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Effect of CD40-CD40 ligand interaction on diacylglycerol-protein kinase C signal transduction pathway and intracellular calcium in cultured human monocytes¹

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KEY WORDS CD40 antigens; CD40 ligand; protein kinase C; diglycerides; monocytes; signal transduction; calcium

ABSTRACT

AIM: To investigate whether CD40-CD40 ligand interaction can activate diacylglycerol (DAG)-protein kinase C (PKC) signaling pathway and intracellular free calcium ($[Ca^{2+}]_i$) in cultured human peripheral blood monocytes (PBMC). **METHODS:** The DAG levels in PBMC were studied with radio-enzymatic assay. Quantitative measurements of ^{32}P -phosphatidic acid were performed by thin-layer chromatography and autoradiography. The activity of PKC and $[Ca^{2+}]_i$ induced by CD40 ligand (CD40L) in PBMC were measured by its ability to transfer phosphate from $[\gamma\text{-}^{32}P]\text{ATP}$ to lysine-rich histone and flow cytometric analysis loading with the Ca^{2+} dye Fluo-3/Am, respectively. **RESULTS:** The DAG levels in PBMC were increased by CD40L in a dose-dependent, biphasic manner. The early phase was rapid and transient, peaking at 20 s; the late phase reached the maximal level at 10 min and then decayed slowly. CD40L increased the PKC total activity in a dose-dependent manner with phase peaking at 12 min, then decreased slowly and maintained for at least 20 min. CD40L induced PKC activity translocation from the cytosol to membrane. Moreover, CD40L also induced biphasic $[Ca^{2+}]_i$ responses including the rapid initial transient phase and the sustained phase. Removal of extracellular Ca^{2+} did not inhibit the rapid phase of CD40L-induced rise in $[Ca^{2+}]_i$, but abolished the sustained phase of $[Ca^{2+}]_i$ response to CD40L. Anti-CD40 monoclonal antibody 10 mg/L significantly suppressed CD40L-induced DAG-PKC signal transduction pathway activation and $[Ca^{2+}]_i$ changes in PBMC. **CONCLUSION:** CD40-CD40 ligand interaction induced a robust stimulation of the DAG-PKC pathway and calcium mobilization from intracellular pool in PBMC.

INTRODUCTION

Activation of DAG-PKC signaling pathway and

intracellular calcium has been shown to induce a variety of cellular changes including regulation of gene expression and seems to be crucial for cellular proliferation or differentiation. DAG, PKC, or $[Ca^{2+}]_i$ is one of important signal transduction elements involved with multiple cell response. Many receptor systems use receptor clustering for transmembrane signaling. It was found that CD40-CD40L interaction mediated the expression of molecules which was thought to be involved in atherosclerosis, such as adhesion molecules, cyto-

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kines, matrix metalloproteinases, and tissue factor^[1,2]. The atherogenic mechanism of CD40 and CD40L interaction is not fully understood. We propose that one potential mechanism that might be responsible for CD40-CD40L interaction-induced cellular response is related to DAG-PKC pathway and $[Ca^{2+}]_i$. Therefore, this study investigates the effects of CD40-CD40L interaction on DAG-PKC signal transduction pathway and $[Ca^{2+}]_i$ changes in PBMC.

MATERIALS AND METHODS

Reagents and instrument RPMI-1640 medium (Gibco, USA); PKC assay system was a Promega product; DAG kit (Code RPN 200) was from Amersham and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from Beijing Institute of Nuclear Research (specific activity $>185\text{ TBq/mmol}$). Fluo-3/Am was from CALBIOCHEM. Others included liquid scintillation counting device LS-5000TD (Beckman, USA) and FACScan (Becton Dickinson, San Jose, CA, USA).

Isolation and culture of monocytes Monocytes were isolated from heparinized blood samples, taken from young healthy volunteers as described before^[3]. Following incubation with hypertonic NaCl thrice, Ficoll 1.077 kg/L gradient centrifugation and adherence, the cells were incubated in RPMI-1640 culture medium at a concentration of 5×10^9 cells/L with the groups of CD40L for indicated period of time.

