Triggering of Human Monocyte Activation through CD69, a Member of the Natural Killer Cell Gene Complex Family of Signal Transducing Receptors

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Summary

The expression and function of CD69, a member of the natural killer cell gene complex family of signal transducing receptors, was investigated on human monocytes. CD69 was found expressed on all peripheral blood monocytes, as a 28- and 32-kD disulfide-linked dimer. Molecular cross-linking of CD69 receptors induced extracellular Ca$^{2+}$ influx, as revealed by flow cytometry. CD69 cross-linking resulted also in phospholipase A2 activation, as detected by in vivo arachidonic acid release measurement from intact cells and by direct in vitro measurement of enzymatic activity using radiolabeled phosphatidylcholine vesicles. Prostaglandin E$_2$ α, 6-keto-prostaglandin F$\text{\tiny{1\alpha}}$, and leukotriene B$_4$ were detected by radioimmunoassay in supernatants from CD69-stimulated monocytes, suggesting the activation of both cyclooxygenase and lipoxygenase pathways after CD69 stimulation. CD69 cross-linking, moreover, was able to induce strong nitric oxide (NO) production from monocytes, as detected by accumulation of NO oxidized derivatives, and cyclic GMP. It is important to note that NO generation was responsible for CD69-mediated increase in spontaneous cytotoxicity against L929 murine transformed fibroblast cell line and induction of redirected cytotoxicity towards P815 FcRII$^+$ murine mastocyte cell line. These data indicate that CD69 can act as a potent stimulatory molecule on the surface of human peripheral blood monocytes.

Materials and Methods

Isolation of Human Monocytes. Human PBMC were isolated from buffy coats by lymphoprep gradient centrifugation (Nycomed Pharma AS, Oslo, Norway) and fractionated on a two-step (43 and 45.5%) Percoll gradient centrifugation. Monocytes obtained from the Percoll interface were usually 80–90% pure as assessed by anti-CD14 staining and flow cytometry analysis. Contaminating T, B, and NK cells were eliminated by negative immunomagnetic selection by using anti-CD2- and anti-CD19-coupled beads (Dynal, Wirral Merseyside, UK). This procedure routinely resulted in a
>95% pure monocyte population, as measured by anti-CD14 staining and flow cytometry analysis.

**Antibodies, Immunofluorescence, and Flow Cytometry Analysis.**

Purified anti-CD69 (anti-Leu23, IgG1) and purified anti-CD45RA (anti-Leu18, IgG1) were from Becton Dickinson (San Jose, CA). Anti-CD69 F(ab')2, and control mouse F(ab')2 were prepared as previously described (16). F(ab')2 goat anti-mouse (GaM) was from Organon Teknika Corp. (Durham, NC). For surface staining, PBMC (1 x 10^7/ml) were incubated for 30 min at 4°C with appropriate amounts of the following directly conjugated antibodies (from Becton Dickinson): PE anti-CD14 (anti-LeuM3), FITC anti-CD69 (anti-Leu23), and isotype-matched FITC and PE control antibodies. Samples were analyzed by flow cytometry on a FACScan® (Becton Dickinson).

**Labeling, Immunoprecipitation, and SDS-PAGE Analysis.**

3 x 10^5 purified monocytes were washed twice and resuspended in Dulbecco's PBS without Ca2+ and Mg2+. 125I labeling and lysis in 0.5 ml 0.5% NP-40 lysis buffer were then performed as previously described (16). Cell lysates were precleared three times with protein G-Sepharose (Sigma Chemical Co., St. Louis, MO) and then incubated for 2 h at 4°C with anti-CD69 bound to protein G-Sepharose. Immunoprecipitates were run on a 10% SDS-PAGE. Gels were fixed, dried, and autoradiographed.

**Ca2+ Flux Measurements.**

Purified monocytes at 10^6/ml were loaded with 1 μg/ml Fluo-3 and 3 μM Fluo-3 (Molecular Probes, Inc., Eugene, OR) for 45 min at 37°C in RPMI-1640 with 1% FCS, which was employed during the whole procedure. Cells were then washed and incubated with 10 μg/ml anti-CD69 F(ab')2 or control mouse F(ab')2. After two more washes at room temperature, cells were kept 10 min at 37°C and immediately analyzed before and after stimulation with 10 μg/ml F(ab')2 GaM by flow cytometry with a FACScan® as previously described (13).

**Arachidonic Acid Release.**

Monocytes were incubated in serum-free medium (RPMI-1640) for 4 h at 37°C with 1 μCi/ml [5, 6, 8, 9, 11, 12, 14, 15-3H]-arachidonic acid (AA) (Amer sham, Bucks, UK). Labeled cells were then washed twice, resuspended in fresh medium containing 0.01% fatty acid-free BSA (Sigma Chemical Co.) and stimulated with 10 μg/ml F(ab')2 anti-CD69 or control mouse F(ab')2 GaM. AA release assay was carried out in a shaking incubator bath at 37°C, and the reaction stopped by centrifugation at 14,000 rpm for 1 min. The 1H content of the supernatant was estimated in a β counter.

