Spreading of HeLa Cells on a Collagen Substratum Requires a Second Messenger Formed by the Lipoxygenase Metabolism of Arachidonic Acid Released by Collagen Receptor Clustering

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HeLa cells attach to a variety of substrata but spread only on collagen or gelatin. Spreading is dependent on collagen-receptor upregulation, clustering, and binding to the cytoskeleton. This study examines whether second messengers are involved in initiating the spreading process on gelatin. The levels of cytosolic free calcium ($[Ca^{++}]_i$), cAMP, and cytoplasmic pH (pH_i) do not change during cell attachment and spreading. However, a basal level of $[Ca^{++}]_i$ and an alkaline pH_i are required for spreading. There is an activation of protein kinase C (PKC) and a release of arachidonic acid (AA) on attachment and before cell spreading. Inhibition of PKC does not block cell spreading, indicating that PKC activation is not essential for spreading. Inhibition of phospholipase A_2 blocks cell spreading, whereas addition of exogenous AA overcomes this inhibitory effect. Among AA metabolic pathways, inhibitors of lipoxygenase (LOX) block cell spreading, suggesting that a LOX product(s) formed from AA initiates spreading. Clustering receptors for collagen with polyclonal antibodies, or with anti-collagen-receptor antigen-binding fragments (Fab) in combination with a secondary antibody, induce AA release. Also, AA is released when cells attach to either immobilized gelatin or immobilized Arg-Gly-Asp (RGD) peptide. Thus, AA is released whenever receptor clustering is observed. Receptor occupancy is not sufficient to release AA; when cells are treated with gelatin or RGD peptide in solution or anti-collagen-receptor Fab fragments without secondary antibody, conditions where receptor clustering is not observed, AA is not released. Thus, a LOX metabolite(s) of AA formed by collagen-receptor clustering is a second messenger(s) that initiates HeLa cell spreading. LOX inhibitors also block the spreading of bovine aortic endothelial cells, chicken embryo fibroblasts, and CV-1 fibroblasts on gelatin or fibronectin, indicating that other cells might use the same second messenger system in initiating cell-substratum adhesion.

INTRODUCTION

The adhesion of cells to an extracellular matrix (ECM)¹ is related to a number of biological processes such as

growth, differentiation, and tumor cell invasion during metastasis (McClay and Etensohn, 1987; Hart *et al.*, 1987). The mechanisms whereby the ECM regulates such cellular functions are under intense investigation. It is clear that cell-surface receptors, such as integrins,

¹ Abbreviations used: AA, arachidonic acid; BAEC, bovine aortic endothelial cells; BAPTA, bis-(*O*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid; BCECF, 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein; BPB, bromophenacyl bromide; BSA, bovine serum albumin; $[Ca^{++}]_i$, intracellular free calcium; CEF, chicken embryo fibroblasts; COX, cyclooxygenase; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGTA, ethylene glycol-bis(β -

aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EOX, cytochrome p450 dependent epoxigenase; Fab, antigen-binding fragment; FCS, fetal calf serum; LOX, lipoxygenase; NDGA, nordihydroguaretic acid; PBS, phosphate-buffered saline; pH_i , cytoplasmic pH; PKC, protein kinase C; PLA₂, phospholipase A₂; PLC, phospholipase C; Tris, tris(hydroxymethyl)aminomethane.

play a critical role in the interaction of cells with the ECM (Akiyama *et al.*, 1990; Albelda and Buck, 1990). HeLa cells, a transformed human epithelial cell line, attach to a variety of substrata, including fibronectin, laminin, and collagen or gelatin, but they spread only on collagen or gelatin (Burke *et al.*, 1983; Fairman and Jacobson, 1983; Lu *et al.*, 1992). We previously identified and characterized collagen receptors that appear to be distinct from the integrin family, although they are Arg-Gly-Asp and Mg^{++} dependent (Lu *et al.*, 1989; Beacham and Jacobson, 1990). The receptors are designated by their molecular mass and by that of any prevalent proteolytic fragments (102/58, 87, and 38/33 kDa). The receptors mediate both cell attachment and spreading on either collagen or gelatin. The receptors are also present inside the cell from where they are transported to the cell surface (exocytotic upregulation) to stabilize cell attachment. Spreading is then initiated by clustering and binding of the receptors to the cytoskeleton (Lu *et al.*, 1992).

Although considerable work has been done on characterizing specific receptors for an ECM, little is known about how the receptors transduce signals across the membrane to initiate adhesion of cells to the ECM. When a cell interacts with a particular ECM molecule via surface receptors, the regulation of adhesion will depend on how the receptors communicate or interact with the adhesion regulatory proteins. One potential mechanism involves the formation of intracellular second messengers. Recent evidence suggests that cell-ECM adhesion is mediated by second messenger systems (reviewed by Watson, 1991). For example, clustering of $\beta 1$ integrin in human carcinoma (KB) cells causes protein tyrosine phosphorylation (Kornberg *et al.*, 1991). Mutants of Chinese hamster ovary cells that do not adhere to fibronectin unless dibutyryl cAMP is added have an altered type I protein kinase, suggesting that cAMP may regulate the adhesion process of these cells (Cheung *et al.*, 1987). Phosphatidylinositol turnover, which generates two second messengers, inositol phosphate and diacylglycerol (DAG), is enhanced in spread BHK-21 (C13) cells (Breuer and Wagener, 1989), although it is not known whether the increased turnover is required for cell spreading. Chemotactic effects of elastin in white blood cells appear to be mediated by an elastin receptor that is activated by a G protein, inositol triphosphate, and DAG (Labat-Robert *et al.*, 1990). In macrophages and neutrophils, changes of intracellular free calcium ($[Ca^{++}]_i$) might be involved in cell adhesion. $[Ca^{++}]_i$ increases before cell spreading, although this increase has been observed when the cells are plated on nonspecific substrata (Kruskal *et al.*, 1986; Kruskal and Maxfield, 1987). A rise in cytoplasmic pH (pH_i) has been correlated with cell adhesion to the ECM (Margolis *et al.*, 1988; Schwartz *et al.*, 1989, 1990; Ingber *et al.*, 1990). The alkalization of pH_i in spread fibroblasts is thought to be regulated by clustering $\beta 1$ -integrin

that enhances the transmembrane Na^+/H^+ antiport (Schwartz *et al.*, 1991). However, it appears that the rise in pH_i is a consequence of cell spreading (Schwartz *et al.*, 1989, 1990; Ingber *et al.*, 1990).

