Retinoic Acid-stimulated Intercellular Adhesion Molecule-1 Expression on SK-N-SH Cells: Calcium/Calmodulin-dependent Pathway

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ABSTRACT

Intercellular adhesion molecule-1 (ICAM-1) is an important cell surface adhesion receptor of the immune system. Its cell surface expression on a wide variety of cells, including cancer cells, is regulated by various proinflammatory cytokines. In the present study, we investigated the role of calcium (Ca++) and calmodulin (CaM) in the retinoic acid and IFN-γ signaling in the human neuroblastoma cell line SK-N-SH for up-regulating ICAM-1 expression. A 24-h incubation in the presence of Ca++-mobilizing agents (A23187 and thapsigargin) resulted in the induction of ICAM-1 expression. Both Ca++-mobilizing agents stimulated ICAM-1 expression additively to IFN-γ but not to retinoic acid, suggesting that IFN-γ does not use Ca++ to stimulate ICAM-1, whereas retinoic acid might use it in part. As a second messenger, Ca++ can be coupled with calmodulin. Using calmodulin inhibitors (W7 and calmidazolium), we found that retinoic acid-stimulated, A23187-stimulated, and thapsigargin-stimulated but not IFN-γ-stimulated ICAM-1 were inhibited. Calmodulin signaling elicited by retinoic acid was an early event occurring within the first h of retinoic acid treatment, providing evidence that they may both be coupled to regulate gene expression. Using a novel CaM kinase II inhibitor, KN-62, we demonstrated that retinoic acid stimulated ICAM-1 expression in a CaM kinase II-dependent fashion. The mechanisms whereby CaM kinase II mediates retinoic acid activity on ICAM-1 expression remain to be elucidated.

INTRODUCTION

The induction of a specific immune response requires lymphocyte adhesion to target cells and involves specific antigen receptors on lymphocytes. Additional antigen-independent interactions between adhesion molecules are also crucial to cell-cell contact-mediated immune reaction (1). ICAM-1 serves as counter-receptor for the leukocyte-integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18; Refs. 2–5), in addition to CD43 (6), and as a receptor for the major group of human rhinovirus and strains of Plasmodium falciparum (7–9). ICAM-1 expression is regulated on a wide variety of cells, including cancer cells, by different proinflammatory cytokines (10–13), PMA (14), and retinoic acid (15, 16).

The adhesion system, constituted by LFA-1 and ICAM-1, plays a crucial role in leukocyte adhesion to target cells (3, 17): (a) it increases the avidity of the interaction between the T-cell receptor and antigen in the context of MHC class II molecules (18); (b) it is crucial to T-cell activation in the absence of high levels of MHC class II molecules on antigen-presenting cells (19); and (c) it is important in cell-mediated cytotoxicity (20). It is also involved in leukocyte adhesion to endothelial cells, extravasation, and migration to inflamed tissue sites (21). The coexpression of ICAM-1 and MHC class I antigens on the cell surface of tumor cells is required for their interaction with autologous T-lymphocytes (22, 23). Furthermore, the cytokine-induced enhancement of ICAM-1 expression increases the vulnerability of tumor cells to tumor-infiltrating lymphocyte-, monocyte-, and natural-killer-mediated lysis (24, 25).

Accordingly, a more complete knowledge of the mechanisms underlying the induction of ICAM-1 expression on target cells is important. The intracellular signaling events that participate in the up-regulation of ICAM-1 are not fully understood. Interleukin-1-induced lymphocyte binding to endothelial cells was reported to be cAMP-dependent, although the specific ligand(s) involved in such binding has (have) not been identified (26, 27). We have previously shown that ICAM-1 expression on human tumor cell lines may be transiently stimulated by cAMP-elevating agents. On the other hand, a 24-h treatment with such agents, including prostaglandin E2, resulted in inhibition of both basal and stimulated ICAM-1 expression (28).