**Phospholipase A2 Activity Assay.**

The cells were treated with anti-CD69 mAb (10 μg/ml) or control anti-Leu18 mAb, and GaM (10 μg/ml) for 10 min at 37°C and lysed by sonication. Radiolabeled phosphatidylcholine (PC) vesicles were prepared by sonication of the radiolabeled phospholipid, 1,2-di-palmitoyl [choline-methyl-32P]PC with a specific activity of 153 mCi/mmol (New England Nuclear Inc., Boston, MA). 100 μg of the whole cell lysate was added to 250 μl reaction buffer (50 mM Tris HCl, pH 8.5, 5 mM CaCl2, 5 mM MgCl2, and 0.1% fatty acid-free BSA) containing the vesicles at 1 μM, and incubated at 37°C for 1 h. Phospholipids were then extracted, applied to a Silica Gel TLC plate (Merck, Darmstadt, Germany), and chromatographed as previously described (19). Labeled lyso-PC released from PC was quantitated and expressed as picomoles of lyso-PC per milligrams of protein.

**RIA of Eicosanoids.**

The cells were incubated as described above in serum-free medium in the presence of 10 μg/ml F(ab')2 anti-CD69, or control mouse F(ab')2, in combination with 10 μg/ml F(ab')2 GaM. Commercial kits for eicosanoids were purchased from New England Nuclear (prostaglandin E2 α [PGE2α] and leukotriene B4 [LTC4]) and from Amersham (6-keto-prostaglandin F1 α [PGF1α]).

**Measurement of Nitric Oxide Synthesis.**

Nitric oxide (NO) synthesis was measured as NO2− accumulation. In aqueous solution, NO reacts rapidly with O2 and accumulates in the culture medium as NO2− and NO3− ions. Monocytes were incubated with 10 μg/ml F(ab')2 anti-CD69 or control mouse F(ab')2, together with 10 μg/ml F(ab')2 GaM, and 100 μl of the culture supernatants were removed for NO2− measurement at the indicated times. NO2− was quantified by a colorimetric assay based on the Griess reaction. The OD was measured at 550 nm using a micro-ELISA reader (Easy Reader EAR 400; Kontron Analytic, Milan, Italy).

**Cyclic GMP Assay.**

Monocytes were incubated with 10 μg/ml F(ab')2 anti-CD69 or control mouse F(ab')2, together with 10 μg/ml F(ab')2 GaM. The reaction was stopped at the indicated times by addition of ice-cold TCA (10%, wt/vol, final concentration). The extraction and the assay were performed as directed in the RIA kit (New England Nuclear).

**Cytotoxicity Assay.**

Assay of monocyte redirected cytotoxicity against murine mastocytoma cell line P815 was performed by incubating monocytes with 5 x 10^5 31Cr-labeled (Na231CrO4; New England Nuclear) tumor cells in triplicate wells, in the presence of 10 μg/ml anti-CD69 or anti-CD45RA mAbs. After 18 h incubation, supernatant was removed from each well and counted. Assay of cytotoxic activity against fibroblast-transformed cell line L929 was performed by incubating monocytes with 5 x 10^5 3H thymidine-labeled (New England Nuclear, Inc.) cells, in the presence of 10 μg/ml F(ab')2 anti-CD69 or control mouse F(ab')2, and 10 μg/ml F(ab')2 GaM. After 18 h incubation, supernatant was removed from each well and counted. The percentage of specific lysis was calculated as follows: 100 × ([experimental release - spontaneous release]/[maximum release - spontaneous release]). The NO synthase inhibitor, L-NAME (N-monomethyl arginine (L-NAME; Wellcome, Beckenham, Kent, UK), was used at 0.5 mM and had no effect on target cell viability or spontaneous [3H]thymidine or 31Cr release.

**Results and Discussion.**

**Monocytes Constitutively Express CD69.**

To determine whether resting monocytes express CD69, freshly purified PBMC were stained with PE anti-CD14 and FITC anti-CD69 mAb. Isotype-matched FITC and PE mouse Ig were used as negative controls. Samples were analyzed on a FACScan® and data were presented in the form of a two-parameter contour plot. Fig. 1 A shows that circulating monocytes, as identified by CD14 expression, constitutively express CD69 molecules. SDS-PAGE analysis of anti-CD69 immunoprecipitates from purified monocytes showed a diffuse band of ~50-60 kD under nonreducing conditions, due to the different dimeric combinations, and two bands of 28 and 32 kD under reducing conditions (Fig. 1 B), indicating that CD69 on monocytes is biochemically similar to that described on lymphocytes and platelets (7, 16).