In this paper we describe a possible second messenger(s) that regulates HeLa cell spreading on a gelatin substratum. Evidence was obtained indicating that collagen-receptor clustering, on interaction of the cells with immobilized gelatin, activates phospholipase A_2 (PLA_2) to release free arachidonic acid (AA). The data also indicate that lipoxygenase (LOX) metabolizes the released AA to a product(s) that serves as a trigger for HeLa cell spreading.

MATERIALS AND METHODS

Materials

$[^3H]AA$ and $[^{32}P]ATP$ were purchased from ICN (Cleveland, OH). Rhodamine-conjugated goat anti-mouse IgG antibody and protein G were obtained from Boehringer Mannheim (Indianapolis, IN). The following chemicals were gifts from The Wellcome Research Laboratory (Beckenham, Kent, England) for BW755C, Takeda Chemical Industries, (Osaka, Japan) for AA861, and The Upjohn Company (Kalamazoo, MI) for piriprost. AA and its metabolites were from Cayman Chemical (Ann Arbor, MI). Acetoxymethyl esters of quin 2 and bis-(O-aminophenoxy)- N,N,N',N' -tetracetic acid (BAPTA), and 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) were purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Culture

HeLa-S₃ (American Type Culture Collection, Rockville, MD) cells were grown in suspension to mid-log phase ($2-4 \times 10^5$ cells/ml) at 37°C in RPMI-1640 medium (K. C. Biologicals, Lenexa, KS) supplemented with 5% calf serum (GIBCO, Grand Island, NY) as described previously (Lu *et al.*, 1989). Bovine aortic endothelial cells (BAEC), chicken embryo fibroblasts (CEF), and transformed fibroblasts (CV-1) from African green monkey kidney were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum.

Substratum Preparation and In Vitro Assay for HeLa Cell Spreading

Type I gelatin from swine skin, bovine serum albumin (BSA), and Gly-Arg-Gly-Asp-Ser pentapeptide were covalently coupled to polystyrene culture dishes (Falcon, Oxnard, CA) as described previously (Lu *et al.*, 1989; Beacham and Jacobson, 1990). Fibronectin was purified from bovine plasma and coated on 35-mm culture dishes (50 μg /ml per dish) as described elsewhere (Ruoslahti *et al.*, 1982). HeLa cells used for attachment and spreading studies were harvested from suspension cultures, washed (2 times), and resuspended in RPMI 1640 medium. Other cell types (BAEC, CV-1, and CEF) had to be detached from culture flasks by trypsin-EDTA, washed (2 times), and resuspended in DMEM. Cell spreading was assayed as previously indicated by Burke *et al.* (1983) and Lu *et al.* (1992) in serum-free medium to minimize the possible contribution of serum factors in cell spreading. HeLa cells do not attach and spread on a gelatin substratum when serum is present (Burke *et al.*, 1983). However, because the cells were out of serum <2 h before plating on substratum, synergistic involvement of residual serum factors on spreading cannot be completely ruled out. HeLa cells cannot be kept in serum-free medium for extended periods of time because they die. The resuspended cells were plated on substratum-coated culture dishes (5×10^5 cells/ml per dish) at 37°C for 30 min before scoring for the percent of cells spread,

unless otherwise indicated. A spread cell is defined as one that is at least two times the diameter of the nucleus (Lu *et al.*, 1992). The maximum number of cells spread occurred after 30-min incubation and did not change up to 2 h unless otherwise indicated. Percentage of cells spread was calculated from the number of spread cells/total number of cells $\times$ 100 from cells in several microscopic fields of view using phase contrast microscopy.

Calcium Measurement

HeLa cells (1×10^6 cells/ml) were incubated with 5 μ M fura 2/AM for 30 min or various concentrations of quin 2/AM for 1 h at 37°C in RPMI 1640 medium. The cells were washed three times and resuspended in RPMI medium. Fluorescence was measured in suspension with a Perkin-Elmer (Norwalk, CT) 650-10S fluorescence spectrophotometer at 37°C with emission wavelength at 505 nm and excitation wavelengths at 340 and 360 nm. $[Ca^{++}]_i$ is expressed as a ratio of excitation fluorescence intensity at 340 and 360 nm, i.e., F340/F360 (Figure 1, A and B). Measurement of $[Ca^{++}]_i$ in individual cells during attachment and spreading was carried out by plating fura 2-loaded cells on gelatin-coated polystyrene plastics (0.8 mm) set on microscopic stage at 37°C and by a digital image analysis of fura 2 fluorescence. The instrumentation has been described previously by Linderman *et al.* (1990). Briefly, cells on the stage of a Zeiss IM-35 inverted microscope (Carl Zeiss, Thornwood, NY) were illuminated alternatively at 334 and 365 nm using a Hg-Xe arc lamp (Hamamatsu, Bridgewater, NJ) and a pair of monochromators (model H20, Instruments SA, Metuchen, NJ). Fluorescence images were detected by a charge coupled device instrumentation camera (Photometrics, Tucson, AZ) every 15 or 20 s up to 30 min as soon as cells attach to a gelatin substratum (<2 min). Image collection and processing was accomplished through a menu-driven software package (Paragon-IPS, Paragon Imaging, Lowell, MA), which has been previously described (Linderman *et al.*, 1990).

