Chloroquine, an Anti-malarial Agent, Acts as a Novel Regulator of β 1-Integrin-Mediated Cell–Cell Adhesion

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Chloroquine is one of the disease-modifying antirheumatic drugs (DMARDs) with anti-malarial effect. In this study, we examined the modulatory effect of chloroquine on the functional activation of β 1-integrins (CD29) using CD29- and CD98 (a functional regulator of CD29)-mediated U937 cell–cell adhesion, comparing macrophage functions and T cell proliferation. Chloroquine effectively suppressed U937 cell–cell adhesion mediated by CD29 and CD98, in a protein kinase (PK) C, PKA, protein tyrosine kinase (PTK), extracellular signal-regulated kinase (ERK) and actin cytoskeleton-independent manner. Other lysomotrophic agents (monesin, methylamine and ammonium chloride) also significantly diminished both CD29- and CD98-mediated cell–cell adhesion, indicating that lysomotrophic character may play a critical role in regulating β 1-integrin functions. Therefore, these results suggest that chloroquine may act as a novel regulator of CD29 function in a lysomotrophic character-dependent novel manner.

Key words chloroquine; cell adhesion; CD29 (β 1-integrins); CD98; lysomotrophic agent

Chloroquine is a disease-modifying antirheumatic drug (DMARDs) that has been used for the treatment of malaria and autoimmune diseases. The therapeutic effectiveness of chloroquine has been thought to act by accumulating within and alkalinizing acidic compartments such as endosomes and lysosomes up to millimolar levels by protonation in low pH environments.¹⁾ Consequently, this compound has been attributed to its ability to kill various parasites causing malaria,²⁾ to increase apoptosis of T lymphocytes³⁾ and to decrease the production of pro-inflammatory cytokines by a NF- κ B-independent pathway composed of Raf/extracellular-signal regulated kinase (ERK).⁴⁾

β 1-Integrins (CD29) are key adhesion molecules in regulating various cell–cell and cell–matrix adhesions, playing a critical role in many different aspects of immunobiology.⁵⁾ Cell adhesion events are involved in cell localization, effector recognition, and activation phenomena, as demonstrated between T cells and antigen-presenting cells, cytotoxic T cells and their targets and lymphocytes and endothelium.⁶⁾ In particular, over-activation of CD29 and subsequent up-regulation of cell adhesion events are found in various cancers and numerous inflammatory and autoimmune diseases.^{7,8)} These facts led us to the assumption that blocking the functional activation of CD29 could therefore be a therapeutic goal for the suppression of relevant diseases.⁹⁾ For these purposes, we established quantitative screening models to develop novel CD29 function regulators using specific agonistic antibodies to CD29 and CD98, a heterodimeric membrane protein closely associated with CD29,^{5,10)} and matrix protein fibronectin to functionally activate CD29 in U937 cells.^{11,12)}

Using the quantitative models, we have previously reported novel natural and synthetic compounds,^{11–13)} some of which are now being tested in some CD29-mediated disease models. Since chloroquine has been found to be a good potential regulator of CD29, we further investigated the inhibitory potency of chloroquine under functional activation of CD29 (β 1-integrins) and its putative mechanism in terms of chemical and pharmacological features. Our current data are therefore the first paper proposing that chloroquine can

act as an effective modulator of β 1-integrins.

MATERIALS AND METHODS

Animals and Materials Chloroquine, genistein, cytochalasin B, methylamine, monensin, and ammonium chloride were purchased from Sigma (St. Louis, MO, U.S.A.). U0126, SB203580, GF109203X (GFX), rottlerin, and KT5720 were obtained from Calbiochem (La Jolla, CA, U.S.A.). Fetal bovine serum (FBS) was obtained from GIBCO (Grand Island, NY, U.S.A.). U937 (a promonocytic cell line) cells were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). All other chemicals were Sigma grade. Aggregation-inducing antibodies to CD98 (ANH-18, purified IgG1), CD29 (MEM 101A, purified IgG1) and CD43 (161-46, ascites, IgG1) and function-blocking antibodies to CD18 (CLB-LFA1, purified IgG1), CD29 (P5D2, purified IgG1), CD43 (148-1B6, ascites, IgG1), CD49c (ASC3, purified IgG1), CD49d (L25, purified IgG1), CD49e (IIA1, purified IgG1), CD98 (BU89, purified IgG1) and CD147 (MEM M6/1, purified, IgG1) were used as reported previously.^{13–15)} FITC-labeled CD29 and CD98 antibodies were obtained from Immunotech (Marseilles, France). Rhodamine phalloidin was purchased from Molecular Probe (Carlsbad, CA, U.S.A.).