Quantitative measurement of DAG levels Cells were seeded in 6-well plates at a density of 5×10^6 cells/well in RPMI-1640 containing 20 % fetal calf serum and allowed to attach overnight. The cells were washed twice with RPMI-1640 and incubated with serum-free RPMI-1640 for another 24 h. The cells were again washed twice and stimulated with CD40L at 37 °C for the indicated periods of time and concentrations. The incubation was terminated by extraction of the culture medium and the immediate addition of 1.0 mL cold methanol. Cells were scraped into this methanol and added to 1.0 mL chloroform. The lipids were then extracted by the method described^[4]. Following centrifugation, the lower chloroform phase was analyzed for DAG within 72 h. Each sample was then added to a sample-reaction buffer as described^[5], containing *Escherichia coli* DAG kinase. The DAG kinase phosphorylates DAG to PA on the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ solution. The chloroform phase of the samples was evaporated under vacuum. Each of the dried samples

was re-dissolved in 20 μL of chloroform:methanol (95:5, v/v), and streaked on to silica gel thin-layer chromatography plates. Plates were placed in a running solvent of chloroform (methanol:acetic acid, 85:15, v/v), and the radioactive spots corresponding to PA were visualized with autoradiography, then scraped and quantified by liquid scintillation counting.

Assay of PKC activity PKC activity was determined by its ability to transfer phosphate from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to lysine-rich-histone. Prepared cells were washed twice with cold PBS buffer, suspended in ice cold extraction buffer (Tris-HCl 20 mmol/L, edetic acid 2 mmol/L, pH 7.4, egtazic acid 10 mmol/L, DTT 2 mmol/L, leupeptin 1 mg/L, aprotinin 1 mg/L). The lysate was centrifugated at 14 000 $\times g$ for 15 min. Supernatants (including the component of cytosolic and membrane) were saved for assay of total PKC activity. The cytosolic component was acquired by centrifugating the supernatants. The deposit was suspended in PKC extraction buffer containing 0.5 % TritonX-100, from which membrane protein was obtained through centrifugation at 14 000 $\times g$ for 15 min. All supernatants were purified by DEAE cellulose column. Reactions were started by the addition of the substrates to tubes containing enzyme and lipid vesicles and terminated after 5 min at 30 °C by spotting 60 μL on to P-81 ion-exchange paper (Whatman). Papers were washed in NaCl 50 mmol/L to remove unchanged ATP and dried. Bound radioactivity was determined by liquid scintillation counting.

Measurement of $[Ca^{2+}]_i$ by flow cytometry $[Ca^{2+}]_i$ was measured by flow cytometry^[6]. Briefly, the cells were loaded with the calcium sensitive fluorescein Fluo-3 in PBS containing CaCl_2 1.3 mmol/L, MgCl_2 1.3 mmol/L and glucose 2.7 mmol/L. Cells ($10^9/\text{L}$) were incubated for 40 min with Fluo-3 3 $\mu\text{mol/L}$. Excitation and emission wavelengths were 488 and 530 nm respectively. The fluorescence intensity recordings were performed by individual cell analysis in a flow cytometer at room temperature. Acquisition and analysis were performed with the FACScan and Chronys Research Software. The calibration procedure includes the obtainment of F_{max} and F_{min} , which was achieved by disruption of the cells with digitonin and the ulterior addition of edetic acid 2 mmol/L respectively. Intracellular free calcium, $[Ca^{2+}]_i$, was calculated by the equation: $[Ca^{2+}]_i = K_d (F - F_{\text{min}}) / (F_{\text{max}} - F)$. Where K_d , the dissociation constant for Ca^{2+} -bound Fluo-3 is 400 and F represents the experimental fluorescence intensity

Data analysis Data were expressed as mean \pm SD

and statistically compared by ANOVA. A difference was accepted as significant when $P < 0.05$.