Protein Kinase C (PKC) Assay

PKC was partially purified by a modified method of Kikkawa *et al.* (1983). Briefly, HeLa cells (6×10^6 cells) were plated on gelatin- or BSA-coated 150-mm culture dishes. After incubation at 37°C for the indicated times, cells were scraped off with a rubber policeman in buffer I (20 mM tris(hydroxymethyl)aminomethane [Tris] buffer, pH 7.5, containing 0.25 M sucrose, 10 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetracetic acid [EGTA], 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin A). The cells were sonicated and centrifuged at 100 000 $\times$ g for 1 h at 4°C, and the supernatant was saved as a cytosolic fraction. The membrane-containing fraction in the pellet was solubilized in the same buffer supplemented with 1% nonidet-40. Both fractions were applied to identical 1-ml DE-52 columns equilibrated with buffer II (20 mM Tris, pH 7.5, containing 50 mM β -mercaptoethanol, 5 mM EGTA, 2 mM EDTA, and protease inhibitors). Columns were washed with buffer II, buffer III (20 mM Tris, pH 7.5, containing 50 mM β -mercaptoethanol, 1 mM EGTA, and 1 mM EDTA), and buffer III containing 30 mM NaCl. PKC was eluted with 1.5 ml of buffer III containing 120 mM NaCl and was used for the *in vitro* measurement of PKC activities. The reaction was initiated by the addition of 50 μ l of PKC extracts to the reaction mixture (250 μ l) containing 20 mM Tris-HCl, 10 mM magnesium acetate, 0.2 mg/ml of histone, 0.1 mM ATP ($1-3 \times 10^6$ cpm of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$) with or without phosphatidylserine (24 μ g/ml), diolein (1.6 μ g/ml), and 0.75 mM CaCl_2 . The incubation was carried out for 15 min at 30°C, and the reaction was stopped by the addition of 1 ml cold trichloroacetate (25% wt/vol). The acid-precipitable materials were collected on a membrane filter, washed, and the amount of radioactivity was determined. PKC activity was calculated from the difference in ^{32}P incorporated into histone in the presence or absence of phosphatidylserine/diolein and CaCl_2 .

AA Assay

HeLa cells were labeled overnight in suspension culture with $[^3\text{H}]\text{AA}$ (0.2 $\mu\text{Ci}/\text{ml}$). During the labeling period, >90% of $[^3\text{H}]\text{AA}$ was incorporated into the cells and >95% of incorporated $[^3\text{H}]\text{AA}$ was recovered in phospholipids. Labeled cells were collected and incubated with RPMI-1640 medium on ice for 30 min. After washing and resuspension of the cells in RPMI medium, equal numbers of cells were allowed to attach and spread on gelatin-coated culture dishes or allowed to attach to BSA-coated culture dishes as a control where the cells did not spread (5×10^5 cells/dish). After incubation at 37°C for the indicated times, cells were scraped off from the culture dish with a rubber policeman and transferred to glass tubes. The dishes were washed with 0.5 ml of RPMI 1640 medium. The lipids were extracted by addition of 3 ml of chloroform:methanol:HCl (1:1:0.01 vol/vol). The lipid-containing lower phase, after centrifugation at 3000 $\times$ g for 1 min, was washed with an equal volume of methanol:water (1:1 vol/vol) and concentrated under nitrogen gas. The lipids were separated on thin-layer silica plates using heptane:diethyl ether:glacial acetic acid (60:40:2 vol/vol) as the chromatography solvent. Individual lipids were visualized by iodine vapor and identified by comigration with standards. Areas of the gel corresponding to AA were scraped into scintillation vials and counted. To determine AA release into extracellular medium on treatment of cells with anti-collagen receptor antibody (see Figure 8), cells were allowed to attach to 65-mm sulfonated culture dishes (2×10^6 cells per 4-ml dish) for 15 min, washed to remove any unbound cells, and treated with the indicated antibody. At the indicated times, aliquots (3 $\times$ 45 μ l) of medium from the same dish were counted. When lipids in the extracellular medium were extracted and separated by thin-layer chromatography, radioactivity was found only in the band corresponding to AA.

Preparation of Anti-38/33 kDa Collagen-Receptor Antibodies and Antigen-Binding (Fab) fragments

Collagen receptors were prepared by affinity chromatography and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (Lu *et al.*, 1989). The electroeluted 38/33-kDa collagen receptor was emulsified in Freund's complete adjuvant and injected intraperitoneally into balb/c mice. Booster shots were given at 3-wk intervals in Freund's incomplete adjuvant. Ascitic fluid was developed with SP2/0 cells, and the mice were tapped 10–15 d after the final booster shot. The antibody in the ascitic fluid was purified on a protein G column as described elsewhere (Harlow and Lane, 1988). For the preparation of Fab fragments, the antibody was incubated with papain (10 μ g/mg antibody) for 2 h at 30°C (Harlow

Table 1. Effects of various treatments on HeLa cell spreading on gelatin-coated culture dishes

Treatment	Percentage of cells spread
Control	90 $\pm$ 2
Dibutyl cAMP (200 μ M, 30 min)	85 $\pm$ 3
8-Bromo-cAMP (200 μ M, 30 min)	84 $\pm$ 5
Staurosporine (1 μ M, 5 min)	86 $\pm$ 3
TPA (0.4 μ M, 18 hrs)*	83 $\pm$ 4

Suspension HeLa cells were treated with various concentrations of each drug for various time periods as indicated. Treated cells were allowed to attach and spread on a gelatin substratum for 30 min at 37°C in the presence of the drugs and percentage of cells spread was scored. The data represent the average of four experiments with the standard deviations.

* TPA-treated cells were washed and allowed to spread for 30 min.