Cell–Cell or Cell–Extracellular Matrix Protein (Fibronectin) Adhesion Assay U937 cell adhesion assay was performed as previously reported.^{12,14)} Briefly, U937 cells maintained in complete RPMI1640 medium (supplemented with 100 U/ml of penicillin and 100 μ g/ml of streptomycin, and 10% FBS) were pre-incubated with chloroquine for 1 h at 37 °C and further incubated with function-activating (agonistic) antibodies (1 μ g/ml) in a 96-well plate. After a 3-h incubation, cell–cell clusters were determined by homotypic cell–cell adhesion assay using a hemocytometer¹²⁾ and analyzed with an inverted light microscope equipped with a COHU high-performance CCD (Diavert) video camera. For the cell–fibronectin adhesion assay, chloroquine-treated U937 cells (5×10^5 cells/well) were seeded on a fibronectin

(50 $\mu\text{g/ml}$)-coated plate and incubated for 3 h.¹⁶ After removing unbound cells with PBS, the attached cells were treated with 0.1% crystal violet for 15 min. The OD value at 540 nm was measured by a Spectramax 250 microplate reader.

Flow Cytometric Analysis Expression of U937 surface adhesion molecules was determined by flow cytometric analysis as previously reported.¹² Stained cells were analyzed on a FACScan (Beckton-Dickinson, San Jose, CA, U.S.A.).

Confocal Microscopy Confocal analysis of actin and CD29 or CD98 co-localization in U937 cells (1×10^5 cells/ml) treated with chloroquine was performed on glass-bottom culture dishes (MatTek Corp., Ashland, MA, U.S.A.) using FITC-labeled antibodies to CD29 or CD98 and rhodamine pallodin. Intensity changes in CD29 and CD98 were imaged by LSCM on a Zeiss LSM 510 META confocal microscope equipped with a Zeiss 37 °C incubation system. Images were analyzed using the Zeiss LSM Image Examiner.

Immunoblotting Cells (5×10^6 cells/ml) were lysed in lysis buffer (in mM: 20 Tris-HCl, pH 7.4, 2 EDTA, 2 EGTA, 50 β -glycerophosphate, 1 sodium orthovanadate, 1 dithiothreitol, 1% Triton X-100, 10% glycerol, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin and 10 $\mu\text{g/ml}$ pepstatin, 1 benzimidazole and 2 hydrogen peroxide) for 30 min rotating at 4 °C. Lysates were clarified by centrifugation at $16000 \times g$ for 10 min at 4 °C. Soluble cell lysates were immunoblotted and phospho-ERK levels were visualized as previously reported.¹²

Statistical Analysis A Student's *t*-test and one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data are expressed as means $\pm$ standard errors (S.E.M.) and the results are taken from at least three independent experiments performed in triplicate. *p* Values of 0.05 or less were considered to be statistically significant.

RESULTS AND DISCUSSION

Before starting our experiments, we confirmed whether U937 cell adhesion induced by CD29 antibody (MEM101A) or fibronectin was mediated by functional activation of $\beta 1$ -integrins using specific function-blocking antibodies to CD29 or other $\beta 1$ -integrin-associated proteins such as CD49, CD98 and CD147. As expected, blocking antibodies to CD29, CD49d, CD49e, and associated proteins (CD98 and CD147) were significantly blocked U937 cell-cell adhesion, while cell-fibronectin adhesion was only suppressed by CD29 blocking antibodies (P5D2 and MAR4) (Fig. 1). Furthermore, ERK and p38 inhibitors U0126 and SB203580 and actin polymerization inhibitor cytochalasin B blocked not cell-fibronectin adhesion but CD29-mediated cell-cell adhesion (Fig. 1). These data therefore indicate that cell-cell and cell-fibronectin adhesions are commonly mediated by molecular activation of CD29, but the involvement of $\beta 1$ -integrin associated molecules (CD98 and CD147), intracellular signaling events, and actin cytoskeleton may be distinct between cell-cell or cell-fibronectin adhesion. Unlike cell-cell adhesion with complicate intracellular signaling process, rather it is thought that cell-fibronectin adhesion may simply require the molecular activation of CD29 itself for binding to fibronectin.