The involvement of PKC activation in the regulation of ICAM-1 expression has also been examined with conflicting interpretations (14, 29–36).

Recent work from different laboratories has demonstrated the involvement of calcium influx and the calcium/calmodulin complex as mediators of IFN-γ activity (37–40). The aim of this study was to investigate the role of calcium and calmodulin in retinoic acid and IFN-γ signaling in the human neuroblastoma cell line SK-N-SH for up-regulating ICAM-1 expression. We found that the retinoic acid-stimulated but not the IFN-γ-stimulated ICAM-1 expression on SK-N-SH cells used calcium/calmodulin-dependent pathways involving the activation of CaM kinase II.

MATERIALS AND METHODS

Reagents and Antibodies. All-trans-retinoic acid (Sigma Chemical Co., St. Louis, MO) was prepared monthly in absolute ethanol as a stock solution of 10 mM, kept in the dark at −20°C, and used at a final concentration of 10 μM. Human recombinant IFN-γ with a specific activity of 100 units/ml was kindly provided by Biogen (Cambridge, MA), stored at −70°C in a 0.125 M sodium phosphate solution containing 25 mg/ml human serum albumin and 25 mg/ml sucrose, and used at a final concentration of 10 units/ml in RPMI 1640 (Sigma) containing 10% fetal bovine serum (ν/v). HyClone Laboratories, Logan, VT). H7 [1-(5-isoquinoline-sulfonyl)-2-methyl-piperazine dihydrochloride] and W7 [N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride] were purchased from Calbiochem-Behring Corp. (Mississauga, Ontario, Canada); calmidazolium (R24571) was from Boehringer-Mannheim (Laval, Quebec, Canada); and TSG was from GIBCO/BRL (Burlington, Ontario, Canada). The calcium/calmodulin kinase II inhibitor KN-62 was from Seikagaku America, Inc. (Rockville, MD); PMA and the calcium ionophore A23187 were from Sigma. All were prepared in dimethyl sulfoxide, stored in the dark at −20°C, and diluted in medium before use. [γ-32P]ATP (5000 cpm/mm) was from New England Nuclear/DuPont (Mississauga, Ontario, Canada), and [35S] methionine (800 cpm/mm) was from Amersham Co. (Oakville, Ontario, Canada). Purified anti-ICAM-1 antibody RR1/1 was a gift from M. Bouillon, A. Tcherenof, M. A. Fortier, E. Leblanc, and M. Audette, Prostaglandin E2 inhibits ICAM-1 expression on tumor cells stimulated by retinoic acid and interferon-γ by a cAMP/protein kinase A-dependent pathway, accepted for publication.
gift from Dr. Robert Rothlein (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT). Antibodies were biotin-labeled using the succinimid e ester method (41).

**Cell Culture and Treatment.** The human neuroblastoma cell line SK-N-SH was obtained from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (HyClone Laboratories), 0.2 mL L-glutamine (Sigma), and 35 µg/mL gentamicin (Sigma) and kept in a humidified atmosphere containing 5% CO₂. Cells were passaged on a weekly basis, and the medium was changed twice a week. Cells were treated in standard growth medium containing agonists and/or inhibitors at the indicated concentrations or with vehicle alone. For experiments involving H7, a 3-min preincubation period was done before treatment with the agonists. All the inhibitors remained present during the entire protocol unless mentioned otherwise.

**Assays for CaM Kinase II Activity.** CaM kinase II activity was assayed by measuring the phosphorylation of a calmodulin-dependent protein kinase synthetic peptide as substrate (Peninsula Laboratories, Belmont, CA). Cell extracts were prepared from 1 × 10⁷ cells, untreated or treated with retinoic acid, IFN-γ, or KN-62 for the indicated periods at 37°C. Control or treated cells were placed on ice and rinsed twice with phosphate-buffered saline (0.9% NaCl-0.2% KCl in 10 mM phosphate buffer, pH 7.25). Cells were collected with a rubber policeman in extraction buffer [20 mM Tris (pH 7.5), 0.5 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM EDTA/2 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride; 0.3 mL/60-mm dish] containing protease inhibitors leupeptin (10 µg/mL) and aprotinin (10 µg/mL). All subsequent steps were performed at 4°C. Cells were disrupted by sonication (six 10-s bursts) and centrifuged at 15,000 × g for 5 min. Supernatants were immediately assayed for CaM kinase II activity.