Figure 1. Role of $[Ca^{2+}]_i$ in HeLa cell spreading on a gelatin substratum. (A) Depletion of $[Ca^{2+}]_i$. Suspension HeLa cells were incubated with varying concentrations of acetoxymethyl ester of BAPTA (●) or quin 2 (■) in the presence of 1 mM EGTA for 1 h at 37°C in RPMI medium. The cells were washed and plated on gelatin in RPMI medium containing 1 mM EGTA. When the cells were incubated with BAPTA in the absence of EGTA in 1 mM calcium-containing medium and allowed to spread, the percentage of cells spread was not affected (○). Incubation of cells with EGTA alone had no effect on spreading (not shown). $[Ca^{2+}]_i$ was monitored with quin 2 fluorescence (□). (B) Increase of $[Ca^{2+}]_i$. Cells were plated on gelatin in the presence of

and Lane, 1988). Any remaining undigested antibody and Fc fragments were removed by running the digested mixture through a protein G column.

Indirect Immunofluorescence Microscopy

HeLa cells attached or spread on substratum-coated polystyrene plastic sheets (0.8 mm, KAMA, Hazleton, PA) were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C for 15 min. Fixed cells were washed and incubated for 45 min with mouse antiserum (anti 38/33 kDa, 1:600) in PBS containing 1 mM $MgCl_2$ and 5% fetal calf serum (FCS) at 4°C. Cells were then washed extensively with PBS/FCS buffer and decorated with rhodamine-conjugated goat anti-mouse IgG antibodies (1:800) for another 45 min in PBS/FCS buffer at 4°C. The specimen samples covered with glycerol were examined with a Nikon Optiphot fluorescence microscope (Nikon, Garden City, NY).

RESULTS

Role of cAMP and $[Ca^{2+}]_i$ Changes in HeLa Cell Spreading

In our attempt to search for signal molecules that initiate HeLa cell spreading on a gelatin substratum, we examined several common second messengers. Treatment of HeLa cells with membrane-permeable cAMP analogs, dibutyryl cAMP or 8-bromo-cAMP, does not affect cell spreading (Table 1). Also, the level of cytosolic cAMP does not change until the cells are fully spread (Lu and Jacobson, unpublished data), suggesting that cAMP is not the trigger for cell spreading. The role of $[Ca^{2+}]_i$ in cell spreading was examined. It is known that extracellular calcium is not required for HeLa cell spreading (Beacham and Jacobson, 1990). When $[Ca^{2+}]_i$ is depleted with the cytoplasm-trapped calcium chelators quin 2 or BAPTA (Rink and Pozzan, 1985), the number of cells spread is reduced in a dose-dependent manner (Figure 1A). Cell spreading is also inhibited by high concentrations, i.e., 1 μM of the calcium ionophores A23187 and ionomycin (Figure 1B), suggesting that a high $[Ca^{2+}]_i$ also inhibits spreading. However, it is possible that the inhibitory effect is due to either cytotoxic effects of the ionophores or modulation of the cytoskeleton by calcium-activated actin-binding proteins such as gelsolin (Forscher, 1989). To examine directly the role of $[Ca^{2+}]_i$ changes in cell spreading, $[Ca^{2+}]_i$ was measured in individual cells every 15 or 20 s by using a digital image

varying concentration of A23187 (●) or ionomycin (■) with 1 mM calcium. Percentage of cells spread was scored 30 min after seeding on plates. $[Ca^{2+}]_i$ was monitored with fura 2 (5 μM) fluorescence (○) in the cells treated with ionomycin. (C) $[Ca^{2+}]_i$ changes in a single cell. $[Ca^{2+}]_i$ (■) was measured in individual cells plated on gelatin every 20 s as described in MATERIALS AND METHODS. Cell area (●) was calculated by measuring four different diameters of a cell every 3 min. The data in A and B represent the average value of five experiments and the error bars indicate the standard deviation. The data in C represent a typical cell from experiments conducted six times with a total 43 cells.

Figure 2. Reduced cytoplasmic hydrogen ion concentration (pH_i) inhibits cell spreading. Spreading assays on a gelatin substratum were performed at the indicated pH in HEPES buffered solution (HBS, 10 mM Hepes; 145 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; and 10 mM glucose) either in the presence or absence of 2 μ M nigericin to alter the pH_i . ○, 30-min incubation on gelatin in 2 μ M nigericin containing HBS. ●, 60-min incubation on gelatin in 2 μ M nigericin containing HBS. □, cells incubated for 30 min on gelatin in 2 μ M nigericin containing HBS were washed (3 times) with HBS and the medium was replaced with HBS, pH 7.2, containing 1 μ M nigericin. Percentage of cells spread was scored after 30-min incubation. ■, 30-min incubation on gelatin in the absence of nigericin. The data represent the average of five experiments with duplicated samples and the error bars indicate the standard deviation.

analysis of intracellular fura 2 fluorescence. No detectable $[Ca^{++}]_i$ changes occur during cell attachment and spreading (Figure 1C). We interpret these results to indicate that spreading of HeLa cells on gelatin requires resting levels of $[Ca^{++}]_i$, but that intracellular increases or decreases of $[Ca^{++}]_i$ are not essential to initiate cell spreading.