RESULTS

Time curves of DAG levels in PBMC increased by CD40L When the cells were treated with CD40L 10 mg/L for different periods of time, DAG levels were increased in a biphasic manner, with a peak at 20 s and a second peak at 10 min. DAG accumulation was consistently sustained above baseline for at least 30 min. However, there were no changes of DAG levels in control PBMC. After cultured PBMC were incubated with indicated concentration (10 mg/L) of anti-CD40 monoclonal antibody (anti-CD40McAb) for 10 min, the cells were then treated with CD40L (10 mg/L) for different periods of time. Anti-CD40McAb significantly reduced the DAG levels increased by CD40L (Fig 1).

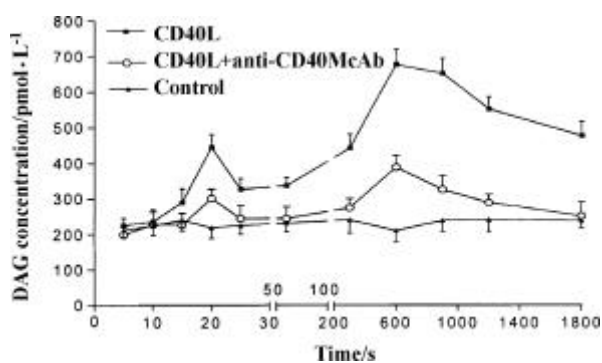


Fig 1. DAG formation by CD40L in PBMC. A biphasic increase of DAG in cultured PBMC induced by CD40. $n=5$. Mean \pm SD.

Effect of different concentrations of CD40L and anti-CD40McAb on DAG levels in PBMC When PBMC were incubated with CD40L (0, 5, 10, 20, and 30 mg/L) at 37 °C for 10 min, CD40L increased DAG from (198 \pm 26) to (768 \pm 58) pmol/L in a dose-dependent manner. The elevated DAG levels were markedly lower than that of the group stimulated by CD40L without anti-CD40McAb pretreatment. DAG levels were not significantly different between anti-CD40McAb 10 mg/L and anti-CD40McAb 20 mg/L pretreatment groups (Fig 2).

Dynamic curve of PKC activity stimulated by CD40L After cells were treated with CD40L 10 mg/L for different periods of time, the PKC total activity was (121 \pm 18) nmol·min⁻¹·g⁻¹(protein) at 0 min and showed a peak [(212 \pm 32) nmol·min⁻¹·g⁻¹(protein)] at

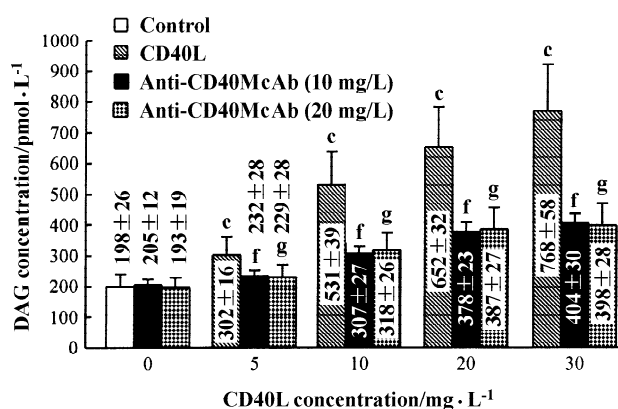


Fig 2. Effect of different concentrations of CD40L and anti-CD40McAb on DAG levels in PBMC. $n=5$. Mean \pm SD. ^c $P < 0.01$ vs control. ^f $P < 0.01$ vs CD40L groups. ^g $P > 0.05$ vs anti-CD40McAb (10 mg/L) groups.

12 min of incubation, which declined to baseline following a sustained phase (20-40 min). However, there was no significant change of PKC levels in control PBMC. When the cells were pretreated with anti-CD40McAb 10 mg/L for 10 min, anti-CD40McAb significantly attenuated the total PKC activity in PBMC induced by CD40L (Fig 3).