Approximately 5 µg of protein were added to a solution containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 5 mM MgCl₂, 100 µM ATP, 0.2 µCi [γ-³²P]ATP (5000 Ci/mmol), 1 mg/mL bovine serum albumin, 40 µM calmodulin-dependent protein kinase substrate with or without 1 mM EGTA, 1 mM CaCl₂, and 3 µM high purity bovine brain calmodulin (Calbioche-Behring Corp.) in a total volume of 50 µL. After 2 min at 30°C, 25-µL aliquots were spotted onto phosphocellulose paper, which was washed twice with 1% (v/v) H₃PO₄ and twice with water. The amount of incorporated [³²P] was then measured as described by Roskoski (42). The phosphorylation ratio in the absence of added Ca²⁺ relative to the total enzymatic activity defined the percentage of autonomous enzymatic activity and was used to assess CaM kinase II activation.

**Assays for PKC Activity.** The PKC assay system was purchased from GIBCO/BRL (Burlington, Ontario, Canada). Cell extracts were prepared from 1.5 × 10⁶ cells, untreated or treated with PMA, H7, W7, or calmidazolium for the indicated times at 37°C. The PKC assay was assayed by measuring the phosphorylation of acetylated myelin basic protein-derived peptide as described previously (36).

**Protein Synthesis Measurement.** We assessed the effects of maximum nontoxic doses of inhibitors and calcium-mobilizing agents on total cellular protein synthesis by measuring [³⁵S]methionine incorporation as described previously (35, 36). The means of triplicate wells are expressed as a percentage of protein synthesis compared with control cells. Data are the mean ± SEM from three independent experiments.

**Enzymoimmunoassay for ICAM-1 Expression.** Cells (1.5 × 10⁶/well) were seeded in 96-well flat-bottomed microtiter plates (Falcon; Biotech Scientific, Inc., Lachine, Québec, Canada) in 200 µL of growth medium and treated for 24 h. The treatments did not significantly affect cell numbers (data not shown). The enzymoimmunoassays were prepared as described previously (36). Inhibition of protein synthesis was taken into account for normalizing ICAM-1 expression. A 100% expression was attributed to nontreated cells. Data are presented as the mean ± SEM of three independent experiments.

**Statistical Analysis.** Data were analyzed using the paired Student t test.

**RESULTS**

Effects of Calcium-Mobilizing Agents on ICAM-1 Expression. To determine whether calcium plays a role in the up-regulation of ICAM-1 expression, SK-N-SH cells were incubated with 0.01 µM A23187 or 1 nM TSG for 24 h. We first established the maximum nontoxic doses for each inhibitor and agonist, used alone or in combination with retinoic acid or IFN-γ. Cell viability was assessed by propidium iodide exclusion staining and was quantified by flow cytometry (data not shown). We then assessed the effects of these maximum nontoxic doses of agonists and inhibitors on total protein synthesis (Table 1). Some experiments were performed with retinoic acid, IFN-γ, and PMA alone or in combination with the above-mentioned agents. Treatment with retinoic acid, IFN-γ, and PMA alone does not affect protein synthesis and does not change the effects of W7, calmidazolium, and KN-62 (data not shown). The resulting inhibition was taken into account for normalizing ICAM-1 expression.

