-linked CD40/CD40 dimer formation was completely abolished in HEK 293 cells expressing CD40-C6Q proteins. Abolishment of disulfide-linked CD40/CD40 dimers in these transfected cells was sufficient to inhibit CD40-induced mRNA expression and secretion of IL-8. This study identifies the extracellular cysteine 6 of CD40 molecules as a potential molecular target to disrupt the expression of CD40-induced pro-inflammatory cytokines by epithelial cells.

Keywords: Receptor dimerization; Cellular activation; Disulfide bond; Cytokine expression

1. Introduction

CD40 molecule is a 45–48 kDa type I phosphorylated glycoprotein that belongs to the tumor necrosis factor receptor (TNFR) superfamily, and was originally described as an antigen expressed on the surface of human urinary bladder carcinomas, and human B cells (Braesch-Andersen et al., 1989). Now it is well established that the expression of CD40 molecules is not restricted to B cells and normal and malignant epithelial cells, but they can also be functionally expressed in various other cell types such as dendritic cells, monocytes, platelets, endothelial cells and fibroblasts (Schonbeck and Libby, 2001; van Kooten and Banchereau, 2000). Subsequently, CD40 plays a major role in humoral and cellular immunity. Therefore, ligation of CD40 results in B cell activation, secretion of a panel of cytokines, up-regulation of co-stimulatory surface molecules, development of long-lived plasma B cells, clonal expansion of memory B cells, and immunoglobulin isotype switching (Ahonen et al., 2002; Grammer and Lipsky, 2000; Guzman-Rojas et al., 2002; Kobayashi et al., 2003). In non-hematopoietic cells, ligation of CD40 enhances the secretion level of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α, up-regulates surface antigen expression, such as ICAM-1 and CD54, and leads to the expression of activated matrix-degrading proteins (Cagnoni et al., 2004; Schonbeck and Libby, 2001; Young et al., 1998).

* Corresponding author. Tel.: +1 418 654 2772; fax: +1 418 654 2765.
E-mail address: walid.mourad@crchul.ulaval.ca (W. Mourad).
[1] Authors contributed equally to this work.
[2] Département de Chimie-Biologie, Université du Québec à Trois-Rivières, CP500 Trois-Rivières, Québec, Canada G9A 5H7.

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doi:10.1016/j.molimm.2004.07.048

In our previous study, we reported that oligomerization of CD40 with soluble trimeric CD154 or with anti-CD40 mAbs cross-linked with a secondary Ab leads to the induction of disulfide-linked CD40/CD40 dimer formation which in turn results in significantly increased expression of B7-2 in human B cells (Reyes-Moreno et al., 2004). CD40-mediated B7-2 expression in human B cells was totally abrogated in conditions in which sulfhydryl groups were blocked by the thiol-alkylating agent iodoacetamide. On the other hand, activation of MAP kinase p38 and B cell homotypic adhesion in response to CD40 ligation is totally independent of disulfide-linked CD40/CD40 dimer formation. Together, these results allow us to propose that high degree of CD40 clustering and subsequent formation of disulfide-linked CD40/CD40 dimers is necessary for certain CD40-induced responses (Reyes-Moreno et al., 2004).