Fig. 1. Effect of Blocking Antibodies to Adhesion Molecules or Signaling Enzyme Inhibitors on Cell-Cell and Cell-Fibronectin Adhesion

(A) U937 cells (1×10^6 cells/ml) pretreated with 10 $\mu\text{g/ml}$ of function-blocking antibodies (to CD29, CD18, CD43, CD44, CD49c, CD49d, CD49e, CD98 or CD147) or inhibitors [U0126 (20 μM), SB203580 (10 μM) and cytochalasin B (5 μM)] were incubated either in the presence or absence of pro-aggregative (agonistic) antibodies (1 $\mu\text{g/ml}$ each) to CD29 (MEM 101A) for 3 h. Quantitative analysis of cell-cell clusters was assessed by quantitative cell-cell adhesion assay as described in Materials and Methods. (B) U937 cells (5×10^5 cells/well) pretreated with 10 $\mu\text{g/ml}$ of function-blocking antibodies (to CD29, CD18, CD43, CD98 or CD147) or inhibitors [U0126 (20 μM), SB203580 (10 μM) and cytochalasin B (5 μM)] were seeded on fibronectin (50 $\mu\text{g/ml}$)-coated plates and further incubated for 3 h. Attached cells were determined by crystal violet assay, as described in Materials and Methods. Data represent mean $\pm$ S.E.M. of three independent observations performed in triplicate. **p* < 0.05, ***p* < 0.01 compared to control.

Interestingly, we observed that chloroquine can act as a novel effective modulator of the functional activation of $\beta 1$ -integrin as assessed by CD29- and CD98-mediated cell-cell adhesion. Thus, this compound dose-dependently blocked cell-cell adhesion induced by agonistic antibodies to CD29 and CD98, but not CD43 (Fig. 2A) with IC_{50} values of 79 and 48 μM , respectively without displaying cytotoxicity (data not shown). However, this compound did not block U937 cell-fibronectin adhesion (Fig. 2B). This suggests that not a molecular process managing a simple interaction between CD29 and matrix (or even other counter surface molecules), but cellular events activating CD29 function for mediating cell-cell interaction may be the favorable target of chloroquine.

To understand the potential pharmacological mechanism, chemical and cell biological features of chloroquine were evaluated. As chloroquine has a lysosmotrophic character and tends to be accumulated in the lysosomes of cells, which is believed to account for its anti-malarial and anti-inflammatory activity,^{2,17} other lysosmotrophic agents such as methylamine, ammonium chloride and monensin were evaluated

Fig. 2. Effect of Chloroquine on Cell-Cell and Cell-Fibronectin Adhesion

(A) Left panel: U937 cells pretreated with chloroquine were incubated either in the presence or absence of pro-aggregative (agonistic) antibodies ($1 \mu\text{g/ml}$ each) to CD29 (MEM 101A), CD98 (ANH-18) or CD43 (161-46) for 3 h. Quantitative analysis of cell-cell clusters was assessed by quantitative cell-cell adhesion assay as described in Materials and Methods. Images of the cells in culture (right panel) were obtained using an inverted phase contrast microscope attached to a video camera. (B) U937 cells pretreated with chloroquine (left panel) were seeded on fibronectin ($50 \mu\text{g/ml}$)-coated plates and further incubated for 3 h. Attached cells were determined by crystal violet assay, as described in Materials and Methods. Data represent mean $\pm$ S.E.M. of three independent observations performed in triplicate. * $p < 0.05$, ** $p < 0.01$ compared to control.

under the same conditions. Similarly, these three agents significantly suppressed CD29- and CD98-mediated cell-cell adhesion up to 60%, without altering cell viability (Fig. 3A), suggesting that the lysomotrophic property may play a critical role in negative modulation of cell-cell adhesion. Next, to judge whether chloroquine was able to diminish the surface level of CD29 or CD98, flow cytometric and confocal analyses of these molecules were performed. As shown in Fig. 3B and 3C, 17 to 20% of surface levels were decreased by chloroquine exposure, although confocal data could not distinguish this difference clearly. Despite this, the data are significant, however, the facts that 1) the inhibition percentages are still so weak as not to fully explain its anti-cell adhesion effect and 2) the binding activity of CD29 to fibronectin were not blocked by chloroquine, suggested the possibility of another mechanism.

Complicated intracellular signaling events, leading to the functional activation of $\beta 1$ -integrins and subsequent cell adhesion have been reported in numerous papers. In particular, we and other groups have previously found that various signaling enzymes are able to positively and negatively modulate CD29 and CD98-induced cell-cell adhesion.^{5,11,12} As Fig. 3D also shows, genistein, a protein tyrosine kinase (PTK) inhibitor, GF109203X, a conventional protein kinase (PK) C inhibitor and KT5720, a PKA inhibitor, remarkably up-regulated CD98-induced cell-cell adhesion, suggesting that these enzymes can act as negative regulators of the cell-cell event. In contrast, the actin polymerization inhibitor cytochalasin B and novel PKC δ inhibitor rottlerin strongly blocked CD98-mediated cell-cell adhesion up to 70 or 65%, indicating that cytoskeleton rearrangement and PKC δ play critical roles in modulating cell-cell clustering, as previously reported.^{12,14} Whether chloroquine-mediated inhibition is due to suppression of actin cytoskeleton rearrangement or PKC δ activation, the pharmacological patterns of these inhibitors were compared using co-treatment of genistein with CD98 function-agonistic antibody (ANH-18). While rottlerin