**CD69 Cross-linking Induces Ca2+ Influx and Phospholipase A2 Activation in Monocytes.**

CD69 cross-linking has been shown to induce [Ca2+]i elevation in lymphocytes, platelets, and granulocytes (14, 16, 20). To establish whether CD69 molecules were able to efficiently transduce a signal in monocytes, we initially measured intracellular [Ca2+] levels after stimulation with F(ab')2 anti-CD69, together with GaM F(ab')2 to maximize antibody cross-linking. Fig. 2 A shows...
that CD69 triggering in monocytes results in rapid and sustained [Ca$^{2+}$]$_i$ elevation. This is most likely due to extracellular Ca$^{2+}$ influx, since EGTA addition to the medium completely abolished CD69-induced intracellular [Ca$^{2+}$]$_i$ elevations.

CD69 stimulation on platelets has been shown to result in selective phospholipase A2 (PLA2) activation, and to be sufficient to lead to platelet degranulation and aggregation (19). To determine whether monocyte CD69 activates PLA2, monocytes were labeled with $^3$H-AA and stimulated with anti-CD69 mAbs. Fig. 2 B shows that $^3$H-AA-labeled monocytes released high amounts of radioactive species in the medium after CD69 stimulation. To directly measure PLA2 enzymatic activity, cell extracts from CD69-stimulated monocytes were incubated with vesicles containing radiolabeled PC. After the in vitro reaction, phospholipids were extracted and chromatographed on TLC plates, and lyso-PC was detected and quantitated. Fig. 2 C shows that cell extracts from CD69-stimulated monocytes caused substantial release of lyso-PC from PC vesicles, demonstrating that CD69 is functionally associated to a PLA2 in monocytes. Taken together, these data indicate that CD69 receptors may operate a very similar signal-transducing apparatus, which includes extracellular Ca$^{2+}$ influx and PLA2 activation, in different cell types.

**CD69 Cross-linking Induces Production of both Cyclooxygenase and Lipoxygenase Metabolites.** Most AA produced after PLA2 activation is oxidized through two main pathways, the lipoxygenase pathway, producing leukotrienes, and the cyclooxygenase pathway, giving rise to prostanoids. Both leukotrienes and prostanoids are important mediators of inflammation (21). We therefore investigated whether AA was undergoing metabolic oxidation in monocytes after CD69 triggering. Fig. 3 shows that CD69 cross-linking in monocytes induced both cyclooxygenase and lipoxygenase products, as indicated by the release in the medium of CD69-stimulated monocytes of amounts of PGE$_2$,$\alpha$, 6-keto-PGF$_1$$\alpha$, and LTB$_4$ comparable with those generated by LPS stimulation. Considering

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**Figure 2.** Anti-CD69-induced Ca$^{2+}$ signaling and PLA2 activation in human monocytes. (A) Purified monocytes were loaded with Fluo-3, labeled with 10 μg/ml anti-CD69 F(ab')$_2$ (open triangles) or control mouse F(ab')$_2$ (open circles) and analyzed by FACS® before and after stimulation at time 0 with 10 μg/ml F(ab')$_2$ GaM. Data from one donor out of six tested, giving similar results, are shown. (B) Monocytes were labeled with $^3$H-AA and stimulated with 10 μg/ml anti-CD69 F(ab')$_2$ (open triangles) or control mouse F(ab')$_2$ (open circles) and radiolabeled with $^3$H-AA before and after stimulation at time 0 with 10 μg/ml F(ab')$_2$ GaM. Data are expressed as percent increase in $^3$H-AA release compared with unstimulated samples. Data from one donor out of six tested, giving similar results, are shown. (C) Cell lysates from unstimulated, anti-CD45RA or anti-CD69 stimulated monocytes, were incubated with radiolabeled PC vesicles for 1 h at 37°C. Then phospholipids were extracted and chromatographed, and radiolabeled lyso-PC was quantitated. Results are expressed as percent increase in lyso-PC release from PC vesicles compared with unstimulated samples. Data from one donor out of two tested, giving similar results, are shown.
in vivo, cross-linking of monocyte CD69 receptors in vivo effects exerted by AA metabolites on several different cell types

A shows that, CD69 cross-linking with F(ab')2 anti-CD69 the wide variety of immunological, vasoactive, and metabolic properties, including the capacity to directly kill parasites, tumor cells, and bacteria (22-25). NO is extremely unstable in aqueous solution, as it is rapidly oxidized to NO2- and NO3-.