As quantified by enzymoimmunoassay, the calcium ionophore A23187 and the Ca²⁺-ATPase inhibitor TSG significantly increased by 1.9-fold the basal level of ICAM-1 expression (Fig. 1). As reported previously (15), 10 µM retinoic acid or 100 units/mL IFN-γ stimulated ICAM-1 expression on SK-N-SH neuroblastoma cells by 2.8- and 2.1-fold, respectively (Fig. 1). When A23187 or TSG was combined with retinoic acid, the resulting stimulation of ICAM-1 expression was less than additive (Fig. 1A), whereas it was additive with IFN-γ (Fig. 1B).

**Effects of Calmodulin on the Cell Surface Expression of ICAM-1.** The calcium/calmodulin complex was reported previously to be involved in IFN-γ-induced expression of HLA class II molecules in HL-60 cells (40). Therefore, we tested the effects of two calmodulin antagonists, W7 and calmidazolium (R24751), on ICAM-1 expression. Because these agents may also inhibit PKC, we first assessed their specificity by measuring PKC activity after a 1-h pretreatment in the presence of 10 µM H7, 7 µM W7, or 1 µM calmidazolium followed by a 15-min treatment with 100 nM of PMA to activate PKC. Table 2 shows that, in untreated cells, 76% of the activity was found in the cytosolic fraction. A 15-min treatment with PMA induced a 7-fold increase in total activity with 88% of the activity in the membrane fraction. The translocation of PKC induced by PMA was not blocked by pretreatment with W7 or calmidazolium, whereas pretreatment with H7 resulted in a 50% decrease in membrane activity.

We next used calmodulin antagonists alone or in combination with A23187, TSG, retinoic acid, or IFN-γ to assess the role of calmodulin in the stimulated cell surface expression of ICAM-1. W7 reduced by 75% the retinoic acid-induced expression of ICAM-1 but had no effect on IFN-γ-induced expression or on the basal level of ICAM-1 expression; it also inhibited A23187 activity by 42% (Fig. 2A). Moreover, W7 almost completely abrogated the TSG-induced ICAM-1 expression.

Calmidazolium (R24571) also significantly inhibited the retinoic acid-, A23187-, and TSG-induced ICAM-1 expression but to a lesser extent compared with W7.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Methionine incorporation (%) of control</th>
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<tbody>
<tr>
<td>0.01 µM A23187</td>
<td>86.78 ± 0.19</td>
</tr>
<tr>
<td>1 nM TSG</td>
<td>75.57 ± 0.53</td>
</tr>
<tr>
<td>7 µM W7</td>
<td>89.46 ± 0.52</td>
</tr>
<tr>
<td>1 µM calmidazolium (R24571)</td>
<td>79.05 ± 7.77</td>
</tr>
<tr>
<td>1 µM KN-62</td>
<td>81.32 ± 6.02</td>
</tr>
<tr>
<td>5 µM KN-62</td>
<td>74.99 ± 1.44</td>
</tr>
<tr>
<td>10 µM KN-62</td>
<td>68.32 ± 3.50</td>
</tr>
<tr>
<td>7 µM H7</td>
<td>79.04 ± 1.98</td>
</tr>
</tbody>
</table>

*Cells were treated for 24 h with calcium-mobilizing agents A23187 or TSG or with protein kinase inhibitors H7, W7, R24571, or KN-62 as described in "Materials and Methods." Protein synthesis was assayed by [³⁵S]methionine incorporation. These results are the mean ± SEM of triplicate determinations from two independent experiments. All treatments were significantly different from nontreated cells (p < 0.05).
CaM IN RETINOIC ACID-INDUCED ICAM-1 EXPRESSION

Involvement of CaM Kinase II in Retinoic Acid-stimulated ICAM-1 Expression. To determine whether CaM kinase II was involved in the expression of ICAM-1, cells were treated with a specific CaM kinase II inhibitor, KN-62. As shown in Fig. 4, a 24-h treatment with KN-62 dose dependently inhibited retinoic acid-stimulated ICAM-1 expression by 87% at 10 μM. The inhibitor had no effect on the IFN-γ-stimulating action except when used at 10 μM, where it slightly (by 12%) inhibited ICAM-1 expression.