and cytochalasin B maintained their inhibitory potency under these conditions, the inhibitory effect of chloroquine was abrogated up to 30% inhibition from 70% (Fig. 3D), indicating that neither PKC δ activation nor actin cytoskeleton may act as the target of chloroquine, and that the PTK inhibition state may be involved in attenuating chloroquine-mediated inhibition. Confocal data (Fig. 3C) also strongly supported an effect of chloroquine on actin cytoskeleton. That is, there was no clear inhibition of co-localization between actin and CD29 or CD98.

According to our results, PTK, PKA, PKC and even cytoskeleton rearrangement may not be the pharmacological target of chloroquine. The previous paper reported that chloroquine acted as a Raf-1 inhibitor suppressing ERK phosphorylation. Since ERK activation is required for CD98-mediated cell adhesion, the potential involvement of the ERK pathway was explored. Several lines of evidence demonstrated that 1) two strong inhibitors, cymaropicrin and rottlerin, known to block the CD98-mediated cell adhesion event *via* blocking ERK activation^{11,14} exhibited a completely different inhibitory pattern during co-treatment of genistein with anti-CD98 antibody (Fig. 3D) and 2) this compound did not diminish CD98-mediated ERK phosphorylation (Fig. 3E), however, seem to exclude the possibility that ERK is a target of chloroquine. Rather, it is regarded that certain acidic compartments such as lysosome may play an important role in regulating CD29- or CD98-mediated cell-cell adhesion. Acidic compartments produce proteolytic enzymes (such as caspases) and various intracellular signaling molecules such as ceramide, involved in regulating cell-cell interaction.³ Although there is currently no clear evidence as to whether lysosome-derived molecules participate in a cell-cell adhesion events, our results suggest such a possibility, requiring additional experiments to prove this assumption.

Conclusively, we found that chloroquine, like other lysomotrophic agents, effectively modulated monocytic cell-cell

Fig. 3. Putative Inhibitory Mechanism of Cell-Cell Adhesion by Chloroquine

(A, D) U937 cells (1×10^6 cells/ml) pretreated with lysosomotropic agents (methylamine, monensin and ammonium chloride) (A left and right panels) or various inhibitors (D) were incubated either in the presence or absence of antibodies to CD98 (A left panel) and CD29 (A right panel) ($1 \mu\text{g/ml}$ each) for 3 h. Quantitative analysis of cell-cell clusters was assessed by quantitative cell-cell adhesion assay as described in Materials and Methods. Data represent mean $\pm$ S.E.M. of three independent observations performed in triplicate. (B) U937 cells (1×10^6 cells/ml) were treated with chloroquine ($100 \mu\text{M}$) for 2 h. Surface levels of CD29 and CD98 were determined by flow cytometry as described in Materials and Methods. Results show one experiment out of three. (C) U937 cells (1×10^6 cells/ml) were treated with chloroquine ($100, 150 \mu\text{M}$) for 2 h. The surface and co-localization levels of actin and CD29 or CD98 were assessed by confocal microscopy as described in Materials and Methods. Results show one experiment out of three. (E) RAW264.7 cells (5×10^6 cells/ml) were stimulated with chloroquine ($100 \mu\text{M}$) in the presence or absence of antibody to CD98 ($1 \mu\text{g/ml}$) for 30 min. After immunoblotting, the levels of phospho-ERK were identified by phospho-specific ERK antibody. Results represent one experiment out of three. * $p < 0.05$, ** $p < 0.01$ compared to control.

adhesion mediated by CD29 and CD98, with lysomotropic character in a novel manner independent of PKC, PTK, PKA, ERK and the actin cytoskeleton. Since there are no reports showing that lysomotropic agents such as chloroquine act as negative regulators of the functional activation of β 1-integrins, our results are believed to open new avenues of relevant scientific studies regarding β 1-integrin-mediated cell-cell adhesion associated with several pathological events, including tumor metastasis, inflammatory states, granuloma formation and blood vessel occlusion.

Acknowledgements This work was supported by a grant (KRF-2006-311-C00455) from KRF, Korea. The author wishes to thank to Miss Joo Young Kim and Mr. Byung Hun Kim for their technical assistance, to The Central Laboratory of KNU, and to the Korea Basic Science Institute (in Chuncheon) for flow cytometric and confocal analyses.

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