CD40 expression in Wehi-164 cell line

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Abstract CD40-CD154 interaction is an important process for cellular and humoral immunity regulation and can be effective in the body's defense against tumors. In the present study, we evaluated the expression of CD40 in Wehi-164 cell line. CD40 expressions on the cell surface and in the cytoplasm were assessed by flow cytometry and intracellular staining assay, respectively. Also, the mRNA expression was identified by real time-PCR. The obtained results showed the high mRNA and cytoplasmic protein expression of CD40 but no surface expression. These results suggest that the Wehi-164 cell line down regulates expression of CD40 on the surface for evasion of immune system.

Keywords Wehi-164 · CD40 · Cytotoxicity

Introduction

One of the members of TNF receptor superfamily is CD40, a 45–50 kDa transmembrane protein which is a TNF receptor homolog (Smith et al. 1994). CD40 is expressed on the surface of the immune cells like B cells, monocytes, macrophages, and dendritic cells (DCs) and non-immune cells such as epithelial, endothelial, and mesenchymal (fibroblasts, myofibroblasts, synoviocytes, stellate cells, etc) cells, as well as platelets (Schonbeck and Libby 2001). Since the importance of CD40-CD40L interaction in humoral immunity is now being obvious, it is concluded that CD40-CD40L interaction is also critical in cell mediated immunity (Grewal et al. 1998a, b; Caux et al. 1994). One of the cellular functions of CD40L is to regulate the co-stimulatory activity of antigen presenting cells (APCs) like B cells and induce the expression of B7.1 and B7.2 in these cells. On the DCs, CD40-CD40L interaction induces the production of various cytokines, such as IL-8, TNF- α , and MIP-1a. The most remarkable one is the induction of IL-12 production by DCs. IL-12 is an important cytokine for the polarization of Th1 immune responses (Cella et al. 1996). Besides, the induction of apoptosis in CD4+ T cells (Blair et al. 2000) and generation of CD8+ T cell memory are mediated by CD40 ligation (Bourgeois et al. 2002). The mentioned molecules are a linkage between the immune cells and numerous types of non-immune cells. That's why the immune and inflammatory responses are amplified. Because information on

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CD40 expression in Wehi-164 is lacking though Wehi-164 is one of the cell lines frequently used for the study of tumor immune response in Balb/c mice, and CD40 plays an important role in tumor immune response, we evaluated the pattern of CD40 expression in this cell line. This research is the first report on the pattern of CD40 expression in Wehi-164 published so far.

Materials and methods

Cell lines

The Wehi-164 (CRL-1751) mouse fibrosarcoma cell line was obtained from National Cell Bank of the Pasteur Institute of Iran. The cells were cultivated in RPMI medium (Gibco, USA) supplemented with 10% FCS, 3 mM L-glutamine, 5 μ M 2ME, 100 IU/ml penicillin and 100 μ g/ml streptomycin, all obtained from Sigma (USA). The BCL1 cell line (TIB-197), a mouse B-cell line that constitutively expresses high levels of CD40, was obtained from National Cell Bank of Pasteur Institute of Iran and was used as a positive control.

Isolation of DCs from spleen

DCs were isolated from mouse spleens using CD11c (N418) microbeads (Miltenyi Biotech, Germany) according to the instruction for isolation of DCs provided by the manufacturer.

Primer design

The primers were designed by Beacon designer software (Primer Biosoft V.7) for CD40 gene (NM_011611) and GADPH gene (NM_008084) as an internal control. Thermodynamic parameters and the secondary structure were determined by mfold software. The primer's positions in relation to exon–exon domains were evaluated by spidey software (www.ncbi.nlm.gov/Spidey, USA) and their specificity was analyzed by BLASTn (www.ncbi.nlm.gov/BLAST, USA).

RNA isolation and cDNA synthesis

RNeasy plus mini kit (Qiagen, USA) was utilized. The BCL1 cell line RNA was extracted according to

the manufacturer's instructions for total RNA isolation from animal cells. The purity of RNA was determined by measuring the optical density 260/280 and its integrity was measured by 1% agarose gel electrophoresis. Genomic DNA traces were removed by DNase digestion using RNase free DNase set (Roche, Germany) and then RNA reverse transcription was done by Superscript III (Invitrogen, USA) and oligo dT.

Real time-PCR

According to the manufacturer's instructions, mRNA expression was quantitatively analyzed by real time-PCR technique using a My-IQ cycler system (Biorad, Milan, Italy) and IQ sybergreen supermix kit (Biorad, Milan, Italy). For DNA amplification, SYBR Green I dye, forward and reverse primers and the template cDNA were added to each tube. Melting curves were analyzed for determination of PCR specificity. For the target gene, the results were analyzed as fluorescent signal intensity and normalized to the internal standard gene GADPH.