Next, CaM kinase II activity was quantified to confirm further its involvement in retinoic acid signaling. As shown in Fig. 5, retinoic acid significantly increased CaM kinase II activity (by 3.5-fold), which was inhibited by 10 μM of KN-62. At 10 min, the inhibition of retinoic acid-stimulated CaM kinase II activity by KN-62 was dose-dependent (1 μM, 20%; 5 μM, 44%; 10 μM, 84%). On the other hand, whereas a 3-min incubation in the presence of IFN-γ slightly stimulated CaM kinase II activity (by 1.6-fold), this IFN-γ-stimulated activity returned to a basal level at 10 min.

DISCUSSION

The intracellular signals required for the up-regulation of ICAM-1 expression are complex and involve several second messengers, according to the stimuli and the cell line examined (14, 26—36). It has extent than W7 (Fig. 2B). R24571 failed to inhibit the relative IFN-γ-induced ICAM-1 expression.

Kinetics of Inhibition by Calmidazolium of Retinoic Acid-induced ICAM-1 Expression. We next questioned the early or late-acting involvement of calmodulin in retinoic acid-stimulated ICAM-1 expression. SK-N-SH cells were coincubated with 1 μM calmidazolium for the first 1, 2, 4, and 8 h of retinoic acid treatment, and the level of ICAM-1 expression was then quantified by enzymoimmunoassay after 24 h. A 1-h coincubation of cells with calmidazolium during the first h of retinoic acid treatment was sufficient to inhibit the retinoic acid-inducing activity by 56% (Fig. 3). The maximal inhibition of retinoic acid activity (77%) was observed within a 2-h coincubation.

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ICAM-1 expression are cell-type specific. A23187 promotes cellular influxes of divalent cations, including Ca\(^{2+}\), from extracellular pools (38, 45). However, it also displays some nonspecific membrane effects that could lead to artificial results. Using TSG, an inhibitor of Ca\(^{2+}/Mg\(^{2+}\) ATPase, resulting in Ca\(^{2+}\) release from intracellular pool (46–48), we found that TSG stimulated ICAM-1 expression, further supporting the fact that Ca\(^{2+}\) by itself is able to stimulate ICAM-1 expression. The levels of stimulation obtained with A23187 and TSG were comparable to those using IFN-\(\gamma\) and weaker than those using retinoic acid. Both Ca\(^{2+}\)-mobilizing agents stimulated ICAM-1 expression additively to IFN-\(\gamma\) but not to retinoic acid, suggesting that IFN-\(\gamma\) does not use Ca\(^{2+}\) to the full extent to stimulate ICAM-1, whereas retinoic acid might use it at least in part.

As a second messenger, Ca\(^{2+}\) may be coupled either with PKC activation or calmodulin. We previously found that PKC activation was not required for stimulation of ICAM-1 expression by IFN-\(\gamma\) and retinoic acid (36), indicating that, in this system, Ca\(^{2+}\) might rather be coupled to calmodulin-dependent signalling. Using W-7 and calmidazolium as calmodulin inhibitors, retinoic acid-stimulated, A23187-stimulated, and TSG-stimulated but not the IFN-\(\gamma\)-stimulated ICAM-1 expression, were inhibited. IFN-\(\gamma\) can induce Ca\(^{2+}\) influx into different cell types (37–40), although this influx may not be coupled to IFN-\(\gamma\)-regulated antigen expression. Indeed, Klein et al. (49) found that HLA-DR expression stimulated by IFN-\(\gamma\) in U937 cells is not inhibited by the calmodulin inhibitor W-7 or by the PKC inhibitor H-7. On the other hand, they found that the cell surface expression of other antigens, such as Mo3, CR3, and Fcy receptor, was W-7 sensitive. Using HL-60 cells, Koide et al. (40) have found that

![Graph](image-url)
Ca\(^{2+}\)/calmodulin complex was involved in IFN-\(\gamma\)-induced expression of HLA class II molecules. However, a significant inhibition was obtained only with 20 and 30 \(\mu\)M of W-7, whereas we are using 7 \(\mu\)M of W-7. We found that in SK-N-SH cells, W-7 concentrations higher than 10 \(\mu\)M significantly inhibited total protein synthesis and were cytotoxic (data not shown). Accordingly, we avoided using such high concentrations of inhibitor. The effect obtained with W-7 and calmidazolium was not due to a nonspecific activity on PKC, because the PMA-induced PKC translocation and activation was not affected by treatments with W-7 and calmidazolium, but it was sensitive to H-7.