The constitutive formation of CD40/CD40 dimers was originally reported to occur at low levels in transitional bladder cancer (TBC) cells and in human B cell lines, such as Raji B cells (Braesch-Andersen et al., 1989). Interestingly, in contrast to Raji B cells, CD40/CD40 dimer formation was reported to be absent in HU549 TBC cells and in HU549 B cells derived from the same patient (Braesch-Andersen et al., 1989). The protein sequences of two CD40 molecules from Raji B cells and HU549 TBC cells differed, in that, the cysteine at position six in Raji CD40 had been replaced by glutamine in HU549 CD40. In addition, there were two conservative changes in positions 15 (a glycine instead a serine) and 19 (a threonine instead a serine). Since disulfide bounds are known to occur mainly between two cysteines residues, we explored the possible implication of cysteine 6 in the CD40/CD40 dimer formation and function. We have established that disulfide-linked CD40/CD40 dimers are formed in CD40-activated epithelial cells. Notably, we have determined that the extracellular cysteine 6 is required for CD40/CD40 dimer formation, as shown in HEK 293 cells transfected with a CD40 mutant gene in which the extracellular cysteine 6 was replaced by a glutamine (CD40-C6Q). Abolishment of disulfide-linked CD40/CD40 dimers is sufficient to completely inhibit the mRNA expression of IL-8 and the protein secretion in CD40-activated CD40-C6Q transfectants. This finding led us to propose that blockade of CD40/CD40 dimer formation could be an attractive target for inhibition of specific biological outcomes triggered by CD40 ligation in different cell types.

2. Material and methods

2.1. Materials

The hybridomas producing the mouse mAbs directed against human CD40 (G28-5; IgG1) and human MHC class I (W632; IgG1) were obtained from ATCC (Rockville, MD, USA). An irrelevant IgG1 isotype control directed against the superantigen staphylococcal enterotoxin A (anti-SEA) was produced in our laboratory and used for flow cytometry analysis. The goat anti-mouse IgG and the FITC- and HRP-conjugated anti-mouse IgG were purchased from Santa Cruz (Santa Cruz, CA, USA). Cell culture agents, phorbol myristate acetate (PMA), HRP-conjugated streptavidin and electrophoresis grade chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Plasmid constructs and site-directed mutagenesis

The CD40-C6Q mutant (cysteine 6 was substituted by glutamine) and the CD40-T234A mutant (threonine 234 was substituted for an alanine) were generated by site-directed mutagenesis using the pCEP4-CD40 wild-type expression vector as template (Reyes-Moreno et al., 2004). Mutations were confirmed by sequencing the inserts. CD40-negative HEK 293 cells (ATCC; Rockville, MD, USA) were cultured at 75% confluency and transfected with the expression vectors pCEP4 alone (mock transfected), pCEP4 CD40-WT, pCEP4 CD40-C6Q, and pCEP4 CD40-T234A by DNA-calcium phosphate precipitation (Clontech, Palo Alto, CA, USA). FACS analysis using the anti-CD40 mAb G28-5 or isotype-matched Abs was performed 24 h post-transfection, and stable transfectants were harvested after 7–10 days of selection in 400 μg/ml Hygromycin (Roche, Montréal, Que., Canada).

2.3. Induction and detection of CD40/CD40 homodimers

T24 bladder cancer cells were kindly gifted by Dr Hélène Larue (Centre de Cancérologie de l'Université Laval, Hôtel-Dieu de Québec, Que., Canada). BJAB B cells, T24 and HEK 293 transfectants were diluted to 1.5×10^6 cells/ml in MEM supplemented with 5% FBS. G28-5 or W632 mAbs were added to a final concentration of 10 μg/ml, and the cells were incubated on ice for 15 min. Washed cells were then resuspended with 5 μg/ml anti-mouse IgG and incubated for 10 min at 37 °C. Cells were lysed in ice-cold TNE buffer (10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100, 2 mM Na_3VO_4, and a cocktail of protease inhibitors for 30 min on ice. Western blot studies were performed using the anti-CD40 mAb G28-5 in non-reducing conditions, as previously described (Reyes-Moreno et al., 2004).

2.4. Two-dimensional non-reduced/reduced SDS-PAGE

In order to confirm that CD40 ligation induces disulfide-linked CD40 homodimers, BJAB B cells (ATCC; Rockville, MD, USA) were processed for biotin labeling prior to CD40 activation, and analyzed by two-dimensional non-reduced/reduced SDS-PAGE (diagonal gel), as previously described by Frank et al. (1994). Briefly, BJAB B cells were washed three times with cold PBS and incubated on ice for 1 h with 5 μg/ml NHS-LC-biotin, according to the manu-