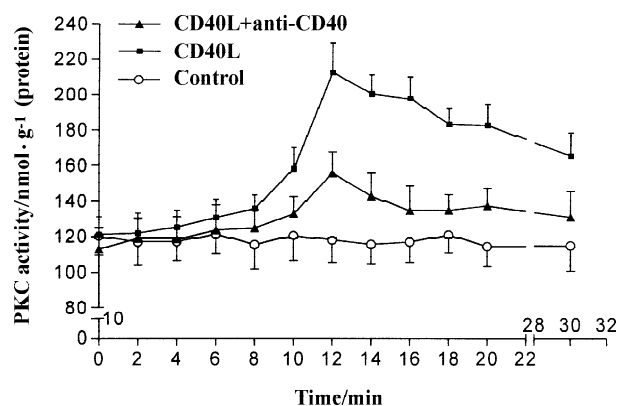


Fig 3. Dynamic curve of PKC activity stimulated by CD40L in PBMC. $n=5$. Mean \pm SD.

Effects of different concentrations of CD40L and anti-CD40McAb on PKC activity in PBMC When cells were incubated with CD40L (0, 5, 10, 20, 30, and 40 mg/L) at 37 °C for 12 min, the total PKC activity was elevated with increasing CD40L concentrations. The total PKC activity was mainly in cytosol at rest. The cell membrane PKC activity was rapidly increased after the cells were stimulated with CD40L (Fig 4A). After pretreatment with anti-CD40McAb 10 mg/L for

10 min, the cell membrane PKC activity was slowly elevated. The PKC activity translocation from the cytosol to membrane was markedly inhibited by anti-CD40McAb (Fig 4B).

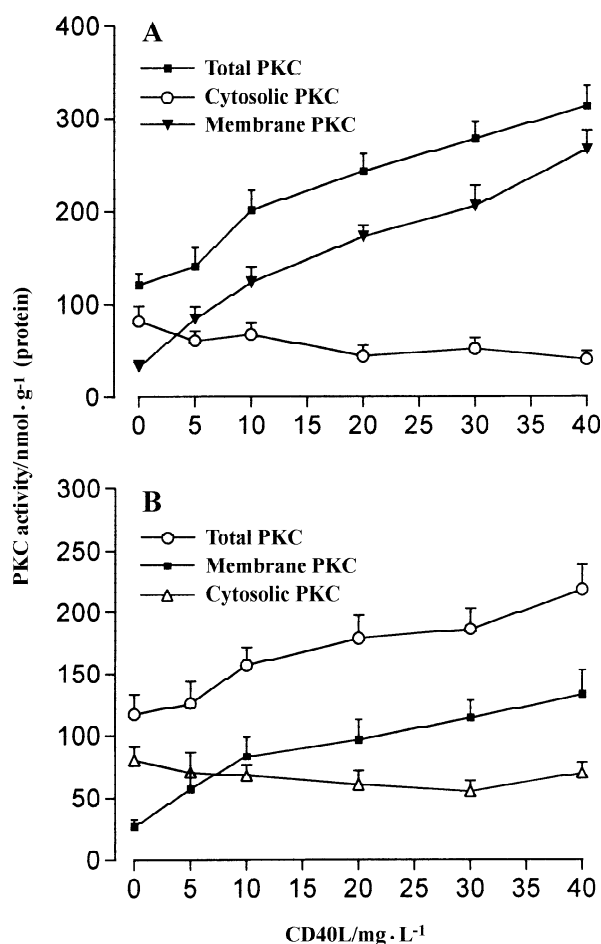


Fig 4. (A) Effects of different concentrations of CD40L on PKC activity in PBMC. (B) Cells were pretreated with anti-CD40McAb 10 mg/L for 10 min and stimulated by the same concentrations of CD40L in PBMC. $n=5$. Mean \pm SD.

Effect of CD40L on intracellular calcium in PBMC When the cells were incubated with CD40L 10 mg/L for different periods of time, CD40L caused a rapid increase in $[Ca^{2+}]_i$ at 50 s and followed by a sustained phase. In anti-CD40McAb-pretreated cells, this biphasic $[Ca^{2+}]_i$ response was significantly depressed. Removal of extracellular Ca^{2+} by addition of edetic acid 1 mmol/L did not significantly affect the initial peak $[Ca^{2+}]_i$ $[(808 \pm 24)$ vs (766 ± 15) nmol/L, $P=0.078$], but markedly inhibited the sustained plateau phase level of $[Ca^{2+}]_i$ (Fig 5).