Very little is known about the relationships between Ca\(^{2+}\), calmodulin, and retinoic acid. Retinoic acid inhibits calmodulin binding to human erythrocyte membranes and reduces membrane Ca\(^{2+}\)-adenosine triphosphatase activity (50). Retinoic acid was able to elicit a rapid rise in [Ca\(^{2+}\)]\(_i\) and to induce phosphorylation of a M\(_{27,000}\) protein in PC12 cells, which was inhibited by W-7. Furthermore, addition of W-7 to culture medium of PC12 cells abolished the choline acetyltransferase activity specifically induced by retinoic acid (51). We provide here further evidence that retinoic acid and calmodulin may be coupled for regulating gene expression. Calmodulin signaling elicited by retinoic acid was an early event occurring within the first h of retinoic acid treatment (Fig. 3), where cotreatment with calmidazolium during the first hour of retinoic acid treatment was sufficient for a 60\% inhibition of retinoic acid activity.

Calmodulin regulates calcium-dependent activation of several proteins, including protein kinases. The multifunctional calmodulin-dependent kinase II (CaM kinase II) has a widespread distribution and is activated by small physiological increases in intracellular Ca\(^{2+}\), which may be overcome by autophosphorylation of the enzyme (52). SK-N-SH cells express a large amount of CaM kinase II (53). Using a novel CaM kinase II inhibitor, KN-62, we found that retinoic acid stimulated ICAM-1 expression in a CaM kinase II-dependent fashion. Furthermore, we confirmed CaM kinase II involvement in retinoic acid signaling by demonstrating that retinoic acid increased by 3.5-fold the CaM kinase II autonomous activity.

Some experiments were done on two cancer cell lines that constitutively express high levels of ICAM-1 (data not shown). In the glioma cell Hs683, as we found in the SK-N-SH cells, the CaM kinase II inhibitor KN-62 abolished the constitutive and retinoic acid-stimulated ICAM-1 expression. The IFN-\(\gamma\)-stimulated expression was not significantly diminished. The melanoma cell line Hs294T also expresses high levels of ICAM-1 and responds to IFN-\(\gamma\) treatment by an increased ICAM-1 expression, but its response to retinoic acid is weak (15). In these cells, KN-62 diminished the constitutive ICAM-1 expression but also inhibited the stimulation induced by IFN-\(\gamma\). This indicates that the signaling mechanisms involved in the regulation of ICAM-1 expression are complex and vary with respect to the cell line examined.

The mechanisms whereby CaM kinase II mediates retinoic acid activity on ICAM-1 expression remain to be elucidated. We found that retinoic acid regulates ICAM-1 gene expression at the transcriptional level, which is dependent on the presence of RAR-\(\beta\) and retinoic acid receptors.\(^5\) It is unlikely that CaM kinase II, a serine/threonine kinase, phosphorylates RAR-\(\beta\) because RAR-\(\beta\) was reported to be phosphorylated on tyrosine residues (54). Furthermore, tyrosine phosphorylation is not required for RAR-\(\beta\) transcriptional activity. Accordingly, a still unidentified early-acting third messenger is probably involved.

\(^5\) F. Aoudjit, M. Bossé, C. Strata, M. Voraberger, and M. Audette, Regulation of ICAM-1 expression by retinoic acid: analysis of the 5' regulatory region of the ICAM-1 gene, in press.
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