Moreover, NO stimulates guanylate cyclase activity with consequent generation of cyclic guanylate (cGMP). NO can be indirectly measured, therefore, by quantitating NO2- accumulation and cGMP generation. To investigate the possibility that stimulation of CD69 in monocytes resulted in NO production, we evaluated NO2- accumulation and cGMP generation in anti-CD69-treated monocytes. Fig. 4 reveals that, CD69 cross-linking with F(ab')2 anti-CD69 plus F(ab')2 GaM, induced increasing amounts of NO2- over time. In contrast, LPS stimulation, alone or in combination with IFN-γ (data not shown), was largely ineffective. CD69-dependent NO2- accumulation was due to NO generation by NO synthase, since NO synthase inhibitor t-NMMA completely abolished NO2- accumulation. Accordingly moreover, unlike LPS (or LPS plus IFN-γ, data not shown), CD69 stimulation caused significant cGMP generation in monocytes (Fig. 4 B). In contrast to the murine system, where the induction of relevant NO production from mononuclear phagocytes is induced by two synergistic signals, including IFN-γ, IFN-α, or IFN-β plus LPS, or IFN-γ plus TNF-α or TNF-β (27, 28), human monocytes are largely unresponsive to cytokines, even in combination with LPS (29). It is interesting to note that human monocytes have been shown to produce NO only after interaction with tumor cells, but not with untransformed cells, although the receptors involved in these interactions remained obscure (29). As we observed that cross-linking of monocyte CD69 receptors induces release of high amounts of NO, this suggested that CD69 cross-linking could also trigger NO-dependent cytotoxicity programs in monocytes.

**Concluding Remarks.** Primary sequence analysis and chromosomal mapping have recently allowed enlistment of CD69 in the NKC family of signal-transducing receptors (32). Members of this family, which includes the NKG2 receptors in humans, the NKR-P1 receptors in the rat, and the Ly-49

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**Figure 3.** Production of PGE2α, 6-keto-PGF1α, and LTB4 upon CD69 stimulation. Purified monocytes were incubated at 10^6/ml in serum-free medium in the presence of 10 μg/ml anti-CD69 F(ab')2 (shaded column), or mouse F(ab')2 control mAb (black column), in combination with 10 μg/ml F(ab')2 GaM. 10 μg/ml LPS (Sigma Chemical Co.) was used as positive control (open column). After 12 h, PGE2α, 6-keto-PGF1α, and LTB4 accumulated in the supernatants were measured by RIA. Data are expressed as mean ± SD from five donors tested.

**Figure 4.** NO production from CD69-stimulated monocytes. (A) Purified monocytes were stimulated with 10 μg/ml LPS (closed circles), 10 μg/ml anti-CD69 F(ab')2 in the absence (closed triangles) or in the presence (open triangles) of 0.5 mM t-NMMA, or mouse F(ab')2 control mAb (open circles), in combination with 10 μg/ml F(ab')2 GaM. NO2- was revealed by the Griess reaction, 4, 12, 24, and 48 h after the stimulation. Data are expressed as mean ± SD from five donors tested. (B) Purified monocytes were stimulated with 10 μg/ml LPS (open triangles), 10 μg/ml anti-CD69 F(ab')2 (closed triangles), or mouse F(ab')2 control mAb (open circles), in combination with 10 μg/ml F(ab')2 GaM. Cell extracts were processed at the indicated time points for cGMP detection by RIA. Data are expressed as mean ± SD from four donors tested.
receptors in the mouse, are expressed mainly on NK cells, share a remarkable sequence homology, similar topology, and molecular assembly. All members of the family, in fact, are disulfide-linked homodimers, formed by type II single transmembrane glycoproteins, with a C-type lectin binding domain in the extracellular portion of the molecule. Moreover, NKC family members are all able to generate an intracellular signal upon molecular cross-linking. However, a number of features clearly distinguish CD69 from other members of the family. NKG2, NKR-P1, and Ly-49 receptors, in fact, are coded for by clusters of crosshybridizing genes, some displaying allelic polymorphism and possible posttranscriptional regulation by alternative splicing, whereas CD69 is the only product of a single nonpolymorphic gene. Moreover, expression of most NKC family receptors appears restricted almost exclusively to NK cells, in a constitutive fashion. On the contrary, CD69 is expressed only upon activation of lymphocytes, including NK cells. Finally, evidence has accumulated indicating that CD69 may be expressed on other hematopoietic cell lineages (14-17).

Here we show that CD69 is constitutively expressed on human circulating monocytes and that it is likely to represent an important route to monocyte activation, as cross-linking of CD69 receptors is sufficient to induce production of key soluble mediators and to trigger NO-dependent cytoxic activity towards transformed cell lines. Only the identification of natural CD69 ligands, however, will reveal the biological significance of monocyte CD69 expression, and the possible relevance in inflammation processes and tumor clearance in vivo.

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