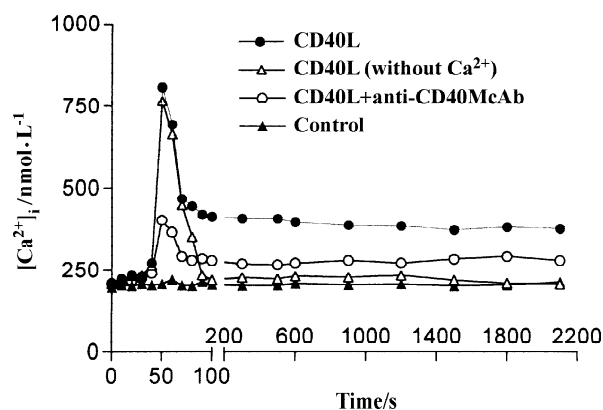


Fig 5. Changes in cytosolic free Ca^{2+} induced by CD40L and anti-CD40McAb in PBMC. $n=3$. Mean \pm SD.

DISCUSSION

The production of DAG is related to two intracellular pathways. One is *de novo* synthesis of DAG from glucose, another is receptor-mediated hydrolysis of membrane phospholipids via G protein, which can activate phospholipase C. As a specific activator, DAG can modulate the molecular conformation of PKC and make PKC viable, which appears to be a key enzyme in signal transduction for the activation of many cellular functions. Moreover, the activation of DAG-PKC signal pathway is related to intracellular free calcium.

The interaction between CD40 and CD40L plays an important role of signal transduction pathways in humoral and cell-mediated immune responses. These signal pathways are mainly focused on NF- κ B and c-jun N-terminal kinase signal pathways, while there is little information concerning the signal transduction of phosphatidylinositol system. Therefore, the effects of CD40-CD40L interaction on DAG-PKC signal pathway and intracellular calcium have not been elucidated.

Our results showed that CD40L not only induced a biphasic formation of DAG and elevation of DAG in a dose-dependent manner, but also mediates CD40L-induced cellular PKC activation. Such a transient and sustained elevation of DAG could presumably occur from phospholipase C-mediated hydrolysis of a major membrane phospholipid such as phosphatidylinositol or phosphatidylcholine. The cellular activation of total PKC activity is mainly due to PKC activity translocation from the cytosol to membrane. All these findings are similar to our previously reports that the activation of DAG-PKC signal pathway is due to the receptor-mediated

pathway via G protein^[7-8].

When PBMC were pretreated with anti-CD40McAb, anti-CD40McAb could significantly attenuate, but not completely, the formation of DAG and the activation of PKC activity. Furthermore, this inhibition was not related to the concentrations of anti-CD40McAb. These results indicated that the changes of DAG levels and PKC activity were induced by the interaction of CD40 and CD40L, anti-CD40McAb and CD40L did not bind to the same site of the cellular surface. We suspected that anti-CD40McAb binding the cells could affect the binding site structure of CD40L. Further studies remained to be done.

IP₃-Ca²⁺ pathway is another signal transduction of receptor-mediated phosphatidylinositol system via G protein. In the present study, we sought to develop a fast FCM kinetic method to evaluate the effect of CD40-CD40L interaction on intracellular Ca²⁺ mobilization in PBMC. The initial peak [Ca²⁺]_i response to CD40L was independent on Ca²⁺ entry and might be due to intracellular Ca²⁺ release from an inositol 1,4,5-triphosphate (IP₃)-sensitive calcium pool. However, whether this effect is mediated by IP₃ remains to be studied.

Recently, many studies have demonstrated that the interaction of CD40-CD40L has been associated with the early formation of atherosclerosis and the long-term atherosclerotic process^[9,10]. It is reasonable to suggest that specific, early interventions directed to the interaction of CD40-CD40L and inhibition of intracellular signal transduction, especially DAG-PKC pathway and cytosolic Ca²⁺ changes, might be a promising new ap-

proach to atherosclerotic disorders.

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