Role of pH_i in HeLa Cell Spreading

To examine whether pH_i changes are involved in HeLa cell spreading, pH_i was measured by loading cells with the pH indicator, BCECF. Nigericin was used to calibrate the pH_i as previously described (Rink *et al.*, 1982). There is no difference in pH_i between cells in suspension and cells spread on gelatin (7.49 ± 0.07 [mean $\pm$ SD] vs. 7.52 ± 0.06 in *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered solution, pH 7.3). This indicates that HeLa cells have an elevated pH_i and that cell spreading does not affect the pH_i , an observation consistent with that found in other transformed cell types (Schwartz *et al.*, 1989). We next examined whether an alkaline pH_i is required for spreading. The pH_i was modulated by adjusting the pH of a buffer (pH_o) containing 145 mM K^+ and nigericin. Under these conditions, the pH_i is equal to the pH_o (Rink *et al.*, 1982). As shown in Figure 2, cell spreading is not affected at pH_i between 7.0 and 7.8. However, spreading is significantly delayed at acidic pH_i . Less than 5% of cells are spread at pH_i 6.6 even after 1 h of incubation (Figure

2). When cells were allowed to spread at an acidic pH_o in the absence of nigericin, where pH_i remained over 7.0 (at pH_o 6.6), >85% of the cells rapidly spread in <30 min. The inhibition of spreading by an acidic pH_i is reversed if the pH_i is returned to 7.2 by replacing the medium with HEPES-buffered solution (HBS, pH 7.2) containing 1 μ M nigericin (Figure 2). This may indicate that an alkaline pH_i rather than an alkalinization of pH_i is required for cell spreading.

Role of PKC in HeLa Cell Spreading

In HeLa cells plated on a gelatin substratum, PKC activity in the membrane fraction is transiently increased in a time period that precedes cell spreading (Figure 3). When the maximum number of cells have spread, PKC activities in the membrane fractions decline. Consistent with the increased PKC activity in the membrane fraction is the decreased cytosolic PKC activity found on attachment of cells to a gelatin substratum. If cells are plated on BSA-coated culture dishes, where cells do not spread, PKC activities in both fractions do not change. To examine whether PKC activation is required for cell spreading, PKC was either inhibited by staurosporine, a potent PKC inhibitor (Tamaoki *et al.*, 1986), or down-regulated by prolonged (18 h) treatment with 12-O-tetradecanoyl phorbol-13-acetate (Ashendel, 1985). Although both conditions indeed block PKC activation in HeLa cells, cell spreading is not affected (Table 1), suggesting that PKC activation is not critical for spreading. It should be noted that the extent of cell spreading is modulated by PKC activity, as will be shown in a more comprehensive paper on the role of PKC in enhancing AA production to amplify cell spreading (Chun and Jacobson, unpublished data).

Figure 3. Activation of PKC before HeLa cell spreading. HeLa cells (6×10^6) were seeded on gelatin-coated 150-mm culture dishes. At the indicated times, both membrane-bound and cytosolic PKC activities were determined as described in MATERIALS AND METHODS. The data represent the mean value of quadruplicate samples from a typical experiment conducted four times and the error bars indicate the standard deviation. ●, cytosolic fraction; ○, particulate membrane fraction; ■, percent cells spread.

Figure 4. Release of AA before HeLa cell spreading. [^3H]AA-labeled cells were plated on a gelatin substratum where cells attached and spread (●) or BSA substratum where cells attached but did not spread (○). At the indicated times, the amount of cellular [^3H]AA was determined as described in MATERIALS AND METHODS. Percentage of cells spread (■) was determined from the cells plated on the gelatin substratum. The data represent the average value of triplicate samples with the standard deviation from a typical experiment conducted more than 15 times.

AA is Released on Attachment and Before HeLa Cell Spreading

When [^3H]AA-labeled cells are plated on a gelatin substratum, the amount of cellular free AA increases before cell spreading (Figure 4). The elevated levels of AA begin to decrease once the maximum number of cells have spread. However, when cells are plated on BSA-coated culture dishes where cells attach but do not spread, only small amounts of AA are released (Figure 4). To examine if cells require AA release to spread, cells were treated with bromophenacyl bromide (BPB), an irreversible inhibitor of PLA_2 (Chang *et al.*, 1987), before the spreading assay. As shown in Figure 5, spreading is blocked in a dose-dependent manner by BPB treatment as is AA release (Figure 5), indicating that AA release is essential for cells to spread.

A LOX Metabolite(s) of Released AA is Required for HeLa Cell Spreading

5,8,11,14-Eicosatetraynoic acid (ETYA), an inhibitor of the three enzymes that uses AA as a substrate, i.e., LOX, cyclooxygenase (COX), and cytochrome p450-dependent epoxigenase (EOX) (Kühn *et al.*, 1984), significantly decreases the percentage of cells spread (Table 2). However, inhibition of cell spreading is not observed when cells are treated with indomethacin and metyrapone, inhibitors of the COX and EOX, respectively (Capdevila *et al.*, 1981). BW755C, an inhibitor of both LOX and COX (Egan and Gale, 1985), reduces the percentage of cells spread. The LOX inhibitors, nordihydroguaretic acid (NDGA) and AA861 (Yoshimoto *et al.*, 1982), are the most potent inhibitors of HeLa cell spreading (Table 2). All of the inhibitors were used at

Figure 5. Dose-dependent effects of BPB on cell spreading (●) and AA release (○). HeLa cells were treated with indicated amounts of BPB for 5 min in suspension, plated on gelatin at 37°C for 30 min, and percentage of cells spread was scored. To determine AA release, [^3H]AA-labeled cells were treated with BPB and plated on gelatin at 37°C. The amount of [^3H]AA was determined as described in MATERIALS AND METHODS after 20-min incubation in which maximum amount of AA is released. The data represent the average of three experiments and the error bars indicate the standard deviation.

concentrations reported to inhibit the respective enzymes. Cell spreading is also blocked, at least partially, by treatment of cells with other agents, such as baicalein,

Table 2. Effects of various AA metabolism inhibitors on HeLa cell spreading

Treatments	Percentage of cells spread
Control (0.5% methanol)	90 ± 2
ETYA (100 μM, 1 hr)	38 ± 12
Indomethacin (200 μM, 30 min)	88 ± 3
Metyrapone (1 mM, 30 min)	88 ± 3
BW755C (200 μM, 1 hr)	31 ± 9
NDGA (25 μM, 5 min)	13 ± 4
AA 861 (100 μM, 10 min)	4 ± 3
NDGA (with washing) ^a	84 ± 4
AA861 (with washing) ^a	80 ± 4
NDGA (to spread cells) ^b	85 ± 3
AA861 (to spread cells) ^b	84 ± 4
Hydroxyanisole (100 μM, 10 min)	87 ± 3
Morin hydrate (100 μM, 10 min)	89 ± 2
L-cysteine (400 μM, 10 min)	88 ± 3

HeLa cells were treated in suspension with drugs at various concentrations for various time periods as indicated. Spreading assays were performed in the presence of the various agents. The data represent the average value of at least five experiments with the standard deviations.