The PCR conditions were as follows:

95°C	3 Min	1X	
-95°C	30 Sec		40x
-60°C	30Sec		
-72°C	30 Sec		
-81°C	10 Sec		
-95°C	1 Min		
-65°C	1 Min		
-65°C	10 Sec	0.5	60

Flow cytometry analysis

In order to analyze the cell surface and the intracellular expression of CD40, FITC-conjugated rat anti-mouse CD40 (Clone 3/23) together with its respective isotype control (all from BD, USA) was used as antibody. For analysis of intracellular expression of CD40 after collection of the cells,

first the cells were fixed with paraformaldehyde, then permeabilized with Triton X-100, and analyzed by flow cytometry.

The FITC-conjugated AnnexinV and PI staining was used for determination of apoptosis /necrosis as previously described by Min et al. (2000).

Fig. 1 CD40 mRNA expression in the mouse splenic DCs, Wehi-164 and BCL1 cell line. The CD40 mRNA expression was measured by real time-PCR and the results were presented as ΔC_t values. A lower ΔC_t means that there is a higher expression of the gene

Results

Evaluation of CD40 expression results in the mRNA level by real time-PCR shows that Wehi-164 expresses CD40 mRNA and these results are corresponding to those obtained with DCs' (Fig. 1). The flow cytometry data of CD40 protein expression in Wehi-164 indicate that they do not express this protein on the cell surface although intracellular staining assay of cytoplasmic CD40 protein and the flow cytometry revealed a high expression of CD40 protein in the cytoplasm (Fig. 2). Collectively, we can conclude that Wehi-164 expresses CD40 mRNA as well as its cytoplasmic protein but not CD40 protein on the cell surface.

Discussion

This cell line was originally established by M. Rollinghoff and N.L. Warner from a fibrosarcoma induced by subcutaneous injections of 3-methylcholanthrene. After actinomycin D pretreatment, the cells were highly sensitive to human cytotoxic monocytes, to human tumor necrosis factor and to lymphotoxin.

Fig. 2 Evaluation of CD40 expression by flow cytometry in Wehi-164 cells. **A** Surface expression of CD40 **B** Cytoplasmic expression of CD40 **C** Isotype control of cytoplasmic expression **D** Isotype control surface expression **E** Surface expression of CD40 in BCL1 **F** BCL1 Isotype control

Thus, for short term chromium release in cytotoxic assays, they can be used as target cells.

Ligation of CD40 on DCs and macrophages leads to production of various cytokines, including IL-8, TNF- α , and MIP-1a. Notably, CD40 stimulation induces IL-12 production by DCs. IL-12 is a cytokine that plays a key role in the polarization of Th1 immune responses and IFN- γ production (Cella et al. 1996).

Sugarman et al. (1985) showed that rTNF- α had cytostatic or cytolytic effects on only some tumor cell lines like Wehi-164. When administered together with rIFN- γ , rTNF- α causes enhanced anti-proliferative effects on a subset of the cell line. Using MTT assay, Espevik and his coworkers showed that TNF as effector molecule can induce high level of cytotoxicity on Wehi-164 (Espevik and Nissen-Meyer 1986). Also, Monastra et al. (1996) reported that TNF- α caused apoptosis in Wehi-164 and this effect was completely inhibited in the presence of anti-mouse TNF- α Ab, while Faraco et al. (1999) showed that TNF- α 's ability for inducing either apoptotic or necrotic cell death in Wehi-164 and the mode of TNF-induced cell death are dependent on the cell cycle stage. Brekke and his coworkers have studied the mechanism of apoptosis in Wehi-164 and suggested that a butylated hydroxyanisole (BHA)-sensitive signaling pathway coupled to arachidonic acid (AA) release is important for TNF-induced cytotoxicity in these cells (Brekke et al. 1999). Lee et al. (1999) have reported that Wehi-164 apoptosis through TNF- α was mediated by NF- κ B. Also, Katsen et al. (1998) showed cell surface and nuclear changes during TNF- α -induced apoptosis in Wehi-164 cells.

In the present study, we showed that Wehi-164 had cytoplasmic expression of CD40 but no expression of CD40 protein on the cell surface. As already mentioned, CD40 ligation leads to the production of various cytokines, such as IL-8 and TNF- α and these cytokines can induce apoptosis in Wehi-164. It seems that non-expression of CD40 on the surface of Wehi-164 is one of the mechanism that helps the evasion of Wehi-164 from immune response especially cytotoxicity and apoptosis due to TNF production by CD40 ligation (Espevik and Nissen-Meyer 1986; Danese et al. 2004). So we can consider this process (cytoplasmic expression of CD40) as a strategy for evasion of cytotoxicity and apoptosis due to TNF- α by CD40.

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