^a Cells attached or spread in the presence of NDGA (25 μM) or AA861 (100 μM) for 30 min were washed (3 times) and the medium was replaced with fresh RPMI medium. Percentage of cells spread was scored after 30-min incubation.

^b Cells were allowed to spread for 30 min and then treated with NDGA (25 μM) or AA861 (100 μM). Percentage of cells spread was scored 30 min after addition of inhibitors.

Figure 6. Effects of exogenous AA on the inhibitory effects of BPB, NDGA, and AA861 on HeLa cell spreading. Suspension HeLa cells were treated with PLA₂ inhibitor, BPB (6 μM), or LOX inhibitors, NDGA (25 μM) or AA861 (50 μM), for 3 min, then allowed to spread on a gelatin substratum either in the absence (□) or presence (▨) of 15 μM AA. Conversely, cells were treated with 15 μM AA for 5 min then with BPB, NDGA, or AA861 for 3 min, then plated on a gelatin substratum (■). Spreading assays were performed in the presence of inhibitors. The data represent the average of five experiments with duplicated samples and the error bars indicate the standard deviation.

piriprost, diphenyl disulfide, and gossypol, which are known to inhibit LOX (Egan and Gale, 1985). These results may indicate that formation of AA metabolite(s) catalyzed by LOX is essential for the initiation of HeLa cell spreading.

The effect of NDGA or AA861 on cell spreading is not attributable to irreversible damage to cells, because the inhibitory effect of NDGA (25 μM) or AA861 (100 μM) is immediately reversed by washing and resuspension of the cells (Table 2). Also, both inhibitors have no effect on spread cells, because addition of NDGA or AA861 to spread cells does not change their morphology (Table 2). Treatment of cells with NDGA at concentrations that inhibit cell spreading does not alter the pattern of AA release as that seen when cells are spreading on gelatin (see Figure 4), indicating that the NDGA effect is not due to inhibition of PLA₂. In addition to inhibiting LOX, NDGA is also a general anti-oxidant. To rule out the possibility that the cell spreading inhibitory properties of NDGA are due to its properties as an anti-oxidant, we determined whether other anti-oxidants that do not inhibit LOX also inhibit cell spreading. Anti-oxidants or free radical scavengers, such as butylated hydroxyanisole, morin hydrate, and L-cysteine, have no effect on HeLa cell spreading (Table 2).

Recovery of Cell Spreading by Exogenous AA

When cells treated with BPB to inhibit PLA₂ are allowed to spread in the presence of exogenous AA, the ability of BPB to inhibit cell spreading is significantly reversed (Figure 6). If cells are treated with AA before BPB, the inhibitory effect of BPB is further reversed. Exogenous AA does not reverse the inhibitory effects of the LOX inhibitors NDGA and AA861 on cell spreading. How-

ever, treatment of cells with AA before NDGA or AA861 significantly abolishes the effects of these inhibitors on cell spreading (Figure 6). This can be explained if a LOX metabolite(s) accumulates in the cells before LOX inhibition. We did attempt to overcome the inhibitory effect of NDGA on cell spreading with stable LOX metabolites. HeLa cells treated with NDGA were allowed to attach to gelatin. They were then exposed to one of the LOX metabolites. As shown in Table 3, a partial recovery of cell spreading is observed by treatment with leukotriene D₄ and 12-hydroxyeicosatetra-endic acid.

Effects of AA Metabolism Inhibitors on the Spreading of Other Cell Types

Effects of inhibitors of AA metabolic pathways on the spreading of various cell types were examined to see whether they share a mechanism in common with HeLa cells. NDGA, but not indomethacin and metyrapone, inhibits spreading of the tested cell types on gelatin or fibronectin substrata (Table 4).

Collagen-Receptor Occupancy is not Sufficient to Release AA

Attachment of cells to immobilized gelatin or immobilized Gly-Arg-Gly-Asp-Ser (GRGDS) pentapeptide induces AA release and cell spreading (Figure 7). However, when cells attached to BSA were exposed to gelatin or the GRGDS pentapeptide in solution, release of AA was not observed. Attachment of cells to a BSA substratum neither affects cell spreading nor AA release (Figure 4). It is known that the RGD sequences in collagen or gelatin are involved in adhesion of HeLa cells to gelatin (Beacham and Jacobson, 1990). The concentration of GRGDS used was sufficient to completely

Table 3. Effects of LOX metabolites on the inhibitor role of NDGA in HeLa cell spreading

Treatments	Percentage of cells spread
Control (0.5% methanol)	90 ± 2
NDGA (25 μM)	14 ± 3
5 (S, R)-HETE	14 ± 5
12 (S, R)-HETE	34 ± 6
15 (S)-HETE	15 ± 4
LTB ₄	11 ± 6
LTC ₄	13 ± 4
LTD ₄	27 ± 5

HeLa cells treated with NDGA for 5 min were allowed to attach and spread on a gelatin substratum for 30 min in the presence of 1 μM of the indicated LOX metabolite. The data represent the average values of percentage of cells spread from four or five determinations with the standard deviations. Treatment of cells with 0.1 μM of the indicated metabolite did not show significant differences. HETE, hydroxy-eicosatetraenoic acid; LT, leukotriene.

Table 4. Effects of AA metabolism inhibitors on the spreading of other cell types

Cell type	Substratum	Control	NDGA	Indomethacin	Metyrapone
BAEC	Gelatin	82 ± 4	2 ± 2	84 ± 5	79 ± 4
CV-1	Gelatin	91 ± 2	7 ± 6	90 ± 3	89 ± 5
	Fibronectin	88 ± 4	2 ± 1	91 ± 3	89 ± 4
CEF	Gelatin	90 ± 4	14 ± 8	92 ± 3	89 ± 5
	Fibronectin	89 ± 4	8 ± 6	89 ± 2	91 ± 3

Cells were treated with NDGA (25 μ M), indomethacin (100 μ M), or metyrapone (1 mM) in suspension before seeding on substratum-coated culture dishes. Spreading assays were performed in the presence of the drugs. The data represent the average of percentage of cells spread from five experiments with the standard deviations. BAEC, bovine aortic endothelial cells; CV-1, transformed fibroblast from African green monkey kidney; CEF, chicken embryo fibroblast.

prevent cells from spreading on gelatin (Beacham and Jacobson, 1990), indicating that the pentapeptide was probably at saturating levels for receptor binding. Therefore, receptor occupancy does not appear to be sufficient to release AA.

Clustering of Collagen Receptors Induces AA Release

Because the above results indicate that AA release requires more than mere binding of ligands to the receptors, we examined the role of collagen receptor clustering in AA release. Clustering of collagen receptors is essential for HeLa cell spreading on gelatin (Lu *et al.*, 1992). Release of AA into the extracellular medium was as-

Figure 7. Collagen receptor occupancy is not the cause of AA release. Cells were allowed to attach and spread either on gelatin- (●) or GRGDS- (■) coated culture dishes. Alternatively, cells were allowed to attach to sulfonated culture dishes for 15 min and then exposed to solubilized 10 mg/ml of gelatin (○) or 0.6 mg/ml of RGDS (□). At the indicated times, AA release into the extracellular medium was determined. The data represent the average of triplicate samples from a typical experiment conducted at least three times. The standard deviations are indicated by the error bars.

Figure 8. Effects of clustering collagen receptors on AA release. (A) Release of [3 H]AA into the extracellular medium was assayed as described in MATERIALS AND METHODS. Cells were incubated with either 25 μ g/ml of control antibody purified from preimmune serum at 4°C (○) or purified anti-38/33-kDa receptor antibody at 4°C (●) or at 37°C (■). (B) Cells were incubated with Fab fragments (25 μ g/ml) of the anti-38/33-kDa antibody (○). Cells treated with Fab fragments for 30 min were rinsed (3 times) and incubated with goat anti-mouse IgG antibody (10 μ g/ml) at 4°C (●). The data represent averages of three experiments and the error bars indicate the standard deviation.

sayed on clustering the 38/33-kDa collagen receptor in the plasma membrane with polyclonal antibody raised against the receptor. As shown in Figure 8A, AA is released when the receptors are clustered with antibody at 4°C (see Figure 9D). AA release begins to increase 9–15 min after antibody addition and remains at the elevated level for the duration of the experiment. However, when receptor clustering is conducted at 37°C where endocytosis occurs, the release of AA is transient (Figure 8A). Although the release of AA with antibody treatment is not extremely large, it is significant. There is no further release of AA when anti-mouse IgG antibody is added to cells treated with mouse anti-38/33-kDa receptor antibody. It should be noted that the anti-38/33-kDa antibody inhibits cell spreading on a gelatin substratum by 70%.

To assess more directly the role of receptor clustering on AA release, monovalent Fab fragments were pre-

Figure 9. Surface distribution of 38/33-kDa collagen receptors. HeLa cells in suspension (A), spread on gelatin for 30 min (B), or attached to gelatin in the presence of 25 μ M NDGA (C) were fixed with 3% paraformaldehyde and decorated with anti-38/33-kDa receptor primary antibody and rhodamine-conjugated secondary antibody. To induce receptor clustering with external cross-linking, cells attached to BSA-coated culture dishes were incubated with anti-38/33-kDa receptor antibody (25 μ g/ml) at 4°C for 30 min either in the absence (D) or presence (E) of 25 μ M NDGA and then fixed and stained with secondary antibody. Spread cells treated with preimmune serum with secondary antibody do not give detectable fluorescence (F). Bar, 10 μ m.

pared. Incubation of cells with Fab fragments does not induce AA release (Figure 8B). However, when Fab-treated cells are subsequently treated with Fab-specific anti-mouse IgG antibody, a pattern of AA release similar to that observed with the intact antibody is found. These results indicate that clustering of plasma membrane collagen receptors is necessary to release AA and initiate cell spreading on a gelatin substratum.

Inhibition of AA Release Does Not Block Clustering of Collagen Receptors

The surface distribution of the 38/33-kDa receptor was examined by immunofluorescence microscopy (Figure 9). Spread cells (B), when compared with suspension cells (A), show a more punctate pattern when one focuses on the basal surface of the cell contacting the gelatin substratum. This indicates that receptors are clustered during cell spreading. To see the role of LOX metabolite formation on gelatin-induced receptor clustering, cells were treated with either BPB to inhibit release of AA or NDGA to inhibit LOX metabolism of AA. As shown in Figure 9C, the receptors on cells attached to gelatin in the presence of NDGA, which in-

hibits spreading, are more punctate than receptors in suspension cells but less punctate than receptors in spread cells. Cells treated with BPB show a similar pattern of surface receptor distribution as NDGA-treated cells. Thus, inhibition of LOX metabolite generation only partially blocks substratum-induced receptor clustering, suggesting that either receptor clustering can occur in the absence of the formation of LOX metabolites or that the inhibitors were not completely effective.

The effects of BPB and NDGA on antibody-induced receptor clustering were also examined. HeLa cell collagen receptors are clustered with a polyclonal antibody to the 38/33-kDa receptor. When cells are treated at 4°C with the antibody before fixation, the receptors aggregate into clusters (Figure 9D). This aggregation is energy independent because it occurs in the cold. When cells are treated at 37°C for 30 min with the antibody, the receptors aggregate into larger clusters, but the total number of clusters is reduced as is the fluorescence intensity. This is consistent with the internalization of the clustered receptors. When the cells are incubated with BPB or NDGA before addition of the antibody to the cells, clustering of the receptors by the antibody is not

affected (Figure 9E). Taken together, these results further support the conclusion that receptor clustering is essential for AA release and that clustering is not dependent on LOX metabolite(s) formation from AA.

DISCUSSION

Release of AA and the Subsequent Formation of LOX Metabolites as the Signal for HeLa Cell Spreading

Based on the observations that (1) AA is released on attachment in a time period that precedes cell spreading, whereas no AA is released on cell attachment to non-specific substrata such as BSA or sulfonated culture dishes (Figure 4); (2) an inhibitor of PLA₂ (BPB) blocks cell spreading (Figure 5), whereas addition of exogenous AA overcomes the inhibitory effect of BPB (Figure 6); and (3) inhibitors of the LOX-mediated pathway of AA metabolism block cell spreading, whereas they do not inhibit AA release (Table 2), it can be concluded that the liberation of AA and the subsequent formation of a LOX metabolite(s) from it is the trigger for HeLa cell spreading on a gelatin substratum.

AA is found in membrane phospholipids in an esterified form that can be released through multiple lipases (Dennis *et al.*, 1991). It is known that the rate-limiting step for eicosanoid production is the release of AA (Needleman *et al.*, 1986; Dennis, 1987). The primary mechanisms for liberation of AA from phospholipid include hydrolytic removal of AA by Ca⁺⁺-dependent PLA₂, or sequential action of phospholipase C (PLC) to produce DAG, and DAG lipase to release AA (Dennis *et al.*, 1991). The involvement of PLA₂ in gelatin-induced AA release in HeLa cells is suggested by the result that an inhibitor of PLA₂ blocks AA release (Figure 5). Data in Figure 1 show that depletion of cytoplasmic calcium inhibits cell spreading, an observation consistent with the need for a Ca⁺⁺-dependent PLA₂. We observed that chelation of [Ca⁺⁺]_i with quin 2 also inhibits AA release in HeLa cells plated on a gelatin substratum. Similar results were observed in human platelets by Simon *et al.* (1986) where chelation of [Ca⁺⁺]_i inhibits activation of PLA₂, whereas PLC is less sensitive to calcium chelation.

Of the three pathways of AA metabolism, i.e., COX, LOX, and EOX pathways, the inhibitor studies reported here (Table 2) indicate that an increase in the amount of a LOX metabolite(s) of AA is an obligatory signal for the initiation of HeLa cell spreading. This is further supported by the observation that treatment of cells with AA before the addition of NDGA or AA861 partially blocks the effects of the inhibitors, whereas AA addition to the NDGA- or AA861-treated cells has no effect (Figure 6). Although 12 (S,R)-HETE and LTD₄ partially reversed the inhibitory effects of NDGA (Table 3), we do not know the nature of the LOX metabolite(s) responsible for HeLa cell spreading. The metabolite(s) could be either stable products, such as leukotrienes or HETEs,

or unstable intermediates. Both stable and unstable LOX metabolites are known to act as second messengers (Piomelli and Greengard, 1990). It is also possible that cell spreading is mediated by a combination of LOX products.

The action of AA metabolites as modulators of cell-cell and cell-substratum adhesion processes has been suggested from work in which cells were pretreated with an AA metabolite and an increase in adhesion was observed. For example, adhesion of Lewis lung carcinoma cells to endothelial cells or to the ECM is enhanced when the tumor cells are treated with the exogenous LOX metabolite, 12-HETE (Grossi *et al.*, 1989; Liu *et al.*, 1991). Human monocytic tumor cell adhesion to endothelium can be promoted by treatment with the epoxigenase product of AA, 14,15-epoxyeicosatrienoic acid (Pritchard *et al.*, 1990).

Receptor Clustering as a Cause of AA Release

The observation that clustering receptors and binding of the clustered receptors to the cytoskeleton is a requirement for cell adhesion to an ECM has been reported in other systems (Cody and Wicha, 1986; Rapraeger *et al.*, 1986). In this report we induced external cross-linking of collagen receptors with antibody to directly assess the role of receptor clustering in PLA₂ activation. The clustering of the receptors and subsequent activation of PLA₂ are apparently energy-independent processes as they occur at reduced temperatures. Energy-independent clustering of other ECM receptors has also been reported (Cody and Wicha, 1986; Rapraeger *et al.*, 1986). However, PLA₂ activation appears not to be totally temperature independent because the rate of AA release during cell spreading is reduced as the temperature is reduced from 37 to 22°C and 4°C. AA release at 22 and 4°C is, respectively, 56 ± 12 and 23 ± 4% that at 37°C. It should be noted that although the maximum amount of AA released by collagen receptor antibody clustering is nearly equal at 4 and 37°C, the initial rate of AA release at 4°C is less than that at 37°C (Figure 8A). This is probably due to the fact that at 4°C, AA reincorporation is dramatically reduced relative to that at 37°C. Release of AA on collagen-receptor clustering with antibodies provides clear evidence that receptor clustering is the cause of AA release. This conclusion is further supported by the observations that inhibition of LOX metabolite formation does not block receptor clustering induced by either a gelatin substratum or an antibody against the 38/33-kDa collagen receptor (Figure 9).

In conclusion, cell spreading is signaled by clustering collagen receptors that activate PLA₂ to produce AA, which is metabolized by the LOX pathway to form a second messenger(s) that turns on the spreading machinery. Although there are no changes in [Ca⁺⁺]_i and pH_i, resting levels of [Ca⁺⁺]_i and an alkaline pH_i are required for spreading.

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